Differential Impact of *In Vivo* CD8$^+$ T Lymphocyte Depletion in Controller versus Progressor Simian Immunodeficiency Virus-Infected Macaques

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ABSTRACT

Numerous studies have demonstrated that CD8$^+$ T lymphocytes suppress virus replication during human immunodeficiency virus (HIV)/simian immunodeficiency virus (SIV) infection. However, the mechanisms underlying this activity of T cells remain incompletely understood. Here, we conducted CD8$^+$ T lymphocyte depletion in 15 rhesus macaques (RMs) infected intravenously (i.v.) with SIV$_{mac239}$. At day 70 postinfection, the animals (10 progressors with high viremia and 5 controllers with low viremia) were CD8 depleted by i.v. administration of the antibody M-T807R1. As expected, CD8 depletion resulted in increased virus replication, more prominently in controllers than progressors, which correlated inversely with predepletion viremia. Of note, the feature of CD8$^+$ T lymphocyte predepletion that correlated best with the increase in viremia postdepletion was the level of CD8$^+$ T-bet$^+$ lymphocytes. We next found that CD8 depletion resulted in a homogenous increase of SIV RNA in superficial and mesenteric lymph nodes, spleen, and the gastrointestinal tract of both controllers and progressors. Interestingly, the level of SIV DNA increased postdepletion in both CD4$^+$ central memory T lymphocytes (T$_{CM}$) and CD4$^+$ effector memory T lymphocytes (T$_{EM}$) in progressor RMs but decreased in the CD4$^+$ T$_{CM}$ of 4 out of 5 controllers. Finally, we found that CD8 depletion is associated with a greater increase in CD4$^+$ T lymphocyte activation (measured by Ki-67 expression) in controllers than in progressors. Overall, these data reveal a differential impact of CD8$^+$ T lymphocyte depletion between controller and progressor SIV-infected RMs, emphasizing the complexity of the *in vivo* antiviral role of CD8$^+$ T lymphocytes.

IMPORTANCE

In this study, we further dissect the impact of CD8$^+$ T lymphocytes on HIV/SIV replication during SIV infection. CD8$^+$ T lymphocyte depletion leads to a relatively homogenous increase in viral replication in peripheral blood and tissues. CD8$^+$ T lymphocyte depletion resulted in a more prominent increase in viral loads and CD4$^+$ T lymphocyte activation in controllers than in progressors. Interestingly, we found T-bet expression on CD8$^+$ T lymphocytes to be the best predictor of viral load increase following depletion. The levels of SIV DNA increase postdepletion in both CD4$^+$ T$_{CM}$ and T$_{EM}$ in progressor RMs but decrease in the CD4$^+$ T$_{CM}$ of controllers. The findings described in this study provide key insights into the differential functions of CD8$^+$ T lymphocytes in controller and progressor RMs.

Several lines of evidence indicate that CD8$^+$ T lymphocytes mediate control of virus replication during both human immunodeficiency virus (HIV) infection of humans and simian immunodeficiency virus (SIV) infection of rhesus macaques (RMs). First, the postpeak decline of viremia in acute HIV infection is coincident with the expansion of HIV-specific T cells (1, 2). Second, during both acute and chronic HIV/SIV infection, immune pressure mediated by HIV/SIV-specific CD8$^+$ T lymphocytes is manifested by viral escape mutations (3). Third, there is a clear association between the presence of certain major histocompatibility complex (MHC) class I alleles and disease progression during both HIV infection of humans and SIV infection of RMs (4, 5). Fourth, HIV-1-infected individuals with polyfunctional HIV-1-specific T cells appear to progress less rapidly than those whose T lymphocytes have more limited functionality (6). While compelling, all of these studies are correlative in nature and fail to establish a direct cause-effect relationship. In this context, the most convincing evidence for a direct effect of CD8$^+$ T lymphocytes in suppressing virus replication came from a series of studies in which these cells were depleted *in vivo* in SIV-infected RMs (7–10). These studies clearly demonstrated that antibody-mediated

in vivo depletion of CD8$^+$ T lymphocytes is consistently associated with increased virus replication and faster disease progression (11). Despite this strong evidence indicating that CD8$^+$ T lymphocytes suppress virus replication during HIV/SIV infections, these cells ultimately fail to prevent disease progression in the vast majority of HIV-infected individuals and SIV-infected RMs.

The mechanisms by which CD8$^+$ T lymphocytes exert an antiviral effect *in vivo* still are incompletely understood. Conceivably, these mechanisms can be summarized by two major functions: (i) CD8$^+$ T lymphocytes may reduce production of virions on a per-cell basis either by direct killing of infected cells or by decreasing the rate of virus production via noncytolytic mechanisms, and/or (ii) CD8$^+$ T lymphocytes may reduce the number of productively infected cells either by inhibiting the spread of infection (i.e., via production of β-chemokines or other cytokines) or by limiting the number of targets (i.e., activated CD4$^+$ T lymphocytes) available for infection. While the exact *in vivo* contribution of these nonmutually exclusive antiviral effects by CD8$^+$ T lymphocytes has not yet been defined, there is preliminary evidence that they all are involved (12–15). Further, there are several basic aspects of how depletion of CD8$^+$ T lymphocytes affects SIV replication that have not yet been fully elucidated. These aspects include (i) the kinetics of virus replication post-CD8 depletion in progressor (i.e., high-viremia) versus controller (i.e., low-viremia) animals; (ii) the anatomic location of productively infected cells that support increased viremia post-CD8 depletion; (iii) how CD8 depletion impacts the frequency of infected cells within the main memory CD4$^+$ T lymphocyte subsets; (iv) the features of the SIV-specific CD8$^+$ T lymphocyte response prior to depletion that correlate best with the increase of virus replication postdepletion; and (v) the impact of CD8$^+$ T lymphocyte depletion on CD4$^+$ T lymphocyte activation.

In this study, we set out to investigate these aspects of the antiviral effect of CD8$^+$ T lymphocytes in 15 SIV-infected RMs (10 progressors and 5 controllers) that underwent CD8$^+$ T lymphocyte depletion at day 70 postinfection. The main results of this study are that (i) CD8$^+$ T lymphocyte depletion is followed by an increase in virus replication that was more prominent in SIV-infected controllers than in progressors, and this increase in virus replication was relatively homogenous in various lymphoid organs and tissues; (ii) the levels of T-bet in CD8$^+$ T lymphocytes prior to depletion predicted the increase in virus replication postdepletion; (iii) the levels of SIV DNA in CD4$^+$ central memory T lymphocytes (T$_{CM}$) increased postdepletion in progressor RMs but decreased in controllers; and (iv) CD8$^+$ T lymphocyte depletion was associated with a greater increase in CD4$^+$ T lymphocyte activation in controllers than in progressors. We concluded that these data reveal a complex role of CD8$^+$ T lymphocytes in controlling virus replication in SIV-infected RMs that includes significant differences between controllers and progressors.

MATERIALS AND METHODS

Animals. Eighteen female adult rhesus macaques were infected intravenously (i.v.) with 3,000 50% tissue culture infectious doses (TCID$_{50}$) of SIV$_{mac239}$. All RMs were Mamu-B*08 and -B*17 negative; three Mamu-A*01-positive RMs were assigned to each experimental group, and two Mamu-A*01 RMs were assigned to the control group. Five animals each were sacrificed at day 3, day 7, and day 14 after CD8 depletion. Mock-depleted RMs were sacrificed 7 days postdepletion. Blood and tissues were collected throughout the study period, and plasma viral loads were monitored on a weekly basis. All animals were housed at the Yerkes National

Primate Research Center and maintained in accordance with NIH guidelines. Studies were approved by the Emory University Institutional Animal Care and Use Committee.

CD8$^+$ T lymphocyte depletion. Ten weeks after SIV infection, 15 RMs were depleted of CD8$^+$ T lymphocytes by i.v. treatment with 50 mg/kg of body weight of the rhesus recombinant monoclonal antibody M-T807R1 (National Institutes of Health Nonhuman Primate Reagent Resource). Three nondepleted control animals were mock depleted with a primatized control IgG1 OKT3 antibody reactive against the human CD3 molecule (NIH Nonhuman Primate Resource Reagent). The efficacy of the depletion in blood was determined by flow-cytometric analysis and complete blood cell counts at multiple time points after administration of the depleting reagent. Depletion efficiency in tissues was determined by flow-cytometric analysis as a fraction of CD8$^+$ T lymphocytes from samples obtained predepletion.

Sample collection and processing. Peripheral blood mononuclear cells (PBMC) were isolated from blood by gradient density centrifugation (Ficoll). Lymphocytes were isolated from freshly obtained lymph node and rectal biopsies by passing them through a 70-μm cell strainer and lysing red blood cells with ACK lysis buffer (Life Technologies).

Immunophenotyping, cytokine responses, and flow cytometry. Multicolor flow-cytometric analysis was performed on lymphocytes isolated from peripheral blood and tissues according to standard procedures. The antibodies used were anti-CD4 allophycocyanin (APC)-Cy7 (clone OKT4; BioLegend), anti-CD4 Pacific blue (clone OKT4; BioLegend), anti-CD8 QDot 705 (clone 3B5; Invitrogen), anti-CD8 brilliant violet 711 (clone RPA-T8; BioLegend), Ki-67 fluorescein isothiocyanate (FITC) (clone B56; BD Biosciences), anti-CD3 Pacific blue (clone SP34-2; BD Biosciences), anti-CD3 APC-Cy7 (clone SP34-2; BD Biosciences), anti-CD95 phycoerythrin (PE)-Cy5 (clone DX2; BioLegend), anti-CCR7 (clone 3D12; BD Biosciences), anti-CCR5 (clone 3A9; BD Biosciences), anti-CD28 ECD (clone 28.2; Beckman Coulter), anti-CD28 PE-Cy7 (clone 28.2; eBioscience), anti-CD16 Alexa 700 (clone 3G8; BioLegend), anti-CD56 QDot 605 (clone MEM-188; Invitrogen), anti-CD20 eFluor 650 (clone 2H7; eBioscience), anti-CD62L PE (clone Sk11; BD Biosciences), anti-interleukin-2 (IL-2) (clone RAT; Life Technologies), anti-MIP-1α PE (clone D21; Fisher), anti-MIP-1β PE (clone 11A3; Fisher), gamma interferon (IFN-γ) APC (clone B27; BD Pharmingen), and tumor necrosis factor alpha (TNF-α) Alexa 700 (clone MAb 11; BD Pharmingen). Samples assessed for Ki-67 expression were surface stained first with the appropriate antibodies and then fixed and permeabilized using BD Perm 2 (BD Pharmingen) and stained intracellularly with Ki-67. Cytokine staining was performed on frozen PBMC after stimulation with pools of 15-mer peptides spanning the sequences of three major antigenic proteins of SIV$_{mac239}$ (gag, pol, and env) as described previously (16). Flow cytometry data were acquired and samples were analyzed on an LSRII flow cytometer driven by the BD FACSDiva software package (version 6.1.3; BD Biosciences). Analyses of the acquired data were performed using FlowJo software (version 9.6.3; Tree Star). Further statistical analyses were performed using Prism 5.0 and Excel (Microsoft Office 2011) software.

Plasma viral loads. The quantitative real-time reverse transcriptase PCR (RT-PCR) assay to determine SIV$_{mac239}$ plasma viral load was performed as previously described (17).

Sorting of CD4$^+$ T lymphocyte subsets. Freshly isolated PBMC were stained with anti-CD4 Pacific blue (clone OKT4; BioLegend), anti-CD3 APC-Cy7 (clone SP34-2; BD Biosciences), anti-CD95 PE-Cy5 (clone DX2; BioLegend), anti-CD28 PE-Cy7 (clone 28.2; eBioscience), and anti-CD62L PE (clone Sk11; BD Biosciences). CD3$^+$ CD4$^+$ T lymphocytes were characterized as either naive (T$_N$; CD28$^+$ CD95$^-$ CD62L$^+$), central memory (T$_{CM}$; CD28$^{+/-}$ CD95$^+$ CD62L$^+$), or effector memory (T$_{EM}$; CD28$^{+/-}$ CD95$^+$ CD62L$^-$) and sorted on a FACSAria III cell sorter (BD Biosciences).

Quantitative PCR for SIV *gag* DNA. Quantification of SIV$_{mac}$ *gag* DNA from sorted naive, central memory, and effector memory CD4$^+$ T lymphocytes was performed as previously described (18). Simultaneous

PCR was performed for monkey albumin gene copy number and used to determine cell number quantifications. Samples with undetectable SIV DNA were assigned a level of half of the lower limit of detection for graphical purposes and statistical analysis.

SIV *in situ* hybridization and quantitative image analysis. SIV *in situ* hybridization was performed on 5-μm tissue sections with SIV-digoxigenin-labeled antisense riboprobes as previously described (19). Quantification of SIV RNA-positive cells was performed by counting positive cells detected by nitrotetrazolium blue–5-bromo-4-chloro-3-indolylphosphate (purple-black color) in 10 randomly selected fields totaling a minimum of 120 mm^2.

Statistical analyses. Statistical analyses were conducted using Graph-Pad Prism 5.0. Kruskal-Wallis one-way analysis of variance tests were performed to determine differences between groups in all of the tissues (see Fig. 2). Mann-Whitney U tests were used to perform analyses between pre- and postdepletion frequencies (see Fig. 2, 4, and 5). Spearman rank correlation tests were used to analyze all correlations (see Fig. 2, 3, and 4). All P values less than 0.05 were defined as significant.

RESULTS

Experimental design. To investigate the *in vivo* role of CD8$^+$ T lymphocytes as mediators of antiviral immune responses during SIV$_{mac239}$ infection of RMs, we depleted these cells from a cohort of 15 adult Indian RMs. All animals were infected intravenously with 3,000 TCID$_{50}$ of SIV$_{mac239}$, and CD8$^+$ T lymphocytes were depleted by i.v. administration of the primatized monoclonal antibody M-T807R1 at day 70 postinfection. Ten RMs were defined as progressors, with high viremia at the time of CD8 depletion, and 5 RMs were defined as controllers due to low viral loads predepletion. The study also included three control SIV-infected, mock-depleted animals. In all animals, several tissues, including blood, bone marrow (BM), lymph nodes (LN), and rectal mucosa via rectal biopsy specimen (RB), were sampled at multiple time points throughout the study (Fig. 1A). The SIV-infected CD8-depleted RMs then underwent selective necropsy at days 3 to 14 after depletion to extensively investigate the effect of this procedure in various lymphoid tissues and cell subsets. As shown in Fig. 1B and C for two representative RMs and for the whole group of animals, respectively, treatment with M-T807R1 resulted in depletion of >99% of circulating CD8$^+$ T lymphocytes. As expected based on previous studies, the level of depletion was lower in tissues than in peripheral blood and ranged between 50 and 70% in lymph nodes and between 86 and 96% in the rectal mucosa (data not shown). Depletion of CD8$^+$ lymphocytes is not complete in all tissues, possibly due to the differential rate of clearance of cells (20). However, since the CD8 molecule is critical for the function of MHC class I recognition, CD8$^+$ lymphocytes are functionally impaired following binding of the depleting CD8 antibody (21). In addition to depleting CD8$^+$ T lymphocytes, administration of CD8-depleting antibody also depletes NK and NKT cells that express high levels of the CD8 molecule (12).

CD8$^+$ T lymphocyte depletion results in a greater increase in SIV viremia in controllers than in progressors. The 15 SIV-infected RMs that were part of this study included 10 normal progressors, with viral loads prior to CD8$^+$ T lymphocyte depletion ranging between 22,200 and 17,200,000 SIV RNA copies/ml of plasma, and five controllers, with viral loads prior to CD8$^+$ T lymphocyte depletion ranging between 50 and 1,300 SIV RNA copies/ml of plasma (Fig. 2A). Consistently with previous studies, treatment with the CD8-depleting antibody resulted in an increase in viral loads in both groups of animals, while, as expected, no

change in the level of viremia was observed in the three animals that were mock depleted. Interestingly, the increase in viral load was more pronounced in the group of controllers than in the progressor RMs, with an average fold increase of 154 in progressors and 5,018 in controllers. At necropsy, however, the viral load of progressor RMs remained higher than that of controllers ($P = 0.0013$) (Fig. 2A). In addition, as shown in Fig. 2B, we found a significant inverse correlation between baseline viremia and the fold change in viremia after CD8 depletion ($P = 0.0004$). Overall, these data are consistent with the established hypothesis that CD8$^+$ T lymphocytes exhibit better suppression of virus replication in controller SIV-infected RMs than in progressors.

CD8$^+$ T lymphocyte depletion results in a homogenous increase in virus production within lymphoid tissues. While several studies have shown that CD8$^+$ T lymphocyte depletion in SIV-infected RMs results in increased virus replication (6–9), very few data are available with respect to the specific effect of this treatment in different anatomic sites. In this study, we examined the levels of SIV RNA in five different tissues at necropsy, including mesenteric LN, superficial LN, lamina propria of the rectum, lymphoid aggregates in the rectum, and the spleen. As these tissues could not be collected longitudinally in the animals, we used the three mock-depleted animals as controls. As shown in Fig. 2C, the levels of SIV RNA were consistently higher in CD8-depleted progressor RMs than in mock-depleted animals, and this effect was most pronounced in the spleen, mesenteric LN, and lymphoid aggregates of the rectum. Controller RMs also exhibited a nonsignificant trend toward higher levels of virus replication than mock-depleted animals. Interestingly, the levels of SIV RNA after CD8 depletion were similar between controller and progressor RMs in lamina propria of the rectum, while higher levels of virus replication were seen at all other sites examined in progressors compared to controllers. Of note, SIV RNA was found in both follicles and paracortex (i.e., T lymphocyte area) in the lymph nodes of CD8$^+$ lymphocyte-depleted controller RMs, similar to the pattern observed in CD8$^+$ lymphocyte-depleted progressors (data not shown). This observation is consistent with two recent studies linking the controller status with a compartmentalization of SIV in the lymph node follicles (22, 23). Overall, these histological data suggest that CD8$^+$ T lymphocyte depletion is followed by a diffuse increase of SIV replication in several lymphoid organs and tissues.

T-bet expression in CD8$^+$ T lymphocytes is the best correlate of the level of viral load postdepletion. Relatively little is known regarding the specific feature(s) of total and/or SIV-specific CD8$^+$ T lymphocyte responses that best correlate with the level of viral rebound after CD8 depletion. To address this issue, we examined a large number of CD8$^+$ T lymphocyte features in our cohort of SIV-infected progressor and controller RMs and investigated any potential correlation with the observed changes in viremia postdepletion in blood and tissues Specifically, we measured the level of Gag-specific cells by tetramer staining in blood, lymph nodes, and rectum; levels of cells producing IFN-γ, TNF, IL-2, or MIP-1β after Gag-peptide stimulation; and the level of CD8$^+$ T lymphocytes expressing Ki-67, T-bet, or Eomesodermin. As shown in Fig. 3, the only significant correlation that we observed was between the levels of T-bet expression in CD8$^+$ T lymphocytes isolated from both lymph nodes and rectal biopsies before CD8$^+$ T lymphocyte depletion and the level of plasma viremia after depletion (Fig. 3A and B). The list of CD8$^+$ T lymphocyte features examined
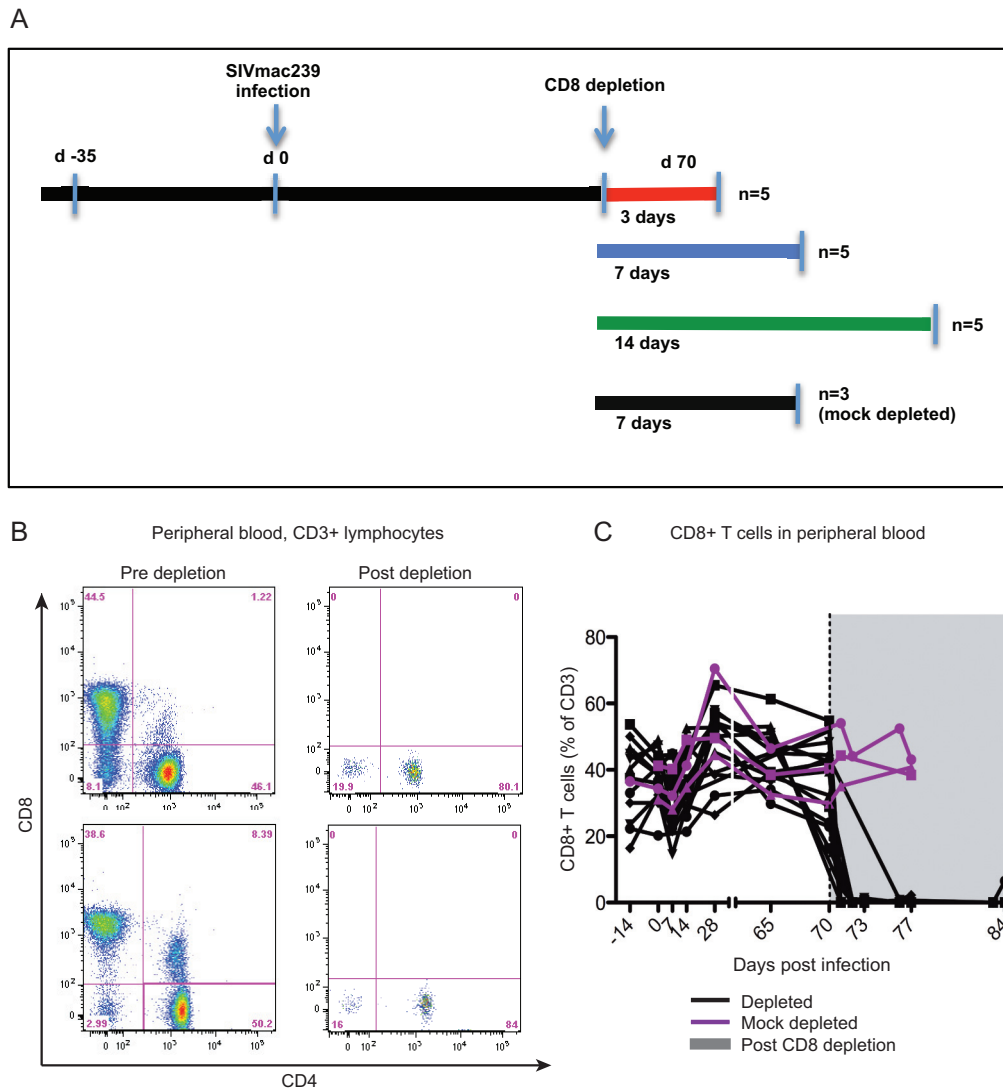
FIG 1 Experimental design and effective depletion of CD8$^+$ T lymphocytes. (A) Study design depicting timeline of SIV infection, antibody-mediated CD8 depletion, and necropsy. Eighteen female rhesus macaques were intravenously infected with 3,000 TCID$_{50}$ of SIV$_{mac239}$. CD8-depleting antibody (M-T807R1) was administered at 70 days postinfection to 15 RMs. Five CD8-depleted animals each were necropsied 3, 7, and 14 days postdepletion. Three RMs were mock depleted using primatized control IgG1 antibody at 70 days postinfection and necropsied 7 days after mock depletion. (B) Representative flow cytometry plots of live CD3$^+$ T lymphocytes isolated from peripheral blood depicting CD8$^+$ T lymphocyte levels predepletion (5 days before depletion) and postdepletion (6 days after depletion). (C) Longitudinal assessment of CD8$^+$ T lymphocyte frequencies (as a percentage of CD3$^+$ T cells) in peripheral blood for all experimentally depleted (black; $n = 15$) and mock-depleted (purple; $n = 3$) animals at pre- and postdepletion (shaded gray) time points. The dotted black line indicates the administration of depleting antibody.

before CD8$^+$ T lymphocyte depletion that did not correlate with the level of viremia postdepletion is shown in Fig. 3E. Taken together, these data suggest a role for the expression of the transcription factor T-bet in CD8$^+$ T lymphocytes of SIV-infected RMs as a predictor of the increase in viremia that follows CD8$^+$ T lymphocyte depletion in these animals. Since the CD8-depleting antibody also leads to depletion of NK cells, we assessed expression of various immunological markers on CD3$^-$ CD8$^+$ NK cells. We found that both T-bet expression on NK cells in lymph nodes and Ki-67 expression on NK cells derived from whole blood correlated with postdepletion viral load (Fig. 3C and D). No other correlations between markers of NK cell activation/differentiation and postdepletion viral load were found (Fig. 3F).

SIV DNA in CD4$^+$ T$_N$, T$_{CM}$, and T$_{EM}$ increases postdepletion in progressor RMs but not in controllers. HIV/SIV replication occurs at different levels in specific CD4$^+$ T lymphocyte subsets (24, 25). Relatively little is known, however, about the role played by CD8$^+$ T lymphocytes in controlling virus replication in the subsets of naive, central memory, and effector memory CD4$^+$ T lymphocytes (T$_N$, T$_{CM}$, and T$_{EM}$, respectively). To address this question, we sorted CD4$^+$ T$_N$, T$_{CM}$, and T$_{EM}$, as determined based on the expression of CD28, CD95, and CD62L, before and after CD8$^+$ T lymphocyte depletion in the 15 SIV-infected RMs included in this study, and measured the level of cell-associated SIV DNA by RT-PCR. As shown in Fig. 4A, we found that the levels of SIV DNA increased almost uniformly in CD4$^+$ T$_N$ (9/10 animals), T$_{CM}$ (10/10), and T$_{EM}$ (10/10) sorted from progressors, while in
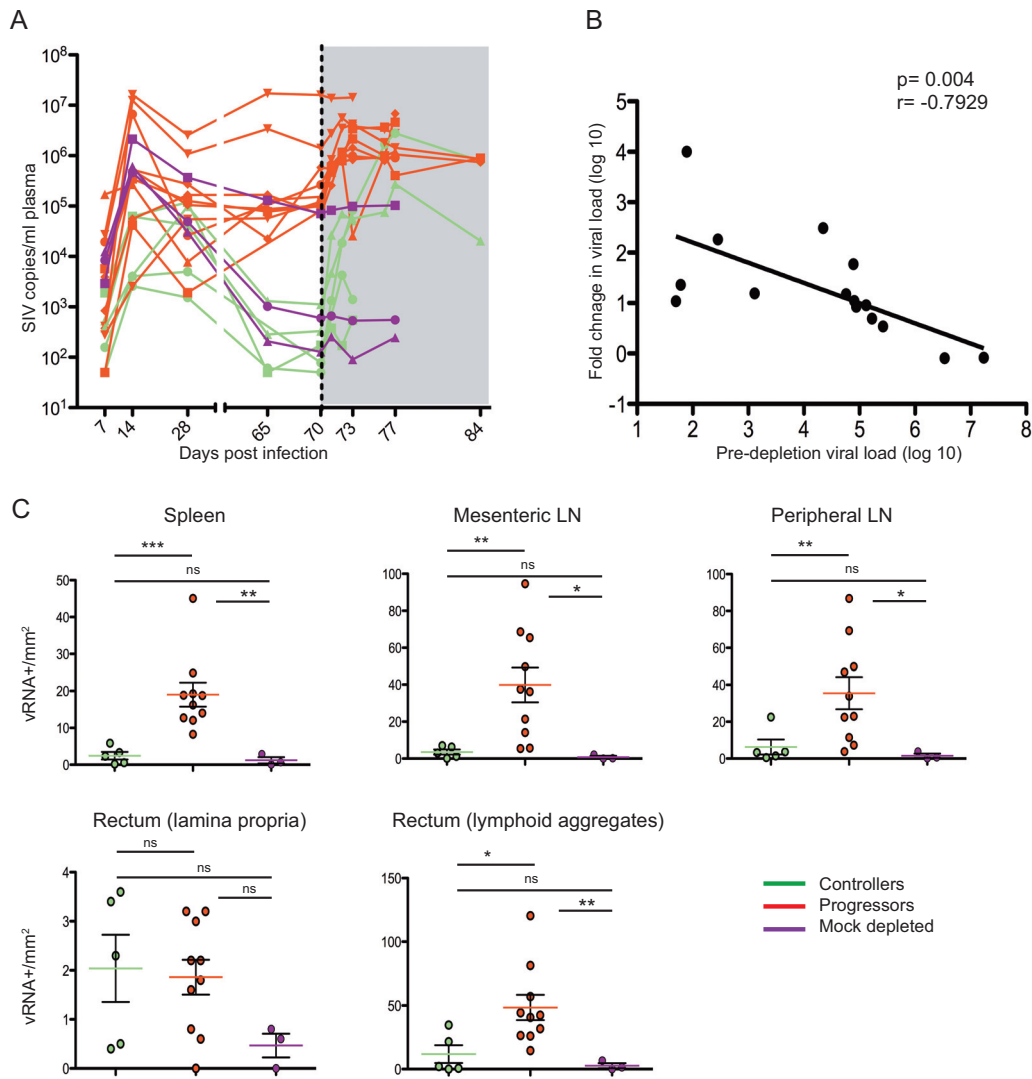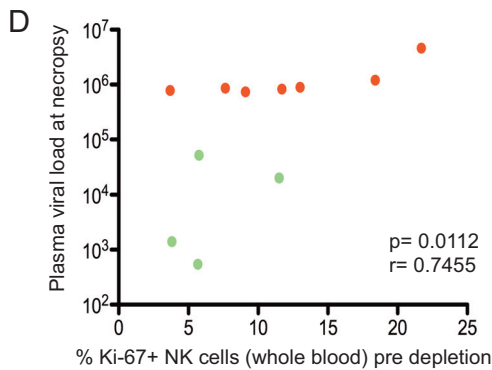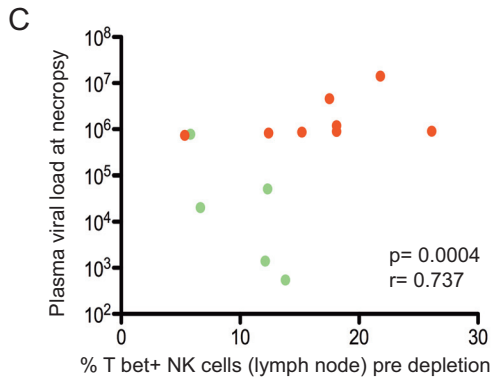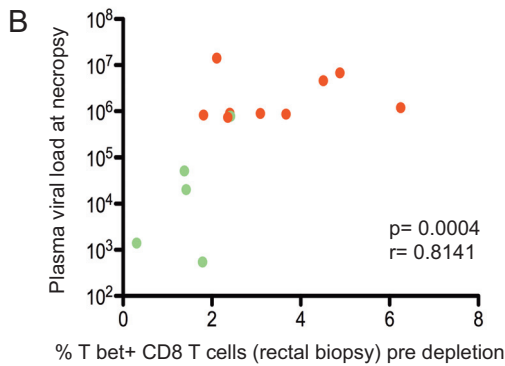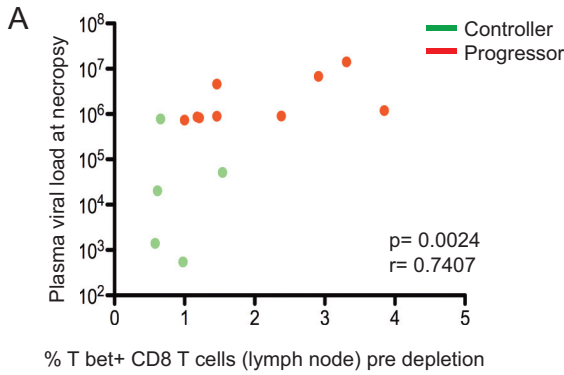
FIG 2 Plasma and tissue viral loads following CD8$^+$ T lymphocyte depletion. Plasma viral load measured longitudinally for each animal. The dotted black line indicates the administration of depleting antibody. The shaded gray area indicates postdepletion time points. (B) Correlation between fold change in viral load (viral load postdepletion/viral load predepletion) with predepletion viral load for all experimentally depleted animals. (C) Number of SIV RNA$^+$ cells within tissues of CD8-depleted controllers ($n = 5$), progressors ($n = 10$), and mock-depleted animals ($n = 3$). Statistical analyses were performed using Spearman rank correlation tests, Kruskal-Wallis one-way analysis of variance, and Mann-Whitney U tests.

controllers the levels of SIV DNA increased in CD4$^+$ T$_{EM}$ of 4/5 RMs but in only 2/5 and 1/5 RMs for the T$_N$ and T$_{CM}$ subsets, respectively. Of note, we found a significant correlation between SIV plasma viremia and the levels of SIV DNA in CD4$^+$ T$_{EM}$ before and after CD8$^+$ T lymphocyte depletion (Fig. 4B and C) and between plasma viremia and SIV DNA in CD4$^+$ T$_{CM}$ after CD8$^+$ depletion (Fig. 4E). However, no correlation was found between SIV viremia and SIV DNA levels in CD4$^+$ T$_{CM}$ before CD8$^+$ depletion (Fig. 4D). Overall, these data are consistent with the possibility that CD8$^+$ T lymphocyte-mediated control of infection operates through different mechanisms in specific CD4$^+$ T lymphocyte subsets.

CD8$^+$ lymphocyte depletion is associated with a greater increase in CD4$^+$ T cell activation in controllers than in progressors. The increase in virus replication that follows CD8$^+$ lymphocyte depletion in SIV-infected RMs may be, at least in part, the result of

increased levels of CD4$^+$ T cell activation that can be caused by factors such as the homeostatic response to CD8$^+$ T cell depletion, increased availability of proliferative cytokines, and reactivation of latent viruses, such as cytomegalovirus (CMV). To address the potential role of CD4$^+$ T cell activation in determining the levels of virus replication before and after CD8$^+$ lymphocyte depletion, we measured the expression of the proliferation marker Ki-67 in CD4$^+$ T$_N$, T$_{CM}$, and T$_{EM}$ isolated from the blood, lymph nodes, and recta (biopsy samples) of our cohort of 15 SIV-infected RMs (Fig. 5). As shown in Fig. 5, we found that Ki-67 expression remained stable overall in CD4$^+$ T cells of progressors (and, in fact, even decreased in LN-derived T$_{CM}$), consistent with the fact that, in these animals, the levels of CD4$^+$ T cell activation was already very high prior to CD8 depletion. In contrast, the level of Ki-67 expression increased in controllers, with a statistically significant difference for both T$_{CM}$ and T$_{EM}$ in peripheral blood and a similar, albeit

FIG 3 T-bet expression in CD8^{+} T lymphocytes is the best correlate of the level of viral load after CD8 depletion. Correlations between T-bet-expressing CD8^{+} T lymphocytes isolated predepletion from lymph nodes (A) and rectal biopsy specimens (B) with plasma viral load at necropsy. Correlations between T-bet expression in NK cells, defined as CD3^{−} CD16^{+} CD8^{+} cells, from lymph nodes (C) and Ki-67 expression in NK cells from whole blood (D) with plasma viral load at necropsy. (E and F) Levels of expression of several markers on CD8^{+} T lymphocytes and NK cells were assessed within peripheral blood, lymph node, and rectal mucosa. None were found to correlate with plasma viral loads after CD8^{+} T lymphocyte depletion. Controllers are shown in green, and progressors are in red. Spearman rank correlation tests were used to analyze all correlations.
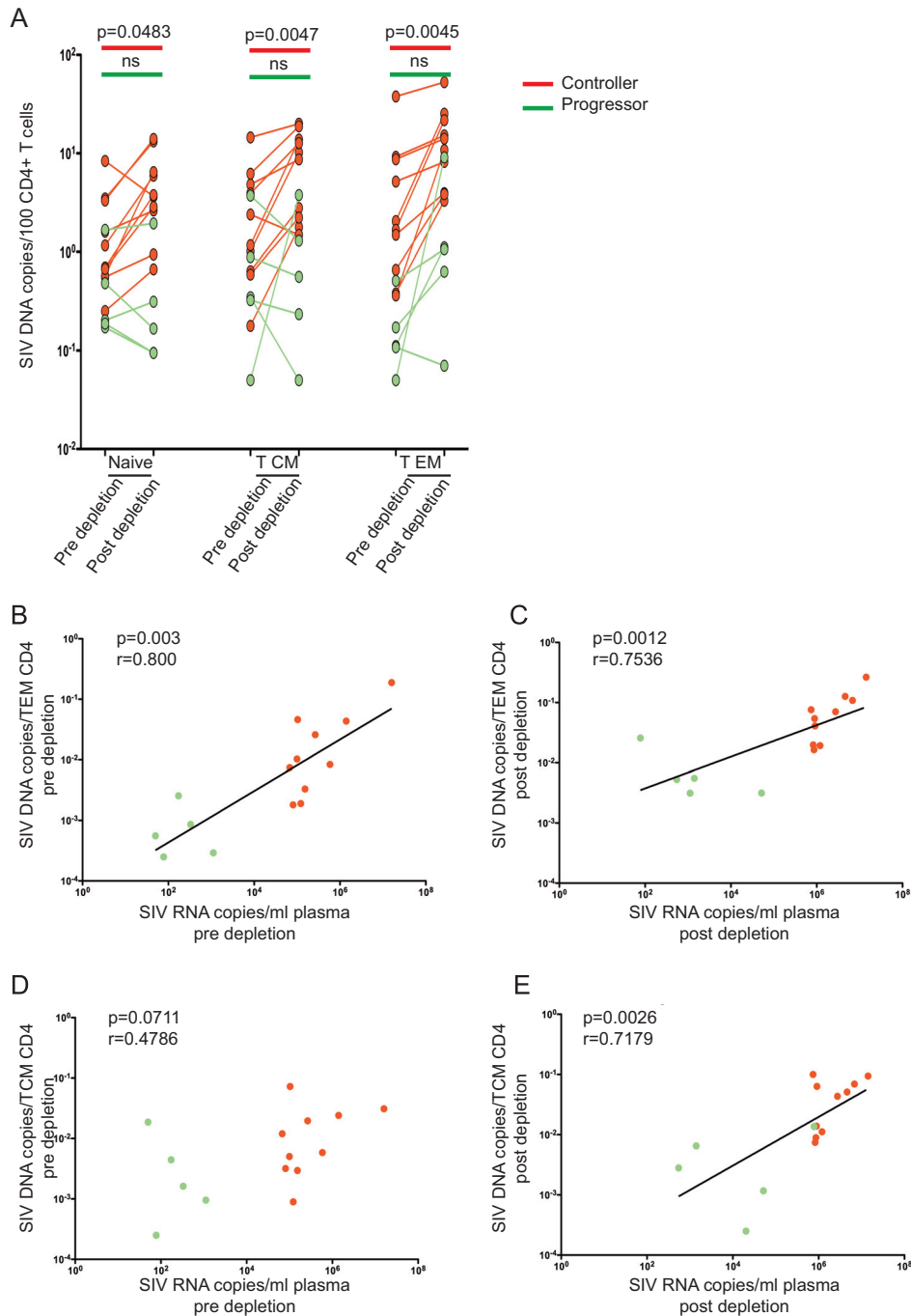
FIG 4 SIV DNA in CD4$^+$ T$_N$, T$_{CM}$, and T$_{EM}$ increases postdepletion in progressor but not controller RMs. (A) Comparison of cell-associated SIV DNA levels in naive, central memory, and effector memory CD4$^+$ T lymphocytes of controllers ($n = 5$) and progressors ($n = 10$) pre- and postdepletion. (B) Correlations between plasma viral load and cell-associated SIV DNA within T$_{EM}$ (B and C) and T$_{CM}$ (D and E) at predepletion and postdepletion time points. Statistical analyses were performed using Mann-Whitney U tests, and Spearman rank correlation tests were used to analyze all correlations. ns, not significant.

nonsignificant, trend in lymph node and rectal biopsy specimens. Overall, these data indicate that CD8$^+$ T lymphocyte depletion is followed by a more pronounced increase in CD4$^+$ T cell activation in controller SIV-infected RMs than in progressors.

DISCUSSION

There are many lines of scientific evidence supporting the role of CD8$^+$ T lymphocytes in controlling virus replication in the setting of HIV or SIV infection. Perhaps the most convincing observation is that *in vivo* experimental depletion of CD8$^+$ T cells in SIV-infected macaques is consistently followed by a significant increase in the level of virus replication. While this observation has been confirmed in numerous studies (8, 12), there are several aspects of this phenomenon that remain relatively poorly understood. For example, there is a lack of evidence regarding (i) the cellular and anatomic origin of the increased virus replication, (ii)
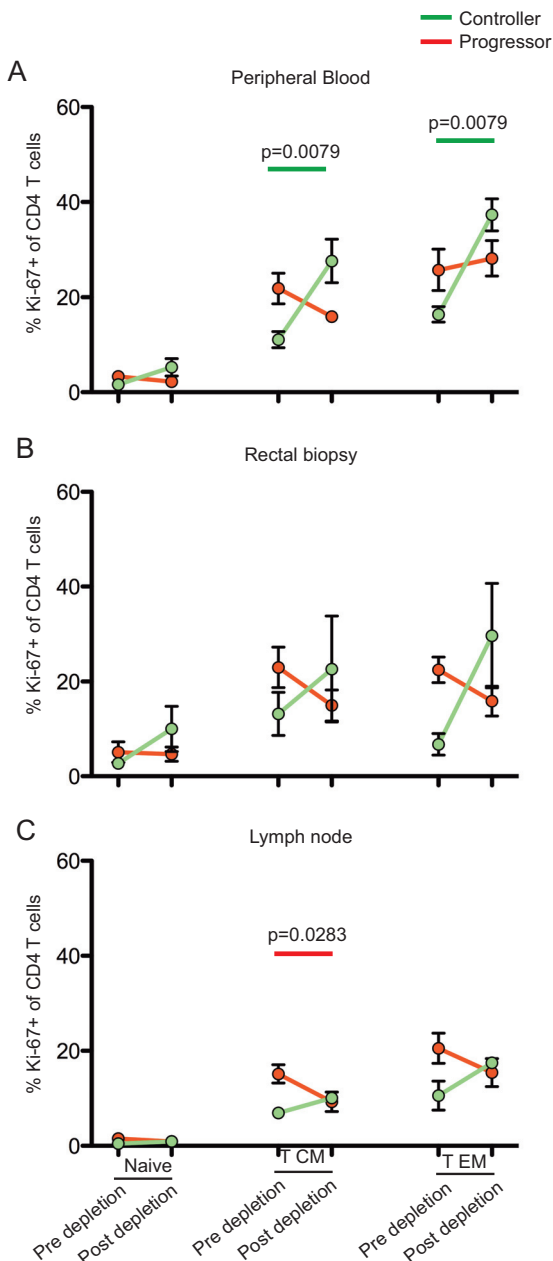
FIG 5 CD8$^+$ T lymphocyte depletion is associated with greater increases in CD4$^+$ T lymphocyte activation in controllers than in progressors. Comparative frequency of Ki-67$^+$ CD4$^+$ T lymphocytes within naive, central memory, and effector memory CD4$^+$ T lymphocytes pre- and postdepletion isolated from the peripheral blood (A), rectal mucosa (B), and lymph nodes (C) of progressors ($n = 10$) and controllers ($n = 5$). Statistical analyses were performed using Mann-Whitney U tests.

The main findings of this study are that (i) CD8$^+$ T lymphocyte depletion was followed by an increase in virus replication that was more prominent in SIV-infected controller than progressor RMs and was relatively homogenous in various lymphoid organs and tissues, (ii) the levels of T-bet expression in CD8$^+$ T lymphocytes prior to depletion was the best predictor of the magnitude of the increase of virus replication postdepletion, (iii) the levels of SIV DNA after CD8$^+$ T lymphocyte depletion increased in all CD4$^+$ T lymphocyte subsets (i.e., T$_N$, T$_{CM}$, and T$_{EM}$) in progressors but only in T$_{EM}$ in controllers, with a decline of SIV DNA in T$_{CM}$ of 4 out of 5 animals, and (iv) CD8$^+$ T lymphocyte depletion was associated with a greater increase in CD4$^+$ T lymphocyte activation in controllers than in progressors. While some of these results overall are confirmatory of previously published work by us and others, several of the above-described findings are novel and deserve particular attention, especially regarding the differential role of CD8$^+$ T lymphocytes in controller versus progressor SIV-infected RMs.

The observation that the increase in viral load following CD8$^+$ T lymphocyte depletion is more pronounced when virus replication is lower prior to depletion is consistent with what was observed in two previous studies (8, 10). In this study, however, we report for the first time a strong inverse correlation between predepletion viremia and fold change in viremia postdepletion. Overall, these data are quite consistent with the hypothesis that CD8$^+$ T lymphocytes are key contributors to the suppression of virus replication that is observed in HIV/SIV-infected elite controllers (both humans and rhesus macaques) and is associated with specific MHC class I alleles (4, 27). CD8$^+$ T lymphocytes contribute less to control in progressors and we see relatively little change in viral load in progressors following CD8$^+$ T lymphocyte depletion, as has been described before (28). Interestingly, a detailed histological analysis of several lymphoid tissues and organs revealed that the frequency of virus-producing cells (i.e., SIV RNA positive by *in situ* hybridization) increases after CD8$^+$ T lymphocyte depletion in a relatively uniform fashion in all examined tissues, with virus found in both B and T cell areas of lymph nodes in the CD8-depleted controllers. The latter observation is consistent with two recent studies showing a specific virus compartmentalization within lymph nodes of controller versus progressor SIV-infected RMs (22, 23).

While numerous studies have linked CD8$^+$ T lymphocyte depletion with increased virus replication (7–12), it is not clear what particular function or phenotypic marker of CD8$^+$ T lymphocytes is best correlated with this effect. In this study, we investigated potential correlations between a number of specific aspects of the CD8$^+$ T lymphocytes, both SIV specific and as bulk populations, prior to CD8$^+$ T lymphocyte depletion and the observed changes in viral load following depletion. We found that expression of the transcription factor T-bet in bulk CD8$^+$ T cells was the only predictor of the levels of viral load after CD8$^+$ T lymphocyte depletion. We did not find any correlation between the level of T-bet expression on SIV-specific CD8$^+$ T lymphocytes and plasma viral load increase. This is a novel finding that is consistent with a possible role of T-bet as a transcription factor that promotes effector CD8$^+$ T lymphocyte functions, including cytotoxic activity and production of multiple cytokines (29, 30), and that represses the expression of the inhibitory receptor PD-1, which suppresses many CD8$^+$ T lymphocyte functions (31), and it has been associated with *in vivo* control of virus replication in HIV-infected hu-

the contribution of cytolytic versus noncytolytic mechanisms of virus control (13, 15), (iii) the role played by CD8$^+$ T lymphocytes versus CD8$^+$ NK cells, which also are depleted by this treatment, and (iv) the contribution of the enhanced activation of CD4$^+$ T lymphocytes that follows CD8$^+$ T lymphocyte depletion in promoting higher levels of virus replication (11, 26). The current study was aimed at providing some insights into these poorly understood aspects of the antiviral role of CD8$^+$ T lymphocytes during HIV/SIV infections.

mans (32, 33). The high predepletion levels of T-bet expression in SIV-infected progressors may reflect the high levels of activation and antigenic load which resulted from the poor control of virus occurring in progressors. Further studies involving *in vivo* blockade of T-bet-expressing $CD8^+$ T lymphocytes would be needed to further delineate the role of T-bet in controlling virus replication in SIV-infected RMs, particularly with respect to whether the levels of T-bet expression in $CD8^+$ T lymphocytes represent a cause or a consequence of prevailing levels of virus replication.

An intriguing novel finding of this study is that the impacts of $CD8^+$ T lymphocyte depletion on the relative proportion of SIV DNA-positive cells within the memory subsets of $CD4^+$ T lymphocytes (T_{CM} and T_{EM}) appear to be different in SIV-infected progressor and controller RMs. As expected, CD8 depletion resulted in increased levels of viral DNA in all $CD4^+$ T lymphocyte compartments of progressors. However, in CD8-depleted controllers, the level of SIV DNA increased only in T_{EM}; in fact, it decreased in T_{CM} of 4 out of 5 animals. Remarkably, this decline of the frequency of SIV-infected $CD4^+$ T_{CM} occurred concomitantly with an increase in plasma viremia. Importantly, this decline in SIV DNA in $CD4^+$ T_{CM} does not appear to be due to trafficking of this cell subset into lymph nodes or the gastrointestinal tract, as the same decline was seen at these sites as well as in peripheral blood. At this time we have not identified a mechanism for this surprising finding, but it is tempting to speculate that, in CD8-depleted SIV-infected controllers, a substantial proportion of SIV-infected T_{CM} differentiated to become T_{EM}, perhaps as a result of the increased $CD4^+$ T lymphocyte activation, or was killed by the reactivated virus in the absence of $CD8^+$ T lymphocytes. The latter possibility would be consistent with the proposed hypothesis that $CD8^+$ T lymphocytes suppress HIV/SIV replication through noncytolytic mechanisms that act at the level of provirus transcription (34).

Several studies have shown that $CD8^+$ T lymphocyte depletion is followed by an increased fraction of activated/proliferating $CD4^+$ T lymphocytes (11, 26). While the relative contribution of this phenomenon to the observed increase in viremia after $CD8^+$ depletion remains unclear, two findings suggest that its role is in fact minor. First, the increased level of $CD4^+$ T lymphocyte activation is observed for the most part at a time when the increase in virus replication has already occurred (12). Second, when the increase of $CD4^+$ T lymphocyte activation following $CD8^+$ T lymphocyte depletion is abrogated through blockade of IL-15 signaling, the increase in SIV replication still is present (11). In the current study, we found that the increase of $CD4^+$ T lymphocyte activation, as measured by the proliferation marker Ki-67, was more pronounced in CD8-depleted controllers than progressors, indicating that, in SIV-infected RMs with high virus replication prior to $CD8^+$ T lymphocyte depletion, a maximal level of $CD4^+$ T lymphocyte activation was present already in the immune system of these animals.

Overall, the current set of results indicates that experimental depletion of $CD8^+$ T lymphocytes during SIV infection of macaques remains a valuable research tool to investigate the mechanisms by which these cells suppress virus replication *in vivo*. Specifically, we have identified a series of differences between SIV-infected progressor and controller RMs with respect to the impact of CD8 depletion on the virology and immunology of SIV infection that provides novel insights into the *in vivo* function of these cells. Ultimately, a better understanding of the mechanisms re-

sponsible for the $CD8^+$ T lymphocyte-mediated protection from HIV/SIV replication may result in interventions that most effectively harness these antiviral functions in the setting of prophylactic and therapeutic vaccines for HIV/AIDS.

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