



Published in final edited form as:

J Immunol. 2009 October 15; 183(8): 5006–5012. doi:10.4049/jimmunol.0900141.

CD8+ cell depletion of SHIV89.6P-infected macaques induces CD4 + T cell proliferation that contributes to increased viral loads

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Abstract

Previous studies have shown that depletion of CD8+ cells during acute and chronic simian immunodeficiency virus (SIV) infection leads to increased viral replication, morbidity and mortality which have been attributed to loss of CD8+ T cell-mediated control of SIV virus. However, these studies did not exclude that CD8+ cell depletion increased homeostatic proliferation of CD4+ T cells, resulting in increased viral targets and therefore viral rebound. Chronically SHIV89.6P-infected cynomolgus macaques were CD8+ cell-depleted and frequencies, cell numbers and phenotype of CD4+ T cells and viral infection were examined using flow cytometry and quantitative real-time PCR. Frequencies and numbers of Ki-67-expressing CD4+ T cells were increased with CD8+ cell depletion. This proliferation of CD4+ T cells occurred even in animals with no rebound of viral loads. Most of the proliferating cells were effector memory CD4+ T cells. Plasma SHIV RNA copies positively correlated with proliferating CD4+ T cells and SHIV DNA copies in Ki-67+ CD4+ T cells. Although this study does not exclude an important role for virus-specific CD8+ T cells in SIV and SHIV infection, our data suggest that homeostatic proliferation is an important contributor to increases in plasma viremia that follow CD8+ cell depletion.

Keywords

T cells; Immunodeficiency Diseases; AIDS; other animals; cell proliferation

Introduction

Although early studies indirectly indicated that CD8+ T cells are involved in controlling HIV infection (summarized in (1)), the strongest direct evidence supporting the important role of CD8+ T cells in controlling both acute and chronic HIV infection has come from a large number of studies examining the effect of CD8+ cell depletion in SIV-infected non-human primates (2–13). These studies have been interpreted to demonstrate that in vivo CD8+ T cells directly protect monkeys during SIV infection as depletion of CD8+ T cells by monoclonal antibodies

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results in rapid increase of viremia, disease progression and reduced survival of animals. One caveat to all these studies however is that depletion of CD8⁺ T cells may perturb homeostasis of other cells and in particular CD4⁺ T cells and therefore the increases in viral load may, at least in part, be due to increased homeostatic proliferation of CD4⁺ T cells and augmented SIV target generation in these animals.

The total numbers of peripheral T cells are constantly maintained due to a balance between death and homeostatic survival and proliferation in the periphery. Naïve CD4⁺ T cells seem to require low-affinity MHC/self-peptide contact and IL-7 whereas long-term survival of memory CD4⁺ T cell seems to be MHC class II independent (summarized in (14)). T cell compartments sense each others size changes most likely due to the common dependency on γ chain cytokines such as IL-7 and IL-15, underscoring the crucial role these cytokines may play for homeostatic proliferation and survival of CD4⁺ and CD8⁺ T cells (14,15). Constant T cell numbers are maintained only on the level of total T cell numbers independent of CD4 or CD8 phenotype, therefore compensatory homeostasis leads to expansion of the reciprocal T cell subsets in CD4⁺ or CD8⁺ T cell depleted mice (16–18) and in humans with decline in CD4⁺ T cells due to HIV infection and chemotherapy (19,20).

Activated CD4⁺ T cells and resting effector memory CD4⁺ T cells are the major sources of progeny virus during acute SIV infection (21–23). At the chronic phase of SIV infection greater than 75% of infected cells are activated CD4⁺ T cells (24). Activated cells are much more efficient at producing virions (23) and are most likely the major source of new virus (25). If CD8⁺ cell depletion alters CD4⁺ T cell homeostasis and therefore activation and proliferation of CD4⁺ T cells this could have an impact on the availability of targets for SIV.

Indeed, we show in this study that CD8⁺ cell depletion results in proliferation of effector memory CD4⁺ T cells, the main target cell population for SIV and possible SHIV89.6P infection. Furthermore, we show a direct correlation between proliferating Ki-67⁺ CD4⁺ T cells and plasma viral loads and also a correlation between frequency of SHIV-infected proliferating CD4⁺ T cells and plasma viral load. This proliferation is not secondary to rebound viral replication-induced activation, as it occurs even in the absence of rebound viremia in CD8⁺ cell depleted animals. Our findings suggest that homeostatic proliferation of CD4⁺ T cells contributes to the increased plasma viremia found in CD8⁺ cell depleted SIV and SHIV-infected macaques. Our studies also underscore the role CD4⁺ T cell homeostatic proliferation may play in viral replication and viral burden in HIV-infected individuals.

Materials and Methods

Animals

Cynomologous macaques were housed at the Bioqual animal facility according to standards and guidelines as set forth in Animal Welfare Act and “The Guide for the Care and Use of Laboratory Animals,” (ILAR, 1996), and according to animal care standards deemed acceptable by the Association for the Assessment and Accreditation of Laboratory Animal Care-International (AAALAC). All experiments were performed following institutional animal care and use committee (IACUC) approval. Macaques were immunized with recombinant DNA vaccine and IL-15 immune adjuvant and challenged with SHIV89.6P two years ago as previously described (26). SHIV RNA levels were measured using SIV gag primers in a quantitative RNA RT-PCR assay (limit of sensitivity 50 SIV RNA copies/ml), which was run on a ABI 7500 Real time PCR cycler (Applied Biosystems, Foster City, CA).

CD8+ cell depletion

Macaques were treated with the CD8+ cell-depleting antibody cM-T807 (provided by the NIH Nonhuman Primate Reagent Resource Center) on day 0 subcutaneous (10 mg/kg) and on days 3, 7 and 10 intravenous (5 mg/kg).

Flow Cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood within 4 hours after blood collection by density centrifugation (Percoll, 1.075 g/ml, Amersham Biosciences, Uppsala, Sweden). Cells from mesenteric lymph nodes (MLN) and peripheral lymph nodes (PLN) were isolated by first mincing the tissues and then incubating in digestion media (0.15 mg/ml DNase (Roche Applied Science, Indianapolis, IN), 3 mg/ml Collagenase A (Roche Applied Science)). Cells were directly stained ex vivo with previously determined appropriate amount of antibodies for 30 min at 4°C and fixed with 1% paraformaldehyde solution as previously described (27,28). For intracellular anti-Ki-67 staining, cells were first surface stained, then fixed/permeabilized (cytofix-cytoperm, BD Biosciences, San Jose, CA) and stained with anti-Ki-67 antibodies or isotype control for 60 min at 4°C. At least 200,000 events were collected per sample on a FACS Aria flow cytometry instrument (BD Biosciences) and analyzed using FlowJo software (Treestar, San Carlos, CA). The following fluorochrome conjugated anti-human monoclonal antibodies that cross-react with cynomolgus macaque antigens were used: anti-CD3 (clone SP34, BD Biosciences), anti-CD4 (L200, BD Biosciences), anti-CD8 (DK25, Dako, Carpinteria, CA), anti-CD45RA (5H9, Invitrogen, Carlsbad, CA), anti-CD62L (SK11, BD Biosciences), anti-Ki-67 (B56, BD Biosciences), and anti-CCR5 (CD195, 3A9, BD Biosciences).

qPCR assay for SIV-gag DNA

For analysis of viral DNA, SIV-gag based assays were used since SHIV-89.6P is composed of the SIVmac239 with the HIV-1 derived auxiliary genes *tat*, *vpu*, and *rev* and the *env* gene derived from HIV-1 89.6 (29). SIV gag DNA was determined in triplicates in sorted Ki-67+ and Ki-67- CD4+ T cells by quantitative real-time PCR method (21). To measure T cell-associated viral DNA, SIV gag primers and probe and a Perkin-Elmer ABI 7700 instrument was used as previously described (6,21,30). The assay was calibrated using a cell line that carried a single copy of proviral SIV DNA as described previously (21).

Statistical Analysis

Statistical analysis was performed using Mann-Whitney U test, Student's t-Test, nonparametric Wilcoxon signed-rank test for paired samples and Shapiro-Wilk W test for normality. P values < 0.05 were considered significant. The JMP statistical analysis program was used (SAS, Cary, NC).

Results

CD4+ T cell numbers are not altered during CD8+ cell depletion

In this study we treated DNA-vaccinated cynomolgus macaques, which were challenged with SHIV89.6P two years ago (26), with depleting anti-CD8 monoclonal antibodies (clone cM-T807) for 10 days. CD8+ T cell numbers were already greatly reduced from peripheral blood of animals by day 3 of CD8 depletion (figure 1A) and slowly recovered after day 14. Since the anti-CD8 monoclonal antibody clone cM-T807 depletes not only CD8+ T cells but also CD3-CD8+ Natural Killer cells (31), the frequency and absolute CD3-CD8+ NK cells were also significantly reduced at day 10 compared to day 0 and reached pre-treatment levels on day 21 (data not shown). Viral replication was undetectable pre-depletion in the peripheral blood of these vaccinated animals, however, viral rebound was observed in 6/21 animals at day 3 and

in 13/21 animals on day 7 with the highest frequency of animals with detectable viral replication on day 14 (71%, 15/21). As CD8+ T cells re-appear, the frequency of animals with viremia decreased to 58% (7/12) on day 21 and to only 19% (4/21) on day 23 (figure 1B). We also examined whether depletion of CD8+ cells altered the absolute CD4+ T cell numbers in these animals. No significant changes were observed for CD4+ T cell numbers before, during and after CD8+ cell depletion (figure 1C).

Proliferating Ki-67-expressing CD4+ T cells are increased during CD8+ cell depletion

Although CD4+ T cell counts were stable in peripheral blood of CD8+ cell depleted animals, this does not exclude proliferation and re-distribution. We therefore analyzed the expression of Ki-67, a proliferation marker, in CD4+ T cells. Indeed, we found a significant increase in proliferating Ki-67+ CD4+ T cells during CD8+ cell depletion in these animals (figure 2A, B). The frequency of Ki-67+ CD4+ T cells increased by 2.2 ± 0.3 fold from day 0 to day 7 and by 2.9 ± 0.3 fold from day 0 to day 10 and remained high on days 21 and 31 (figure 2A, B). The frequency of Ki-67+ CD4+ T cells was significantly lower in both peripheral lymph nodes (PLN) and mesenteric lymph nodes (MLN) compared to peripheral blood at day 31, the time of necropsy (figure 2B). When absolute Ki-67+ CD4+ T cell numbers were calculated in the peripheral blood, significant increases were found when day 0 was compared with day 7 (4.8 ± 1.2 fold increase, $p < 0.05$), day 10 (2.9 ± 0.4 fold increase, $p < 0.001$) and day 21 (3.6 ± 0.6 fold increase, $p < 0.001$) (figure 2C).

Proliferation of CD4+ T cells in CD8+ cell depleted animals is independent of viral rebound

Next we examined whether proliferation of CD4+ T cells in these animals was dependent on viral rebound. Five of the CD8+ cell depleted animals did not show rebound of viral replication (figure 3A). In these animals, the frequency of Ki-67+ CD4+ T cells nevertheless increased from $9.0 \pm 1.1\%$ on day 0 to $24 \pm 6.1\%$ on day 10 (figure 3A). When the frequencies of Ki-67+ CD4+ T cells on day 10 were compared between animals without rebounded viremia ($n=5$) and viremia ($n=16$) during CD8+ cell depletion, comparable frequencies were found (Figure 3B). These findings indicate that CD4+ T cell proliferation in these CD8+ cell depleted animals is independent of rebound in viral replication.

Viral load correlates with frequency of Ki-67+ CD4+ T cells and SHIV-infected Ki-67+ but not Ki-67-CD4+ T cells

Increased proliferating Ki-67+ CD4+ T cells at effector sites may result in increased plasma viremia, since the replication of SIV in Ki-67+ CD4+ T cells is four to seven times higher than in Ki-67- CD4+ T cells (24). This high replication in Ki-67+ CD4+ T cells could contribute to the increase in viral load in CD8-depleted animals we and others have seen (3–6,8–13). To test whether proliferation of CD4+ T cells and viral replication is directly related, we sorted Ki-67+ CD4+ T cells and Ki-67- CD4+ T cells and determined the SHIV DNA copy numbers in these cells on day 7. We found a significant correlation between day 7 plasma viral load and day 7 SHIV DNA copy numbers in Ki-67+ CD4+ T cells ($R^2 = 0.65$, $p = 0.016$) (figure 3C) but not Ki-67- CD4+ T cells ($R^2 = 0.32$, $p > 0.05$). This indicates that although both CD4+ T cell populations can be infected, proliferating CD4+ T cells are the source of rebound viremia, and this underscores the important effect homeostatic proliferation of CD4+ T cells may have on viral replication in these CD8+ cell-depleted animals. We also examined whether frequency of Ki-67+ CD4+ T cells and viral replication correlate during viremia. We analyzed animals with detectable viremia on days 3, 14 and 21. Significant positive correlation was found for all 3 time points (day 7: $R^2 = 0.39$, $p = 0.0035$, $n = 20$; day 21: $R^2 = 0.52$, $p = 0.0054$, $n = 13$ (figure 3D); day 10 frequency of Ki-67+ CD4+ T cells/day 14 viral loads: $R^2 = 0.4$, $p = 0.0005$, $n = 26$).

CD8+ cell depletion induces preferentially proliferation of effector memory CD4+ T cells

To further evaluate whether homeostatic proliferation of CD4+ T cells can enhance the SIV and SHIV target pool, we examined the effect this proliferation has on memory subpopulation distribution. We have shown that the total CD4+ T cell numbers do not change during CD8+ cell depletion (figure 1C), however, the composition of CD4+ T cells still could be altered with an important effect on SIV and SHIV replication as effector memory CD4+ T cells are important targets for SIV (21, 22) and R5X4 SHIV infections. Although the frequency of memory CD4+ T cell subpopulations remained relatively unchanged up to day 10 post-depletion (figure 4A), effector memory CD4+ T cells showed significantly increased frequency of Ki-67 expression on day 10 with the CD45RA+CD62L- effector memory CD4+ T cells showing the highest increase (4.5±0.8 fold) (Figure 4B, C). When we calculated absolute cell numbers in these subgroups, we found a 2.4±0.9 fold increase between day 0 and day 10 for Ki-67+CD45RA-CD62L- effector memory CD4+ T cells and a 5.8±0.5 fold increase for Ki-67+CD45RA+CD62L- effector memory CD4+ T cells (Figure 4D). These data indicate that proliferation of both effector memory CD4+ T cell subpopulations is significantly increased in CD8+ cell depleted animals and this suggest that CD8+ cell depletion indeed could increased the target pool for SIV and SHIV89.6P infection.

Discussion

Numerous studies examining the potential role of CD8+ T cells in controlling HIV and SIV infection have employed the depletion of CD8+ T cells from SIV-infected animals to demonstrate a rapid rebound or increase of viral replication (2–13). This effect of CD8+ cell depletion on viral replication has been interpreted to provide direct evidence that CD8+ T cells play a critical role in controlling SIV replication. This depletion however also affects CD3-CD8+ NK cells (31) and this may also contribute to viral control. Although cytotoxic CD8+ T cells undeniably play an important role in suppressing viral replication, the experiments employing CD8+ cell depletion suffer from one serious caveat. If depletion of CD8+ cells from the periphery creates homeostatic space that is sensed by CD4+ T cells, these cells may undergo homeostatic proliferation that provides targets for SIV infection and replication, and thus the viral rebound may not be exclusively due to loss of cytotoxic CD8+ T cell control, but also increases in viral targets. Indeed, in this study we show that depletion of CD8+ cells from cynomologous macaques results in rapid and massive proliferation of CD4+ T cells and this we believe raises the question whether targets are being generated by the ensuing homeostatic proliferation that follows CD8+ cell depletion.

The increased proliferation of CD4+ T cells in CD8+ cell depleted animals could be due to homeostatic proliferation or virus-induced activation and/or inflammation of CD4+ T cells. Importantly, we found an increase in Ki-67+ CD4+ T cells in a subgroup of animals without viral rebound. Also, frequencies of Ki-67+ CD4+ T cells were not different between animals with and without viral rebound. These findings suggest that this proliferation is independent of viral replication and virally induced activation or inflammation and that CD8+ cell depletion per se induces CD4+ T cell homeostatic proliferation. Although we have not examined the effect of CD8+ cell depletion on CD4+ T cell homeostatic proliferation in uninfected macaques, it is well established that the depletion of one subset of T cells, i.e. CD4+ or CD8+ T cells, leads to homeostatic proliferation that preserves the total T cell numbers and this is supported by studies in both mice (16–18) and humans (19,20). Recently, such homeostatic proliferation of CD4+ T cells was also conformed in CD8+ cell depleted uninfected rhesus macaques (32).

The dramatic decrease of HIV-infected Ki-67+ CD4+ T cells after the initiation of HAART has suggested that the bulk of plasma virus comes from such proliferating cells (24,33). Therefore it is reasonable to assume that providing more proliferating Ki-67+ CD4+ T cells at

effector sites may result in increased plasma viremia. It was shown before that replication of SIV in Ki-67+ CD4+ T cells is four to seven times higher than in Ki-67- cells (24). This high replication in Ki-67+ T cells could contribute to the increase in viral load in CD8+ cell depleted animals we and others have seen (2–13). A direct correlation between proliferating Ki-67+ CD4+ T cells and viral loads was shown before in CD8+ cell depleted naturally infected sooty mangabeys, also indicating that the transient increased viral replication was due to the expanded target pool (34). The authors of that study suggested that the increased viral loads in these animals are rather due to an increased target pool for SIV, possible due to cytomegalovirus reactivation, than the depletion of CD8+ T cells. Our findings in this study with macaques also suggest that increased viral targets for SIV infection may be one mechanism leading to increased viral loads, and that the underlying reason for this increased pool of SIV targets is due to homeostatic proliferation of CD4+ T cells. This is specifically supported by positive correlations we found between the frequency of Ki-67+ CD4+ T cells and viral loads in these animals during the anti-CD8 antibody treatment and also between SHIV plasma viremia and viral DNA in Ki-67+ CD4+ T cells but not viral DNA in Ki-67- CD4+ T cells.

A remaining question is whether other factors, specifically neutralizing antibodies, could have bias the results of this study. Rasmussen et al. showed that high titer neutralizing antibodies in vaccinated macaques could lower the plasma viral loads in CD8+ cell-depleted animals, however, all animals had viral rebound due to CD8+ cell depletion (9). In our study, only 3 out of 21 vaccinated animals showed low titer neutralizing antibodies (data not shown) and these animals were comparable in viral rebound with the animals which were negative for neutralizing antibodies. This suggests that neutralizing antibodies did not play a significant role in our study.

Our study examining the effect of CD8+ cell depletion on CD4+ T cell homeostasis was part of a vaccine study (26,35), which used SHIV-89.6P as a challenge virus. The original SHIV89.6 is composed of the SIVmac239 with HIV-1 derived auxiliary genes *tat*, *vpu*, and *rev* and the dual-tropic *env* gene derived from HIV-1 89.6 (29). The pathogenic clone SHIV89.6P was generated by serial in vivo passage (36). The question whether SHIV89.6P is indeed using the co-receptors CCR5 and CXCR4 to infect target cells remains unclear. An early study using in vitro cellular inhibition assays suggested that SHIV89.6P used preferentially CXCR4 in macaque PBMC although single CCR5-transfected cells could also be infected (37), a more recent study indicated that CXCR4 inhibitors may also inhibit the binding of the virus to CCR5 by a yet unknown mechanism (38). We have observed in our study that during CD8+ cell depletion the frequency of CCR5+ CD4+ T cells increased (data not shown). An increase of CCR5 expression was also observed on proliferating Ki-67+ CD4+ T cells, specifically in the PLN and MLN (data not shown). However, CXCR4 expression was not analyzed on these cells. CXCR4 is preferential expressed by naïve and central memory T cells (39), but can be upregulated through T cell activation (39,40). Importantly, the majority of CD4+ T cells in the GALT, which are effector memory cells, express both, CCR5 and CXCR4, and could therefore be preferential target populations for R5, R5X4 and X4 viruses (41–43). Although the question of co-receptor usage by SHIV89.6P in vivo remains open, the presence of a high number of proliferating CCR5 (and possible CXCR4) expressing effector memory CD4+ T cells would present a major target population for SHIV89.6P, SIV and HIV.

The animals used in our study were cynomolgous macaques, whereas previous CD8+ cell depletion studies used mostly Indian rhesus macaques (2,4–6,8–12) but also chimpanzees (3) with a variety of viruses used for infection including SHIV89.6P (2,9). Additional studies are necessary to further elucidate the effect of CD8+ cell depletion on CD4+ T cell homeostatic proliferation and viral rebound and to verify that the macaques and virus used in this study do not pose, although unlikely, a potential bias. Also, although SHIV89.6P was suggested to be a suboptimal challenge virus in CTL-based vaccine studies (commented on in (44)),

pathogenicity of the virus was not the subject of this study. As discussed earlier, our data suggest that CD4⁺ T cell homeostatic proliferation induced by CD8⁺ cell depletion is independent of viral replication and therefore most probably independent of pathogenicity of viral infection.

The anti-CD8 monoclonal antibody clone cM-T807 depletes CD8 expressing cells which includes CD8⁺ T cells and CD3-CD8⁺ NK cells (31). Therefore, as expected, we saw a reduction not only in CD8⁺ T cells but also in CD8⁺ NK cells in the treated animals during CD8⁺ cell depletion (data not shown). Similar depletion of CD3-CD8⁺ NK cells were also observed in other studies, however, a role of NK cells in controlling viral replication was deemed rather unlikely as no NK cells were detected in the blood of the animals at time of re-appearance of CD8⁺ T cells and subsequent decline in viremia (2).

Our studies are limited to blood and this may be not the best compartment to examine infected cells. Further studies are necessary to analyze the SIV RNA and DNA content of CD4⁺ T cells in tissues as it was shown recently that in rhesus macaques the highest amount of SIV RNA was found predominately in mesenteric lymph nodes (45). Such sites may be where proliferating cells get infected. Our results also indicate that although both Ki-67⁺ and Ki-67-CD4⁺ T cell populations can be infected, proliferating CD4⁺ T cells are the source of rebound viremia. Within CD4⁺ T cell subpopulations, memory CD4⁺ T cells harbor and produce more viruses compared with naïve CD4⁺ T cells upon activation (46). Furthermore, vaccine-induced proliferation of memory CD4⁺ T cells was correlated with increased viral replication in rhesus macaques (25). We show here that in CD8⁺ cell-depleted animals, mainly effector memory CD4⁺ T cells undergo proliferation, and this further supports the notion that proliferating memory CD4⁺ T cells can indeed increase viral replication in these animals.

A previous study that examined peripheral blood Ki-67⁺ CD4⁺ T cells during CD8⁺ cell depletion found a 3 fold increase in absolute peripheral blood Ki-67⁺ CD4⁺ T cell numbers, but concluded that these cells cannot account for the viral rebound (4). However, the authors did not examine the phenotype of these cells. In our study, we have shown that these expanded Ki-67⁺ CD4⁺ T cells generated during CD8⁺ cell depletion are predominantly effector memory CD4⁺ T cells, cells which support viral replication (46). Also, such changes in peripheral blood absolute numbers may not be representative of changes at effector sites where effector memory CD4⁺ T cells would migrate. Future studies examining the effect of CD8⁺ cell depletion on GALT and LN lymphocyte populations would address this question.

In our study we observed a slow re-appearance of CD8⁺ T cells after day 14, which was followed by regain of viral replication control by the majority of the animals on day 23. Reduced viral replication was already observed on day 21, despite high frequency of proliferating CD4⁺ T cells. These findings clearly implicate that in the presence of (functional) CD8⁺ T cells and NK cells, viral replication is controlled, even if the viral target population, proliferating CD4⁺ T cells, are still available.

Interestingly, CD4⁺ T cell proliferation in our study was not accompanied by a substantial increase in absolute numbers of peripheral blood CD4⁺ T cells. This increase in proliferation without an accompanied increase in absolute numbers can be explained by either an increased turnover of CD4⁺ T cells due to increased viremia or a rapid redistribution of CD4⁺ T cells from blood into sites that have “open” space like LN and GALT. The most likely reason for the constant CD4⁺ T cell numbers despite CD4⁺ T cell proliferation is migration of CD4⁺ T cells out of peripheral blood and redistribution in other tissue. It is now well established that during acute SIV-infection, CD4⁺ T cells, and in particular effector memory CD4⁺ T cells, are massively depleted from the GALT (21,22,42,47,48). In rhesus macaques that progress normally, GALT effector memory CD4⁺ T cell numbers stabilize after the loss of acute

infection, and even partially rebound but never fully recover (49,50). During the chronic phase, effector memory CD4⁺ T cells gradually decline and when they fall below a threshold, animals become clinically sick (49). Therefore in acutely or chronically SIV infected animals the massive depletion of CD4⁺ T cells creates space in GALT and probably other tissues, which could be replenished by newly generated CD4⁺ T cells. We found that CD8 depletion induced significant increased frequencies of CCR5⁺ CD4⁺ T cells in peripheral blood and this would support redistribution of CCR5⁺ cells out of the peripheral blood into tissues. The importance of CCR5 expression for SIV infection was underlined by data from natural SIV hosts which have much lower CCR5 expression on CD4⁺ T cells than non-natural hosts including humans and macaques (51). Besides recruitment of CCR5⁺ CD4⁺ T cells to non-lymphoid tissue, CCR5⁺ CD4⁺ T cells which often also express CXCR3 (52,53) could also be recruited to lymph nodes from HIV infected individuals by the chemokines CXCL10 and 11 (54).

A study, published while our manuscript was in review, examined the effect CD8⁺ cell depletion has on CD4⁺ T cell proliferation and expansion during acute SIV_{mac239} infection of rhesus macaques (32). In line with our study, the authors also report an increase in percentage of proliferating effector memory and transitional memory CD4⁺ T cells. The authors also observed an expansion of memory CD4⁺ T cell numbers in peripheral blood, something we have not seen, indicating a possible difference between acute and chronic SIV infection. Okoye et al. suggested in this study that in acute infection, increased CD4⁺ T cell proliferation induced by CD8⁺ cell depletion was not responsible for the increase in viral replication. However, the effect of CD8 depletion could be different in acute infection compared to chronic infection as viral replication is only transiently increased in CD8 depleted chronically SIV infected animals and returns to pre-treatment levels after reappearance of CD8⁺ T cells (our study and (4)). An important difference between the Okoye et al. study and ours is that CD8⁺ cell depletion during acute infection resulted in a prolonged CD8⁺ T cell depletion for 84 days whereas in our study the CD8⁺ T cell levels were restored by day 23. This may suggest that in addition to CD4⁺ T cell proliferation, depletion of CD8⁺ cells during acute infection results in a sustained depletion of SIV-specific CD8⁺ T cells and this leads to lack of viral control. IL-15 clearly is responsible for CD4⁺ T cell homeostatic proliferation in CD8⁺ cell depletion during acute infection (32). Whether it also drives this proliferation during CD8⁺ cell depletion of chronic infection remains to be established. We have shown that IL-15 is transiently elevated during acute infection but remains undetectable during the chronic phase (55). Therefore, IL-15 may play a more dominant role during CD8⁺ cell depletion of acute infection. Both the Okoye et al. study and ours (data not shown) found that CCR5⁺ CD4⁺ T cells are increased with CD8⁺ cell depletion. The results from our and Okoye et al. studies show that CD8⁺ cell depletion induces CD4⁺ T cell homeostatic proliferation in rhesus macaques and cynomolgous macaques infected with different SIV viruses (SHIV89.6P and SIV_{mac239}), and indicate the general nature of CD8⁺ cell depletion-induced CD4⁺ T cell homeostatic proliferation.

In conclusion, our findings suggest that CD8⁺ cell depletion leads to homeostatic proliferation of CD4⁺ T cells and specifically effector memory CD4⁺ T cells. Frequencies of Ki-67⁺ CD4⁺ T cells correlate positively with viral loads during treatment with anti-CD8 antibodies. SHIV infection of proliferating Ki-67⁺ CD4⁺ T cells also positively correlate with viral loads. This homeostatic proliferation of CD4⁺ T cells is independent of viral replication as it was also observed in animals without viral rebound and the frequency of Ki-67⁺ CD4⁺ T cells in these animals was comparable to animals with viral rebound. Our study does not disprove the role of CD8⁺ T cells and potential CD8⁺ NK cells controlling viral replication in SIV and HIV infection. However, our data indicates that the increased viremia found in CD8⁺ cell depleted SHIV and SIV infected macaques is at least in part due to CD8⁺ cell depletion-induced homeostatic proliferation of CD4⁺ T cells. This also raises the question whether baseline homeostatic proliferation is an important contributor to viremia in HIV-infected patients.

Acknowledgments

The authors declare no conflict of interests.

This study was supported by grants NIH R01 AI62437 and AI46719 to PDK, NIH R01 A1-071886 to JDB, NIH K22 AI07812 and R21 DE018339 to J.M. DBW was supported in part by grants from the NIH.

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Abbreviations

PLN, Peripheral lymph nodes; MLN, Mesenteric lymph nodes.

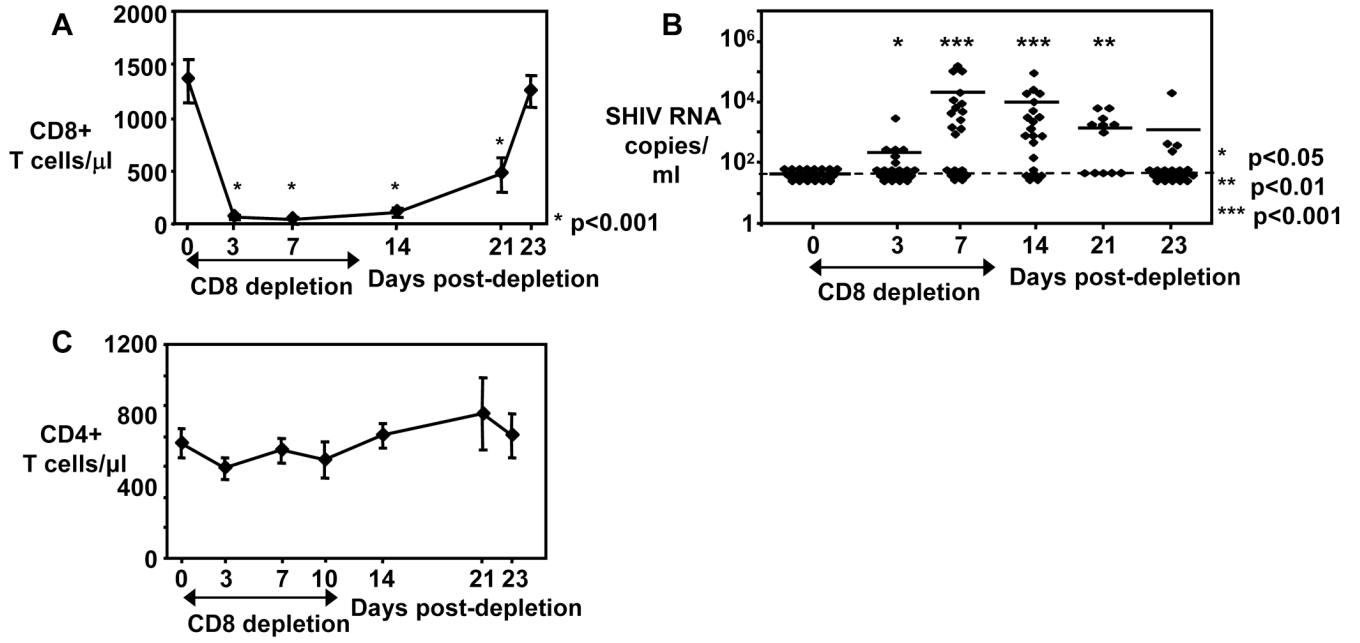


Figure 1. CD4+ T cell numbers are stable during CD8+ cell depletion
A) Absolute CD8+ T cell numbers shown before, during and after CD8+ cell depletion. Mean ± SE depicted for n = 13 – 21. **B)** Viral loads (Plasma SHIV RNA copies/ml) shown before, during and after CD8+ cell depletion. Each diamond represents one animal. Line depicts mean. Dashed line indicates limit of SHIV RNA detection. **A–B):** Asterisks depict statistical significant changes of CD8+ T cell numbers (A) and viral loads (B) compared to day 0. **C)** Absolute CD4+ T cell numbers shown before, during and after CD8+ cell depletion. Mean ± SE depicted for n = 13 – 21. The nonparametric Wilcoxon Signed-Rank test for paired samples was used for statistical analysis.

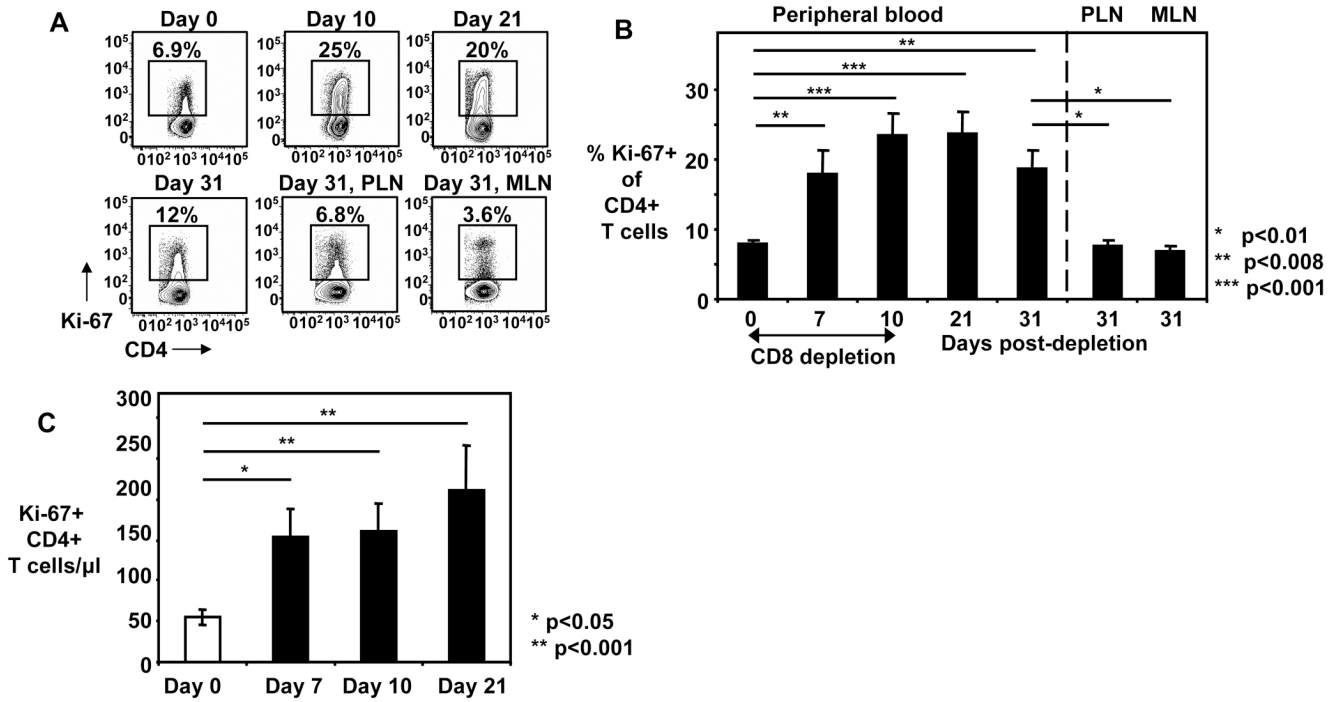


Figure 2. Ki-67+ CD4+ T cells are increased during CD8+ cell depletion

A) Representative FACS plots shown for expression of Ki-67 on peripheral blood CD4+ T cells on day 0 up to day 31 and PLN and MLN CD4+ T cells on day 31 from one animal; numbers indicate the percentage of Ki-67+ cells of CD4+ T cells. **B)** Frequency of Ki-67+ cells of CD4+ T cells were measured before (day 0), during (days 7, 10) and after (days 21, 31) CD8+ cell depletion in peripheral blood and PLN and MLN (day 31). Bars show mean \pm SE of peripheral blood (n=9–21), PLN (n=9) and MLN (n=8). **C)** Absolute Ki-67+ CD4+ T cell numbers show for days 0, 7, 10, and 21. Bars show mean \pm SE. Lines and asterisks depict statistical comparison. The nonparametric Wilcoxon Signed-Rank test for paired samples was used for statistical analysis.

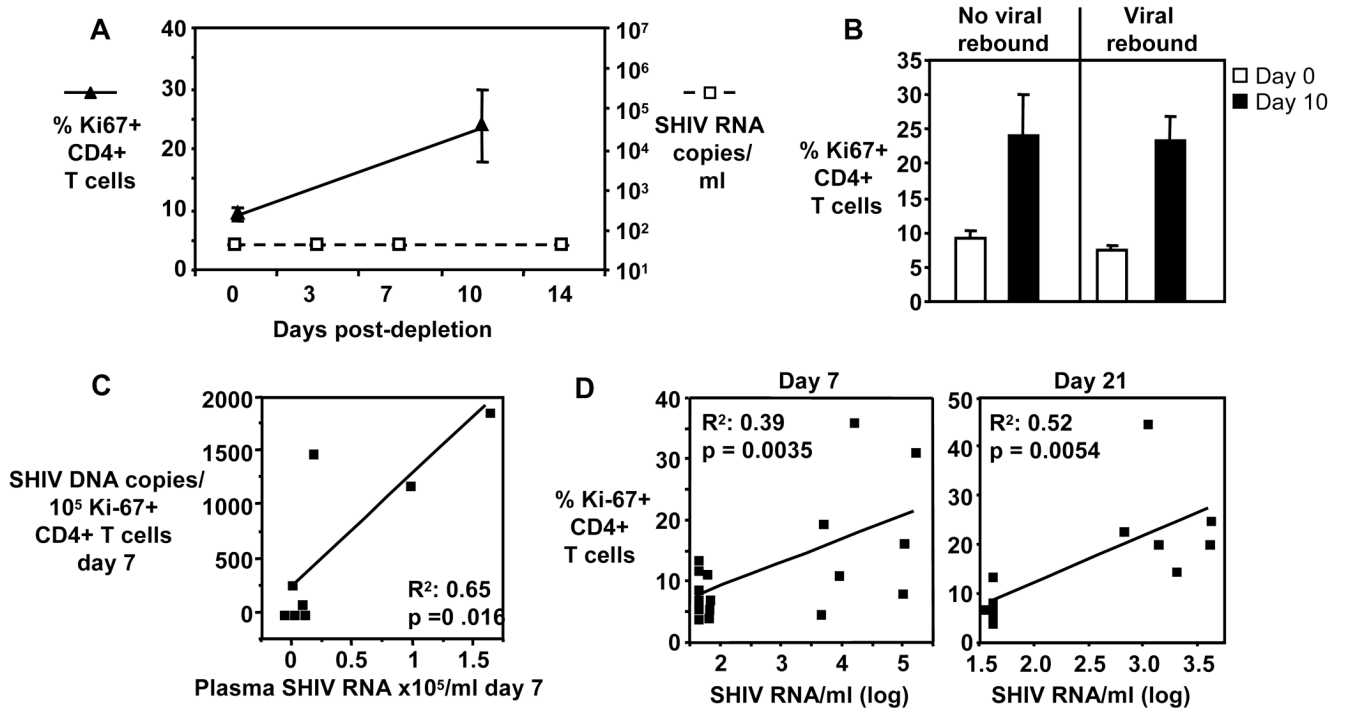


Figure 3. CD4+ T cell homeostatic proliferation is independent of viral replication

A) Frequency of Ki-67+ CD4+ T cells (filled triangles) and viral loads (open squares) shown for $n = 5$ animals which had undetectable viral loads during CD8+ cell depletion. **B)** Pooled data showing frequency of Ki-67+ CD4+ T cells on day 0 and 10 for animals without viral rebound ($n = 5$) and animals with viral rebound ($n = 16$). Bars show mean \pm SE. **C)** SHIV-infected Ki-67+ CD4+ T cells correlate with viral loads. Correlations between SHIV DNA copies/ 10^5 Ki-67+ CD4+ T cells and viral loads (plasma SIV RNA copies/ml) shown. Each square represents one animal. **D)** Frequencies of Ki-67+ CD4+ T cells correlate with viral loads (plasma SHIV RNA/ml) during CD8+ cell depletion. Viral loads and frequencies of Ki-67+ CD4+ T cells shown for day 0/day 7 ($n=20$) and day 0/day 21 ($n=13$). C and D: ANOVA regression analysis was used to determine significant correlations.

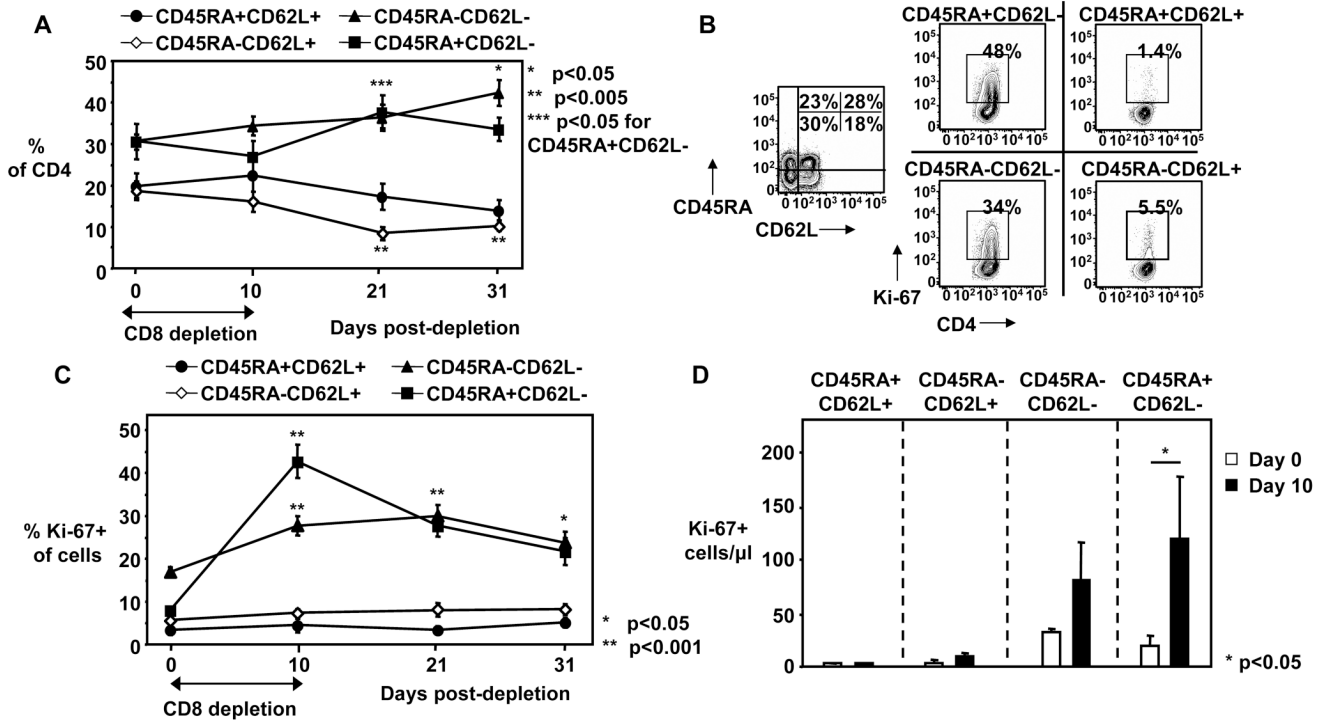


Figure 4. Effector memory CD4+ T cells are proliferating during CD8+ cell depletion

A) Frequency of naïve (CD45RA+CD62L+), central memory (CD45RA-CD62L+), and effector memory (CD45RA-CD62L- and CD45RA+CD62L-) CD4+ T cells shown from day 0 to day 31. Mean ± SE shown for n = 13 – 21. **B)** Representative FACS plots from CD4+ T cells day 10 shown, left plot depicts expression of CD45RA and CD62L on CD4+ T cells, right plots depicts expression of Ki-67 on CD4+ T cell subpopulations. **C)** Frequency of Ki-67+ cells of CD4+ T cell subpopulations were measured in peripheral blood before (day 0), during (day 10) and after (days 21, 31) CD8+ cell depletion in macaques. **D)** Absolute Ki-67+ memory CD4+ T cell subpopulation numbers shown for day 0 and day 10. A–D: Mean ± SE show for n = 9–21. Asterisks depict statistical significant changes compared to day 0 (A, C) or indicate statistical comparison (D). The nonparametric Wilcoxon Signed-Rank test for paired samples was used for statistical analysis.