Lineage-specific T-cell reconstitution following *in vivo* CD4+ and CD8+ lymphocyte depletion in non-human primates

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Abstract

Many features of T-cell homeostasis in primates are still unclear, thus limiting our understanding of AIDS pathogenesis, in which T-cell homeostasis is lost. Here, we performed experiments of in vivo CD4+ or CD8+ lymphocyte depletion in two non-human primate species, i.e. rhesus macaques (RM)s and sooty mangabeys (SMs). While RMns develop AIDS following infection with simian immunodeficiency virus (SIV), SIV-infected SMs are typically AIDS-resistant. We found that, in both species, most CD4+ or CD8+ T-cells in blood and lymph nodes were depleted following treatment with their respective antibodies. These CD4+ and CD8+ lymphocyte depletions were followed by a largely lineage-specific CD4+ and CD8+ T-cell proliferation, involving mainly memory T-cells, which correlated with interleukin-7 plasma levels. Interestingly, SMs showed a faster repopulation of naïve CD4+ T-cells than RMns. In addition, in both species CD8+ T-cell repopulation was faster than that of CD4+ T-cells, with CD8+ T-cells reconstituting a normal pool within <60 days and CD4+ T-cells remaining below baseline levels up to day 180 post-depletion. While this study revealed subtle differences in CD4+ T-cell repopulation in an AIDS-sensitive versus an AIDS-resistant species, such differences may have particular relevance in the presence of active SIV replication, where CD4+ T-cell destruction is chronic.
Introduction

The size and composition of the pool of mature T-lymphocytes are tightly regulated by complex homeostatic mechanisms, with the total number of T-cells remains relatively constant overtime in healthy individuals \(^1-^3\). The numeric homeostasis of mature T-cells is required for a normal immune function, with several human diseases being associated with failure of T-cell homeostasis. In the presence of an acute or chronic depletion of T-cells, the overall homeostasis of the T-cell compartment can be maintained essentially in three ways: (i) by generation of naive CD4+ or CD8+ T-cells from thymic precursors, (ii) by an increased longevity of T-cells, or iii) by peripheral expansion, i.e., proliferation of mature T-lymphocytes \(^4,^5\). The latter phenomenon is defined as “homeostatic proliferation” or “lymphopenia-induced proliferation”, i.e., a spontaneous, antigen-independent proliferation of mature T-cells, and appears to be the most rapid component of T-cell immune reconstitution following experimental depletion \(^4,^6\).

Despite its central role in maintaining immune function, the mechanisms regulating the homeostasis of T-cells \textit{in vivo} are still largely unclear. The vast majority of the relevant studies were conducted in mice, where lymphocyte depletion is induced experimentally (i.e. using antibody-mediated depletion or irradiation) or by genetically engineering mice that are unable to produce mature T-cells (i.e. RAG \(-/-\)). Of note, it is still unclear what molecular and cellular mechanisms trigger the proliferation of mature T-cells in the event of depletion. It is well established that cytokines, such as IL-7, IL-15 and others, are crucial for T-cell homeostasis. Indeed, plasma concentration of IL-7 and IL-15 is higher in condition of T-cell depletion, a phenomenon that may be related to decreased consumption of these cytokines or, alternatively, to increased production by as yet undefined cell type(s) that senses T-cell depletion \(^7-^11\). However,
it is not clear if the requirements for T-cell homeostasis are different for CD4+ and CD8+ T-cells, and how the homeostasis of the different CD4+ and CD8+ T-cell subsets, namely naive, effector, and memory cells, is regulated. In addition, it has not been determined to what extent the homeostatic response that follows T-cell depletion is lineage specific (i.e., whether only the depleted cell type reconstitutes the compartment) or blind (i.e., whether non-depleted cells may occupy the “available space”). These questions are even more difficult to answer in primates, either human or non-human, because transgenic/knock out systems are not available to aid experimental approaches. As a result, our knowledge of the regulation of homeostasis of T-cells in primates is still very limited.

Pathogenic HIV infection in humans, as well as SIV infections in non-naturally adapted hosts, such as rhesus macaques (RMs), are characterized by progressive CD4+ T-cell depletion and AIDS (reviewed in 12,13). An important factor contributing this CD4+ T-cell depletion is an insufficient CD4+ T-cell reconstitution, i.e., a substantial failure of the regenerative capacity of the lymphoid system to produce enough T-cells to compensate for their loss 14-20. The role of an insufficient CD4+ T-cell reconstitution in AIDS pathogenesis has been recently emphasized by the observation that disease progression is ultimately associated with homeostatic failure at the level of central-memory CD4+ T-cells, even though the majority of CD4+ T-cells that are either killed by the virus or succumb to apoptosis show an effector or effector-memory phenotype 21. The mechanisms responsible for the failure of T-cell reconstitution during HIV infection are complex, and likely involve the presence of aberrant chronic immune activation, extensive recruitment of naïve and central memory CD4+ and CD8+ T-cells into the effector compartment, reduced input of naïve T-cells from the thymus, and disruption of the bone marrow (BM) function.
and lymph node (LN) microenvironment. Failure of CD4+ T-cell homeostasis precedes the development of clinically-defined AIDS by approximately 1-2 years, and appears to be a critical step in HIV disease progression. Important advances in our understanding of the pathogenesis of HIV-infection in humans have been provided by studies of primate species that are natural hosts for SIV, such as sooty mangabeys (SMs). In marked contrast to HIV/SIV-infected humans or RMs, SIV-infected SMs generally maintain normal CD4+ T-cell counts and do not progress to AIDS. Extensive characterization of SIV infection of SMs has shown that high levels of virus replication, lack of immune control, short lifespan of infected cells in vivo, tropism for CD4+ T-cells, and depletion of mucosal CD4+ T-cells are all common features of progressive and non-progressive HIV/SIV infections. Based on these findings, the current paradigm maintains that the continuous replication of a cytopathic virus that targets activated/memory CD4+ T-cells is not sufficient to cause AIDS during a primate lentiviral infection, and predicates that additional cofactors are critical in HIV pathogenesis. Of note, SIV infection of SMs is typically associated with the preservation of a normal lymphoid regenerative compartment and low levels of immune activation, which may account, at least in part, for the preservation of T-cell homeostasis in these animals. In addition, we recently showed that, in non-human primates, the BM is a preferential site for T-cell proliferation under depleting conditions, and that in naturally SIV-infected SMs a preserved BM function is instrumental in maintaining CD4+ T-cell homeostasis.

In the current study, we attempted to further elucidate the features of T-cell homeostasis in non-human primates, and investigate whether the capacity to maintain T-cell homeostasis differs
between SMs and RM.s, by conducting experiments of *in vivo* antibody-mediated depletion of either CD4+ or CD8+ lymphocytes.
Materials and methods.

Animals.

Six SIV-uninfected SMs and six SIV-uninfected RMs, housed at the Yerkes Primate Research Center, Atlanta, GA, were included in this study. The SMs and RMs of the CD4+ lymphocyte depletion study were on average 5.1 and 4.3 year-old, respectively; the SMs and RMs of the CD8+ lymphocyte depletion study were on average 4.3 and 3.9 year-old. The ages of these SMs were similar to those at which they become naturally infected with SIV. The average lifespan of healthy, SIV-uninfected RMs and SMs is approximately 20 years (Else, personal communication). All animal studies were approved by the University of Pennsylvania and Emory University IACUCs.

Lymph node biopsies.

Lymph node biopsies and isolation of LN-derived T-cells were conducted as described previously [28].

Depletion of CD4+ or CD8+ lymphocytes in vivo.

Depletion of CD4+ and CD8+ lymphocyte were performed using 10 mg/Kg i.v. anti-CD4 mAb (OKT4A) on day -10 and 5 mg/Kg on days -7, -3, and 0, while for CD8+ lymphocyte depletion animals were treated with 4 mg/Kg i.v. anti-CD8 mAb (OKT8F) on days -2, -1, and 0, a protocol that has been shown to deplete CD4+ and CD8+ lymphocytes in vivo in both RMs and SMs [30-32]. We have previously shown that the depleting antibodies used in this study did not mask staining with the fluorescent-labeled anti-CD4 or anti-CD8 antibodies used to assess the levels of these cells [30,33]. Of note, the protocol used for CD4+ lymphocyte depletion does not deplete monocytes...
or dendritic cells (data not shown). Blood and LN collection were performed at baseline and at different time points following the last antibody administration.

**Flow cytometry.**

The immunophenotype of lymphocytes derived from PB and LN was analyzed by 4- and 7-color flow cytometric analysis. The staining was performed according to standard procedures using a panel of monoclonal antibodies that have been shown to be cross-reactive with SMs and RMs\(^ {29,30}\). The antibodies used in the study included: anti-CD3 FITC, PerCP, AlexaFluor-700 and APC (SP34); anti-CD4 PerCP and PerCP-Cy5.5 (L200), and anti-CD4 APC-Cy7 (SK3); anti-CD8 APC and PE (OKT8), and anti-CD8 Pacific Blue (RPA-T8); anti-CD28 FITC and PE-Cy7 (CD28.2); anti-CD95 APC (DX2); anti-CD62L PE (SK11); anti-CCR7 FITC and PE (150503), and anti-CCR7 PE-Cy7 (3D12); anti-Ki67 FITC (B56); anti-CD127 PE (R34.34). Data were acquired on a FACScaliber cytometer driven by the CellQuest software (4-colors), and on a LSRII flow cytometer driven by the DiVA software package (7-colors) (Becton Dickinson). Analysis of the acquired data was performed using FlowJo software (Tree Star, Ashland, OR).

**Plasma levels of IL-7 and IL-15.**

IL-7 and IL-15 quantitation was performed as described in\(^ {34}\).

**Statistical analysis.**

The performed analyses include the two-tailed Student t test or the Mann-Whitney test for comparisons between groups. Partial correlations that take into account the longitudinal structure of the study (i.e., adjusted for repeated measure of the same animal) were computed between
variation of IL-7 and CD4+ T-cell counts and fraction of CD4+Ki-67+ T-cells. All analyses were performed using Prism (GraphPad Software Inc.) or SAS 9.1 (SAS Institute Inc.) software. A $P$ value <0.05 was considered significant.

**Mathematical model.**

Reconstitution data from individual animals were fitted to a bilinear model, in which a first and second rate of increase of cell numbers was distinguished. The moment at which the model switches from the first to the second slope was determined by the fitting procedure. For the reconstitution curves, all time points at which cell numbers were not yet consistently increasing were ignored. The best fits to the experimental data were determined using least square minimization, using the DNLS1 subroutine\textsuperscript{35}. Ninety-five percent confidence intervals for the inferred parameters were then determined using a bootstrap method\textsuperscript{36}, where the residuals to the optimal fit were resampled 500 times.
Results.

Antibody-mediated CD4+ and CD8+ lymphocyte depletion in non-human primates.

To directly assess the homeostatic regulation of T-lymphocytes in non-human primates, we utilized an experimental system in which a transient but severe depletion of CD4+ or CD8+ T-cells is induced by administering OKT4A or OKT8F monoclonal antibody, respectively. The OKT4A MAb was administered four times in a ten-day period (days -10, -7, -3, 0) in three SIV-uninfected RMs and three SIV-uninfected SMs, and the OKT8F MAb was administered for three consecutive days (days -2, -1, 0) in three additional SIV-uninfected RMs and SMs. In all cases, we used flow cytometry to assess CD4+ and CD8+ T-cell depletion and reconstitution in peripheral blood (PB) and lymph nodes (LN), and observed a profound depletion of the targeted T-cell subset (see Figure 1A and 2A for representative examples of the levels of CD4+ and CD8+ T-cells pre-depletion, and at an early and a later time point post-depletion). More specifically, the pre-depletion percentage of CD3+CD4+ T-cells in PB was 20.6%±7.9% (mean ± SD) in SMs and 30.8%±3.4% in RMs; and in LNs it was 47.9%±14.3% in SMs and 41%±4.7% in RMs. The percentage of CD3+CD8+ T-cells at baseline in PB was 25.3%±1.9% in SMs and 17.3%±3% in RMs; in LNs it was 19%±0.3% in SMs and 17.9%±4.6% in RMs. As shown in Figures 1 and 2, the depleting antibodies induced a rapid, lineage-specific (i.e., mainly involving CD4+ T-cells in the event of CD4+ lymphocyte depletion and CD8+ T-cells in the event of CD8+ lymphocyte depletion), and profound depletion of CD4+ or CD8+ T-cells. For circulating CD4+ T-cells, the nadir value post-depletion was 15.1±8.9% of baseline levels in SMs and 3.6%±3.3% of baseline levels in RMs (figure 1B), which corresponded to 56.1±29.1 cells/μl in SMs and 33.3±30.7 cells/μl in RMs (figure 1C). For LN CD4+ T-cells the nadir value was 36.7%±6.7% and
33.4%±8.6% of baseline in SMs and RMs, respectively (figure 1D). For circulating CD8+ T-cells, the nadir value was 0.1%±0.1% of baseline in SMs and 3.8%±1.5% of baseline in RMs (figure 2B), which corresponded to 0.8±0.1 cells/μl in SMs and 18.4±10.3 cells/μl in RMs (figure 2C). For LN CD8+ T-cells the nadir value was 2.1%±0.6% and 10.6%±4.1% of baseline in SMs and RMs, respectively (figure 2D). In general, we observed a less efficient depletion in LNs than in blood, a finding that was particularly evident for CD4+ T-cells. Of note, none of the experiments depleted the non-targeted populations (i.e., CD8+ T-cells during anti-CD4 treatment or CD4+ T-cells during anti-CD8 treatment). Collectively, these data indicate that both anti-CD4 and anti-CD8 antibodies are effective in depleting T-cells in primates, with the loss of CD8+ T-cells being more profound than that of CD4+ T-cells, even though the anti-CD8 antibody was infused less frequently than the anti-CD4 antibody.

Replication of CD4+ T-cells after antibody-induced depletion is slow and occurs with similar kinetics in RMs and SMs.

As described above, infusion of anti-CD4 antibody results in a similarly severe depletion of CD4+ T-cells in both SMs and RMs. Given the different outcomes of SIV infection in these two species, we hypothesized that the AIDS-resistance of SMs is related to an intrinsically better ability to repopulate the CD4+ T-cell pool. To address this issue, we investigated the kinetics of CD4+ T-cell repopulation in SMs and RMs. As shown in figure 1B-D, the kinetics of CD4+ T-cell repopulation post-depletion in PB and LN were very similar in SMs and RMs. Importantly, the repopulation of CD4+ T-cells was relatively slow and incomplete in both species, with the last available experimental time point showing a level of CD4+ T-cell reconstitution reaching only approximately 50% of baseline levels (figure 1B-D).
Taken together, these findings suggest that an intrinsically reduced ability of CD4+ T-cells to repopulate their pool after depletion may be involved in the HIV/SIV-associated CD4+ T-cell depletion. On the other hand, the observation of comparable kinetics of CD4+ T-cell reconstitution in RMs and SMs does not support the hypothesis that the AIDS resistance of SMs is caused by a more effective homeostatic response to the depletion of CD4+ T-cells associated with SIV infection.

**The repopulation of CD8+ T-cells is more rapid than that of CD4+ T-cells.**

We then investigated the kinetics of CD8+ T-cell repopulation following Ab-mediated CD8+ lymphocyte depletion. As shown in figure 2, the repopulation of CD8+ T-cells was relatively rapid in both species; already at day 11 post-depletion, the fraction of CD3+CD8+ T-cells in PB returned to 45.3%±1.4% of baseline in SMs and to 50.6%±14.1% of baseline in RMs. In terms of absolute numbers, we observed a significant increase of CD8+ T-cells between day 11 and 21 in both species (Figure 2C). In LNs, the fraction of CD3+CD8+ T-cells returned to 79.1%±7.8% and 91.5%±19.9% of baseline values in SMs and RMs, respectively (Figure 2D), a pattern of reconstitution considerably faster than that observed for CD4+ T-cells (figure 1). Interestingly, peripheral CD8+ T-cells seem to repopulate similarly in the two studied species.

To compare the rates of T-cell reconstitution after depletion in SMs and RMs, we fitted the reconstitution data from individual monkeys to a bilinear model. This enabled us to compare the absolute rates of T-cell reconstitution (in cells/μl blood per day) between different T-cell subsets and monkeys. These fits revealed an initial rapid rate of reconstitution, and a second slower rate of reconstitution, where the initial rate of reconstitution may in part reflect the relatively rapid process of T-cell redistribution after depletion. These analyses reconfirmed that the CD8+ T-cell
pool reconstitutes more quickly after depletion than the CD4+ T-cell pool. During the first rapid phase of reconstitution, CD8+ T-cells increased in the blood at a median rate of 100 cells/μl blood per day in SMs and 88 cells/μl blood per day in RMs, compared to 17 CD4+ T-cells/μl blood per day in SMs and 6 CD4+ T-cells/μl blood per day in RMs. During the slower second phase CD8+ T-cells also reconstituted more rapidly than CD4+ T-cells: 4.6 and 5.8 CD8+ T-cells/μl blood per day in SMs and RMs, respectively, compared to 0.6 and 1.5 CD4+ T-cells/μl blood per day in SMs and RMs.

**CD4+ and CD8+ T-cell repopulation post-depletion involves both naïve and memory T-cells.**  
Naïve and memory T-cell subsets of RMs and SMs can be classified based on the surface expression of CD28 and CD95, which allows to identify the naïve (CD28+CD95-), memory (CD28+CD95+), and effector (CD28-CD95+) subsets of CD4+ and CD8+ T-cells \(^{39,40}\). To assess the phenotype of T-cells that reconstitute the CD4+ and CD8+ T-cell compartments after depletion, we longitudinally measured the fraction of naïve, memory, and effector CD4+ and CD8+ T-cells in the blood and LN. The gating strategy is shown in figures 3A and 4A, respectively. In both SMs and RMs, the average fraction of memory cells within the total CD4+ T-cell pool increased from ~50% (pre-depletion) to ~75% by day 21 post-depletion (figure 3B). This relative expansion of memory CD4+ T-cells may reflect the fact that these cells were depleted less effectively than naïve T-cells (Figure 3C), and/or be caused by an intrinsically stronger proliferative potential of residual memory CD4+ T-cells after depletion \(^{41,42}\). In terms of absolute numbers, however, CD4+ T-cells of SMs appear to recover both naïve and memory subsets with similar rates, while CD4+ T-cells of RMs show a more rapid reconstitution of the memory pool (figure 3C). Indeed, in SMs the number of naïve CD4+ T-cells became similar to
pre-depletion by day 120 post-depletion, while in RMs this number remained significantly lower than baseline throughout the study (p<0.01 for all time points). This latter finding suggests that SMs may be better able to replenish their naïve CD4+ T-cell compartment. The depletion-induced enrichment in memory CD4+ T-cells was more dramatic in LN, where the fraction of memory cells increased from approximately 20% pre-depletion to 60% and 75% at day 0 (i.e., the last day of depleting treatment) in SMs and RMs, respectively (figure 3D). Similarly to what we observed in blood, SMs tended to restore the pre-depletion proportion of naive and memory CD4+ T-cells in LNs faster than RMs. Depletion of CD8+ lymphocytes was followed, in both species, by more subtle changes in the relative proportion of naive, memory, and effector CD8+ T-cells, with the baseline proportion being restored by day 28 in blood and by day 11 in LNs (figure 4B-D).

Fits of the reconstitution of the different T-cell subsets in individual animals demonstrated that the relative contribution of memory and naïve T-cells to both CD4+ and CD8+ T-cell reconstitution was similar, both in SMs and in RMs. With respect to CD4+ T-cells, we found that during the initial rapid phase of reconstitution following CD4+ lymphocyte depletion, naïve CD4+ T-cells increased in the blood at a median rate of 9 cells/μl blood per day in SMs and 1.1 cells/μl blood per day in RMs, compared to memory CD4+ T-cells which increased by 5 cells/μl blood per day in both SMs and RMs (Figure 5). The second slower phase of reconstitution also consisted of almost equal numbers of naïve and memory CD4+ T-cells: naïve CD4+ T-cells increased in the blood at a median rate of 0.5 cells/μl blood per day in SMs and 0.2 cells/μl blood per day in RMs, compared to memory CD4+ T-cells which increased by 0.5 cells/μl blood per day in SMs and 1 cells/μl blood per day in RMs. In all, these findings indicate that naïve and memory T-cells contributed almost equally to the repopulation of the CD4+ and CD8+ T-cell pools.
Ab-mediated CD4+ and CD8+ lymphocyte depletion in SMs and RMs is followed by a rapid, largely lineage-specific increase in T-cell proliferation.

To determine how the depletion of CD4+ and CD8+ lymphocytes influences the proliferation of the residual T-cells in RMs and SMs, we longitudinally assessed the fraction of these cells expressing the proliferation marker Ki-67 in both PB and LN. We found that, in both SMs and RMs, the depletion of CD4+ T-cells was followed by a dramatic increase of their levels of proliferation, whose kinetics were similar (no significant difference at any time point post depletion) in the two species (figure 6A). Indeed, in both species, the fraction of CD4+Ki-67+ T-cells was three times higher compared to pre-depletion levels by day 4 post-depletion, and remained higher until day 28 post-depletion (figure 6A). Consistent with the slow and incomplete reconstitution of CD4+ T-cells (figure 1), which may have triggered a prolonged homeostatic response, CD4+ T-cell proliferation remained higher than baseline throughout the follow-up period in both species (figure 6A). A similar albeit less dramatic increase in the levels of proliferating CD4+ T-cells was found in the LNs of the CD4+ lymphocyte depleted animals (Supp. figure 1A). We next analyzed the levels of memory CD4+ T-cell proliferation in the blood. As shown in figure 6B, in both RMs and SMs the depletion of CD4+ lymphocytes was followed by a rapid (i.e., starting at day 4 post-depletion) increase in the memory CD4+ T-cell proliferation, with comparable kinetics in the two species. Of note, in both species the fraction of memory CD4+Ki67+ T-cells returned to pre-depletion levels by day 60 post-depletion (figure 6B). This increase in memory CD4+ T-cell proliferation was specific for circulating lymphocytes, since no changes were found in the LNs of the same animals (figure 6B).
We next analyzed the proliferative CD8+ T-cell response in CD8+ lymphocyte depleted animals and found that, in both species, the Ab-mediated depletion was followed by a rapid increase in the fraction of circulating CD8+Ki-67+ T-cells (figure 6C). At day 7 post-depletion, this proliferative response was much higher in SMs compared to RMs: PB-derived CD8+ T-cells of CD8+ lymphocyte depleted SMs expressed 10 times higher levels of Ki-67 than pre-depletion, while in RMs the increase was only 2.3 fold (figure 6C). As observed for CD4+ T-cell proliferation, the increase in CD8+ T-cell proliferation was more limited in LN, with the fraction of CD8+Ki-67+ cells being slightly increased only at day 0 post-depletion (Supp. figure 1B). As expected based on the fast reconstitution of the peripheral CD8+ T-cell compartment, the fraction of proliferating CD8+Ki-67+ T-cells returned to pre-depletion levels in both species by day 11 post-depletion and in both blood (figure 6C) and LN (Supp. figure 1B). Of note, in both SMs and RMs, the proliferation that followed CD4+ or CD8+ lymphocyte depletion was predominantly lineage-specific, with an increase in CD4+ T-cell proliferation and only limited changes in CD8+ T-cell proliferation when CD4+ lymphocytes were depleted (figure 6A), and an increase in CD8+ T-cell proliferation with only limited changes in CD4+ T-cell proliferation (particularly so for SMs) when CD8+ lymphocytes were depleted (figure 6C).

Taken together, these data indicate that the increased level of proliferation that follows a rapid and severe depletion of CD4+ or CD8+ lymphocytes is quantitatively similar in RMs and SMs, involves by-and-large lineage-specific circulating T-cells, and appears to be shaped by the ongoing level of CD4+ or CD8+ T-cell reconstitution.
CD4+ and CD8+ lymphocyte depletions are followed by an early plasmatic increase of interleukin-7 (IL-7).

Cytokines such as interleukin-7 (IL-7) and interleukin-15 (IL-15) play a critical role in promoting T cell homeostasis. To investigate the role of IL-7 and IL-15 in the reconstitution of CD4+ and CD8+ T-cells that follows antibody-mediated depletion, we longitudinally assessed the plasma levels of these cytokines. Consistent with previous studies, baseline (i.e., pre-depletion) plasma levels of IL-7 and IL-15 were higher in RMs (2.36±1.5 pg/ml and 7.54±0.1 pg/ml, respectively) than in SMs (0.30±0.1 pg/ml and 0.65±0.3 pg/ml, respectively). To account for these different baseline levels, we chose to present plasma levels of IL-7 and IL-15 as a percentage of their pre-depletion values.

As shown in figure 7A-B, in both species plasma levels of IL-7 increased rapidly after CD4+ (figure 7A) and CD8+ (figure 7B) lymphocyte depletion. Interestingly, in RMs plasma IL-7 levels after CD4+ lymphocyte depletion remained significantly higher than baseline throughout the follow up period, while in SMs they returned to pre-depletion levels by day 60 despite an incomplete reconstitution of the CD4+ T-cell compartment. Of note, in SMs the restoration of baseline levels of IL-7 by day 60 post-depletion temporally correlated with the restoration of pre-depletion levels of both proliferating memory CD4+ T-cells (figure 6B) and the frequency of memory CD4+ T-cells (figure 3B). In contrast, the kinetics of plasma IL-7 levels after CD8+ lymphocyte depletion were very similar in SMs and RMs, with IL-7 remaining significantly higher compared to baseline throughout the follow-up period (figure 7B). Finally, hardly any significant changes were observed in plasma IL-15 levels after CD4+ or CD8+ lymphocyte depletion, with the exception of a late (i.e., starting at day 60) increase of IL-15 levels in SMs after CD4+ lymphocyte depletion (figure 7A-B).
To further elucidate the role of IL-7 in CD4+ T-cell homeostasis of non-human primates, we next investigated the relationship between plasma levels of IL-7 and either CD4+ T-cell counts or the fraction of proliferating (i.e., Ki-67+) CD4+ T-cells. We found that changes of plasma IL-7 (ΔIL-7, i.e., percentage variation compared to baseline) correlated inversely with the changes of CD4+ T-cell counts (p=0.0008, data not shown) and directly with the changes in the fraction of CD4+Ki-67+ T-cells (p=0.0068, figure 7C). These findings suggest a role of IL-7 in regulating CD4+ T-cell homeostatic proliferation, and are consistent with two recent published studies showing a beneficial effect of IL-7 in inducing CD4+ T-cell proliferation and reconstitution in HIV-infected humans. The moderate and transient increase in the levels of proliferation of the non-depleted subsets (see figure 6) may also be related to the increased availability of circulating IL-7 following CD4+ or CD8+ lymphocyte depletion.

Finally, we longitudinally assessed the levels of CD127, i.e., the α-chain of the IL-7 receptor, on CD4+ T-cells of the CD4+ lymphocyte depleted animals. In both SMs and RMs, the depletion of CD4+ T-cells and the concomitant increase in plasma levels of IL-7 were followed by a rapid decrease in the fraction of CD4+ T-cells expressing CD127 (figure 7D). While the kinetics of CD127 expression was similar in the two species, the observed fluctuations were slightly higher in RMs (nadir value post-depletion was 42.4±6.2% of baseline levels) than SMs (nadir value post-depletion was 67.6±7.7% of baseline levels). These findings suggest that, in both SMs and RMs, IL-7-dependent mechanisms may play an important role in determining the increased levels of CD4+ or CD8+ T-cell proliferation that follow the antibody-mediated depletion of these cells.
Discussion.

Despite the crucial role of CD4+ and CD8+ T-cells in maintaining immune function, the mechanisms regulating their homeostasis in vivo are still poorly understood, and particularly so in primates, where inbred transgenic/knock out animal strains are not available. This relative lack of knowledge has a negative impact on our understanding of the pathogenesis of HIV infection, in which CD4+ T-cells are depleted during chronic infection. Basic unanswered questions regarding T-cell homeostasis in primates include: (i) is the homeostasis regulated similarly between CD4+ and CD8+ T-cells? (ii) is the homeostasis of the naive, memory and effector T-cell subsets regulated in the same way? (iii) is the homeostatic proliferation of CD4+ and CD8+ T-cells lineage-specific? (iv) what molecular mechanisms and anatomic compartments support the homeostatic proliferation of T-cells? In the context of AIDS pathogenesis, an additional key question is whether CD4+ T-cell homeostasis is regulated in a different way in species that experience pathogenic HIV or SIV infections (i.e., humans and RMs) versus species that experience a non-progressive SIV infection (i.e., SMs). This latter question is important since elucidating the mechanisms responsible for the preservation of CD4+ T-cell homeostasis in natural hosts for SIV may help developing therapeutic approaches aimed at preventing or treating CD4+ T-cell depletion in HIV-infected humans.

In this study, we conducted experiments of CD4+ or CD8+ lymphocytes depletion in non-human primates. We selected two species that experience either progressive (RMs) or non-progressive (SMs) SIV infection, to test the hypothesis that the AIDS-resistance of SMs may be related to a species-specific, genetically determined ability to preserve CD4+ T-cell homeostasis in the
presence of a depleting event. To the best of our knowledge, this is the first time that a comparative, longitudinal analysis of CD4+ and CD8+ T-cell repopulation after in vivo depletion has been performed in these two species of non-human primates. The main results of this study are the following: (i) in both RMs and SMs, the depleting antibodies induced a rapid, profound, and largely specific (i.e., without impacting the non-targeted populations) depletion of CD4+ or CD8+ T-cells; (ii) in both species, CD4+ and CD8+ lymphocyte depletions were followed by a rapid proliferation which was largely lineage specific, i.e., involving predominantly the depleted subset of T-cells; (iii) in both species, the reconstitution of CD4+ T-cells was significantly slower than that of CD8+ T-cells, even though the used antibodies were more effectively depleting CD8+ than CD4+ T-lymphocytes; (iv) in both species, CD4+ and CD8+ lymphocyte depletion was followed by increased plasma levels of IL-7 which, in turn, correlated with increased proliferation of the depleted T-cell subset. In addition, the increase in plasma levels of IL-7 that followed CD4+ lymphocyte depletion was associated with reduced expression of CD127 (IL-7R) on CD4+ T-cells.

These data indicate that the kinetics of CD4+ and CD8+ T-cell reconstitution post-depletion are relatively similar in SMs and RMs, therefore not supporting the hypothesis that SIV-infected SMs maintain healthy CD4+ T-cell counts due to an intrinsically more rapid homeostatic response to depletion. Nonetheless, an interesting qualitative difference between SMs and RMs in CD4+ T-cell repopulation was that only the former were able to recover to the baseline ratio of naïve and memory cells. It is tempting to speculate that this ability of SMs to better reconstitute a naïve-rich CD4+ T-cell compartment may help protect these animals when infected with SIV. However, the antibody-mediated depletion of CD4+ lymphocytes is clearly different in many ways from that induced in vivo by SIV infection. For instance, the antibody-mediated depletion is more rapid,
involves virtually all CD4+ T-cell subsets, and based on other studies \(^{30}\), involves predominantly CD4+ T-cells in the blood and LN. In contrast, the SIV-induced depletion is slower, involves predominantly CD4+CCR5+ T-cells, and affects mucosal tissues more than blood and LN \(^{46,47}\). For these reasons, the hypothesis that a more effective preservation of CD4+ T-cell homeostasis plays a role in the AIDS resistance of naturally SIV-infected SMs (and possibly other natural SIV hosts) needs to be tested with further and more targeted interventions.

Despite the relatively low number of animals included in each experimental setting, the current study provides useful information regarding the basic features of CD4+ and CD8+ T-cell homeostasis in non-human primates, which is an understudied field of research. First, our study indicated clearly that, in both SMs an RMs, the CD4+ T-cell repopulation after antibody-mediated depletion is significantly less efficient compared to that of CD8+ T-cells. In addition, CD8+ T-cells reconstitute more rapidly the pre-depletion ratio of naive and memory cells. These observations are consistent with the fact that, in humans undergoing hematopoietic stem cell transplantation, CD4+ T-cell reconstitution is much slower than that of CD8+ T-cells \(^{37,38}\). The possibility that CD4+ T-cell homeostasis is intrinsically more fragile than that of CD8+ T-cells is compatible with the notion that the immunological damage caused by the chronic immune activation of pathogenic HIV/SIV infections affects CD4+ T-cells more than the CD8+ T-cells, above and beyond the fact that these viruses preferentially infect and kill CD4+ T-cells \(^{14}\). Second, our study indicates that the homeostatic proliferation of T-cells which follows antibody-mediated depletion is by-and-large lineage-specific, i.e. higher in the depleted compared to the non-depleted compartments; this is particularly evident in the setting of CD4+ lymphocyte depletion. This finding does not support the model, originated from mouse studies, that T-cell homeostasis is blind
to the distinction between CD4+ and CD8+ T-cells. The current study emphasizes our limited understanding of the molecular mechanisms underlying the lineage specificity of the T-cell proliferation that follows antibody-mediated depletion. Interestingly, our results are consistent with the observation that in idiopathic CD4 lymphopenia (ICL), a rare syndrome associated with low CD4+ T-cell counts in absence of HIV, only CD4+ but not CD8+ T-cells are activated and cycling. In contrast to ICL, however, antibody-mediated CD4+ (or CD8+) lymphocyte depletion is not associated with increased microbial translocation (as assessed by sCD14 levels) at any of the studied experimental points in either species (data not shown). Third, our study indicates that, in both species, CD4+ and CD8+ lymphocyte depletion is followed by a rapid increase in plasma IL-7, coincident with the peak of homeostatic T-cell proliferation. This finding suggests that, in healthy non-human primates, IL-7 plays an important role in the homeostatic response to the experimental depletion of CD4+ and CD8+ T-cells. Interestingly, the increase of plasma IL-7 that follows CD4+ lymphocyte depletion was more prolonged in RMs than in SMs, even in the setting of a similarly incomplete reconstitution of the CD4+ T-cell pool. This pattern was observed previously following the acute phase of SIV infection during the initial early decline in CD4+ T-cell counts which is associated with increased levels of plasma IL-7 and proliferating T-cells. The similarity between antibody-mediated depletion and acute SIV infection was further indicated as the increases in plasma IL-7 and T-cell proliferation were lower and more transient in SMs compared to the RMs. The fact that SMs may have a more attenuated homeostatic response to CD4+ T-cell depletion could, paradoxically, facilitate their ability to preserve CD4+ T-cell homeostasis during chronic SIV infection, especially since proliferating CD4+ T-cells may serve as targets for SIV replication.
In conclusion, this analysis of the kinetics of CD4+ and CD8+ T-cell repopulation after antibody-mediated depletion provided important preliminary information on the regulation of T-cell homeostasis in non-human primates. Further studies of T-cell homeostasis in non-human primate species that experience either a progressive or a non-progressive SIV infection may improve our basic understanding of AIDS pathogenesis and help in designing therapeutic intervention aimed at preserving CD4+ T-cell homeostasis in HIV-infected individuals.
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Authorship

Contribution: M.P., B.C., and G.S. designed the study and wrote the paper, with contributions from the other authors as appropriate; J.C.E. and B.C. performed the immunophenotypic analyses and prepared the figures; J.A.M.B. and R.J.B. fitted experimental data to a mathematical model and contributed to the writing of the paper; S.N.G., A.C., and N.R.K. helped in processing the samples and in analyzing the results; N.R.K. and J.M.B. provided the data on dendritic cells and sCD14; R.S.M. provided the depleting antibodies; J.E. supervised the housing and care of the animals and contributed to the design of the study; D.L.S. provided the IL-7 and IL-15 data and contributed to the design of the study.

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References


Figure legends

Figure 1. CD4+ T-cell reconstitution following Ab-mediated CD4+ lymphocyte depletion in non-human primates. Peripheral blood (PB) and lymph node (LN) derived mononuclear cells were obtained from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RM). (A) Flow cytometric staining of CD3+CD4+ T-cells in PB of a representative SM (left panels) and RM (right panels) as measured before and at days 7 and 120 after Ab-mediated CD4+ lymphocyte depletion. (B) Fraction of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells, expressed as percentage variation from baseline, in the PB of SMs and RM following CD4+ lymphocyte depletion. (C) Absolute number of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells in the PB of SMs and RM following CD4+ lymphocyte depletion. (D) Fraction of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells, expressed as percentage variation from baseline, in the LN of SMs and RM following CD4+ lymphocyte depletion. In (B-D) values are means ± SD, SMs and RM are shown in black squares and red triangles, respectively, and the dotted lines indicate the timing of anti-CD4 monoclonal antibody administrations.

Figure 2. CD8+ T-cell reconstitution following Ab-mediated CD8+ lymphocyte depletion in non-human primates. Peripheral blood (PB) and lymph node (LN) derived mononuclear cells were obtained from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RM). (A) Flow cytometric staining of CD3+CD8+ T-cells in PB of a representative SM (left panels) and RM (right panels) before and at days 4 and 60 after Ab-mediated CD8+ lymphocyte depletion. (B) Fraction of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells, expressed as percentage variation from baseline, in the PB of SMs and RM following CD8+ lymphocyte depletion. (C) Absolute number of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells in the PB of
SMs and RMs following CD8+ lymphocyte depletion. (D) Fraction of CD3+CD4+ (left graph) and CD3+CD8+ (right graph) T-cells, expressed as percentage variation from baseline, in the LN of SMs and RMs following CD8+ lymphocyte depletion. In (B-D) values are means ± SD, SMs and RMs are shown in black squares and red triangles, respectively, and the dotted lines indicate the timing of anti-CD8 monoclonal antibody administrations.

**Figure 3.** Naïve, memory, and effector CD4+ T-cell reconstitution following Ab-mediated CD4+ lymphocyte depletion. Peripheral blood (PB), and lymph node (LN) derived mononuclear cells were obtained from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RMs). (A) Gating strategies used to identify naïve (—), effector (——), and memory (——) CD4+ T-cells are shown in a representative animal. (B-D) Fractions (B, D) and absolute numbers (C) of naïve, effector and memory cells in the CD4+ T-cell pool were longitudinally assessed following antibody-mediated depletion of CD4+ lymphocytes in the PB (B, C) or LN (D) of SMs (left graphs) and RMs (right graphs). In (B-D) values are means ± SD, and the dotted lines indicate the timing of anti-CD4 monoclonal antibody administrations.

**Figure 4.** Naïve, memory, and effector CD8+ T-cell reconstitution following Ab-mediated CD8+ lymphocytes depletion. Peripheral blood (PB), and lymph node (LN) derived mononuclear cells were obtained from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RMs). (A) Gating strategies used to identify naïve (—), effector (——), and memory (——) CD4+ T-cells are shown in a representative animal. (B-D) Fractions (B, D) and absolute numbers (C) of naïve, effector and memory cells in the CD8+ T-cell pool were longitudinally assessed following antibody-mediated depletion of CD8+ lymphocytes in the PB (B, C) or LN (D) of SMs (left graphs)
and RMs (right graphs). In (B-D) values are means ± SD, and the dotted lines indicate the timing of anti-CD8 monoclonal antibody administrations.

**Figure 5. Quantitative kinetics of T-cell subset reconstitution following Ab-mediated CD4+ or CD8+ lymphocyte depletion.** The data on the reconstitution of total CD4+ (A) or CD8+ (B) T-cell pools, as well as their naïve, memory, and effector subsets in individual SMs and RMs were fitted to a bilinear model. In this model, an initial rapid phase (Phase 1, left graphs) and a second slower phase (Phase 2, right graphs) of increase of cell numbers was distinguished. The levels of T-cell reconstitution are graphed as cells per μl blood/day for each of the 3 uninfected SMs and 3 uninfected RMs included in the study, with bars representing the 95% confidence interval on the estimated parameter.

**Figure 6. T-cell proliferation following Ab-mediated CD4+ and CD8+ lymphocyte depletion in non-human primates.** Peripheral blood (PB) and lymph node (LN) derived mononuclear cells were obtained from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RMs). (A) Levels of circulating CD4+ (left graphs) and CD8+ (right graphs) T-cells of SMs (—) and RMs (—) that express the proliferation marker Ki-67 following Ab-mediated CD4+ lymphocyte depletion. (B) Levels of memory (i.e., CD28+CD95+) CD4+ T-cells that express Ki-67 in the PB (left graph) or LN (right graph) of SMs (—) and RMs (—) following Ab-mediated CD4+ lymphocyte depletion. (C) Levels of circulating CD4+ (left graphs) and CD8+ (right graphs) T-cells of SMs (—) and RMs (—) that express Ki-67 following Ab-mediated CD8+ lymphocyte depletion. In all the graphs levels of Ki-67+ T-cells are shown as percentage variation from baseline (mean ±
SD), and the dotted lines indicate the timing of anti-CD4 (A, B) or anti-CD8 (C) monoclonal antibody administrations.

**Figure 7. Variation of IL-7 and IL-15 plasma levels as well as IL-7 receptor (CD127) expression following Ab-mediated CD4+ and CD8+ lymphocyte depletion in non-human primates.** (A, B) Plasmas isolated from healthy SIV-uninfected sooty mangabeys (SMs) and rhesus macaques (RMs) were tested for levels of IL-7 (—) and IL-15 (—). Levels of these cytokines are expressed as percentage variation from baseline (mean ± SD) in SMs (left graphs) and RMs (right graphs) following Ab-mediated CD4+ (A) and CD8+ (B) lymphocyte depletion. (C) Correlation between change over time (Δ, i.e. percentage variation compared to baseline) of plasma IL-7 and those of proliferating CD4+Ki67+ T-cells. (D) Levels of CD4+CD127+ T-cells (expressed as percentage variation from baseline) in SMs (—) and RMs (—) following Ab-mediated CD4+ lymphocyte depletion. The dotted lines in the graphs indicate the timing of anti-CD4 monoclonal antibody administrations.
Figure 1

CD4+ lymphocyte depletion

A. SM vs RM

B. Graph showing % CD3+CD4+ in lymphogate (as % of baseline) over days for SMs and RMs.

C. Graph showing # CD3+CD4+ cells/mm^2 for SMs and RMs.

D. Graph showing % CD3+CD4+ in lymphogate (as % of baseline) for SMs and RMs over days.
Figure 2

CD8+ lymphocyte depletion

A

SM

RM

d -14  
d 4  
d 60

CD3

CD8

B

% CD3+CD4+ in lymphocyte (as % of baseline)

Days

SMs  
RMs

% CD3+CD4+ in lymphocyte (as % of baseline)

Days

SMs  
RMs

C

# CD3+CD4+ cells/mm³

Days

SMs  
RMs

# CD3+CD4+ cells/mm³

Days

SMs  
RMs

D

% CD3+CD4+ in lymphocyte (as % of baseline)

Days

SMs  
RMs

% CD3+CD4+ in lymphocyte (as % of baseline)

Days

SMs  
RMs
Figure 3
CD4+ lymphocyte depletion

CD3+CD4+

A

CD28

CD95

SMs

RMss

B

% of CD3+CD4+

Days

Effector 0.31
Memory 0.48
Naive 0.50

C

# CD3+CD4+ cells/mm^3

Days

D

% of CD3+CD4+

Days
Figure 4

CD8+ lymphocyte depletion

A

CD3+CD8+

CD28

CD95

B

SMs

RM

Days

Days

C

D

Effector

Memory

Naive

Days

Days

Effector

Memory

Naive

Days

Days

% of CD3+CD8+

% of CD3+CD8+

# CD3+CD8+ cells/mm³

# CD3+CD8+ cells/mm³

% of CD3+CD8+

% of CD3+CD8+

Effector

Memory

Naive

Effector

Memory

Naive

Naive 20.8

Memory 28.2

Effector 49.3
Figure 5
T-cell reconstitution in cells/day

Phase 1

Phase 2

CD4

CD8

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Figure 6

CD4+ lymphocyte depletion

A

B

PB

LN

CD8+ lymphocyte depletion
Figure 7

A: SMs
CD4+ lymphocyte depletion

B: CD8+ lymphocyte depletion

C: $r = 0.33$
$p = 0.0068$

D: %CD127+ among CD4+ (as % of baseline)
Lineage-specific T-cell reconstitution following in vivo CD4+ and CD8+ lymphocyte depletion in non-human primates

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