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Low levels of SIV infection in sooty mangabey central-memory CD4+ T-cells is associated with limited CCR5 expression

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Abstract

Naturally SIV-infected sooty mangabeys (SMs) do not progress to AIDS despite high-level virus replication. We previously showed that the fraction of CD4+CCR5+ T-cells is lower in SMs compared to humans and macaques. Here we found that, after *in vitro* stimulation, SM CD4+ T-cells fail to up-regulate CCR5, and that this phenomenon is more pronounced in CD4+ central-

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Contribution: M.P., B.C., and G.S. designed the study and wrote the paper, with contributions from the other authors as appropriate; M.P., B.C., and E.R.A. performed the immunophenotypic analyses, analyzed results, prepared the figures; L.M., A.M.O., and A. C. helped in processing the samples and analyzing the data; C.V. and J.M.B. performed the quantitative PCR for SIV gag DNA; S.N.G. provided the data on experimentally SIV-infected SMs and RMs; S.E.B. and N.F. determined CCR5 mRNA levels; P.L.H. performed the sorting experiments; T.S., M.L.C., and M.P.D. contributed to the design of the study and statistical analyses; J.E. supervised the housing and care of the animals and contributed to the design of the study and sample collection; J.M and F.K. provided the GFP-expressing SIV reporter virus; C.A., I.P., N.E.R., F.K., and R.G.C. contributed to the study design and preparation of the manuscript.

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memory T-cells (T_{CM}). CD4+ T-cell activation was similarly uncoupled from CCR5 expression in SMs *in vivo* during (i) acute SIV infection and (ii) following antibody-mediated CD4+ T-cell depletion. Remarkably, CD4+ T_{CM} of SMs that express low levels of CCR5 demonstrated reduced susceptibility to SIV infection both *in vivo* and *in vitro* when compared to CD4+ T_{CM} of RMs. These data suggest that low CCR5 expression on SM CD4+ T-cells favors the preservation of CD4+ T-cell homeostasis and promotes an AIDS-free status by protecting CD4+ T_{CM} from direct virus infection.

Introduction

Human Immunodeficiency Virus type-1 (HIV-1) infection of humans and Simian Immunodeficiency Virus (SIV) infection of rhesus macaques (RMs) lead to a progressive immune deficiency known as AIDS. In contrast, SIV infections of African “natural host” species, such as sooty mangabeys (SM) and African green monkeys (AGMs), are typically non-pathogenic despite high levels of virus replication[1,2]. Understanding the reasons why “natural” SIV infections are non-progressive is a key priority in contemporary AIDS research, with significant implications for HIV pathogenesis, therapy, and vaccines[3].

Previous studies showed that SIV_{smm} infection of SMs is characterized by: (i) peak of viremia at day 10–15 post-infection, followed by a post-peak decline to set-point levels of 10^4–10^6 copies/ml of plasma[4,5]; (ii) a vigorous but transient immune response to the virus, with marked activation of the type-I Interferon response[6,7]; (iii) early depletion of mucosal CD4+ T-cells with relative preservation of Th17 cells[4,8]; (iv) maintenance of healthy peripheral CD4+ T-cell counts in ~80–90% of animals[9]; (v) short *in vivo* lifespan of productively infected cells[10]; (vi) absence of microbial translocation and mucosal immune dysfunction[4,11]; (vii) low levels of immune activation throughout the chronic phase of infection[4,12,13].

The limited immune activation of SIV-infected SMs represents a striking difference with the generalized immune activation that characterizes pathogenic HIV and SIV infections[14,15]. Several hypotheses have been proposed to explain this phenotype, including SIV_{smm}Nef-mediated down-modulation of CD3, rapid up-regulation of PD-1 in lymph nodes, lower plasmacytoid dendritic cells response to TLR ligands, and lack of microbial translocation[11,16–19]. Of note, the fact that SIV infection of SMs is associated with a transient phase of strong immune responses to the virus suggests the involvement of immune regulatory mechanisms, as opposed to a genetically determined inability to sense the virus[6]. Currently, the mechanisms by which SIV-infected SMs avoid chronic immune activation remain poorly understood, and it remains unclear to what extent this lack of immune activation represents a cause rather than a consequence of preserved immune function.

We previously reported that in natural SIV hosts the fraction of circulating and mucosal CD4+ T-cells expressing the SIV co-receptor CCR5 is markedly lower than in humans and RMs[20]. However, the interpretation of this observation has been complicated by the high viremia of naturally SIV-infected SMs[13,21]. In this study, we discovered that central memory CD4+ T-cells (T_{CM}) of SMs are exquisitely resistant to CCR5 up-regulation upon *in vivo* and *in vitro* activation, and that this pattern of reduced CCR5 expression is

associated with lower *in vivo* and *in vitro* susceptibility of CD4+ T_{CM} to direct SIV infection. These data define a novel and potentially critical mechanism of AIDS resistance in SIV-infected SMs characterized by selective protection of CD4+ T_{CM} from virus infection due to limited CCR5 up-regulation.

Results

The fraction of CD4+CCR5+ T_{CM} is significantly lower in SMs than RMs

We previously showed that the fraction of CD4+CCR5+ T-cells is lower in natural SIV hosts compared to humans and RMs[20]. To further investigate this phenomenon, we first assessed the fraction of naive (T_N, CD28+CD95−), central memory (T_{CM}, CD95+CD62L+) and effector memory (T_{EM}, CD95+CD62L−) CD4+ and CD8+ T-cells of uninfected RMs and SMs that express CCR5 on their surface (Fig. 1a,b)[9,22,23]. We found that the fraction of CCR5+ cells was significantly lower in all CD4+ T-cell subsets of SMs when compared to RMs ($p<0.0001$ for T_N, T_{CM}, and T_{EM}, Fig. 1c). Of note, in both species CD4+ T_{EM} cells expressed higher levels of CCR5 when compared to T_{CM} ($p<0.001$), with the lowest CCR5 expression on T_N (Fig. 1c). Lower fractions of CCR5+ cells were also observed for all subsets of CD8+ T cells of SMs as compared to RMs ($p=0.0084$ for T_N; $p<0.0001$ for T_{CM} and T_{EM}, Figure 1c). Collectively, these data indicate that reduced expression of CCR5 is a phenomenon that involves all CD4+ T-cell subsets of SMs, including the CD4+ T_{CM}, that, in contrast to RM CD4+ T_{CM}, are by and large CCR5 negative.

Limited CCR5 expression upon *in vitro* activation in CD4+ T-cells of SMs

To determine how *in vitro* activation affects CCR5 expression, we next measured the fraction of CD4+CCR5+ T-cells in PBMCs isolated from uninfected SMs and RMs that were stimulated with mitogens (ConA/IL-2) for eight days. CD4+ T-cells from SMs and RMs became activated and proliferated to similar extent, as determined by the expression of the proliferating marker Ki-67 (Fig. 2a) and the activation marker CD69 (data not shown). In contrast, the fraction of CD4+ T-cells expressing CCR5 was remarkably different between SMs and RMs. At baseline, the fraction of CD4+CCR5+ T-cells was higher in RMs, but a transient down-regulation of CCR5 expression in RMs resulted in similar fractions of CD4+CCR5+ T-cells by 24-hours post-stimulation. Importantly, at later time points the fraction of CD4+CCR5+ T-cells increased only in RMs, becoming significantly higher than in SMs by 120 hours post-stimulation (Fig. 2b,c). This phenomenon was not restricted to CD4+ T-cells, as a similar, albeit less dramatic difference in activation-induced CCR5 up-regulation was observed for CD8+ T-cells (Fig. S1).

The reduced fraction of SM CD4+CCR5+ T-cells upon *in vitro* activation was also evident when double staining for Ki-67/CCR5 at 120 hours post-stimulation (Fig. 2d). To further define the relationship between cell proliferation and CCR5 up-regulation, we CFSE-labeled PBMCs and analyzed CCR5 expression on cells expressing various levels of CFSE dilution at 120 hours post-stimulation. SM and RM CD4+ T-cells diluted CFSE at similar levels, i.e., underwent the same number of cell divisions, but only in RM did a sizeable fraction of CD4+ T-cells that cycled once or twice up-regulate CCR5 expression (Fig. 2e). Intriguingly, most of the RM CD4+ T-cells that divided several times were CCR5-negative. This result

suggests either that cycling CD4+CCR5+ T-cells proliferate more slowly than CD4+CCR5− T-cells, or that a fraction of cycling CD4+CCR5+ T-cells down-regulates CCR5 expression at later time points.

To next determine how stimuli other than mitogens affect CCR5 expression on CD4+ T-cells in SMs and RMs, we treated PBMCs with recombinant IL-7 (10ng/ml) as well as anti-CD3/CD28. Treatment with IL-7 resulted in a moderate increase of CD4+ T-cell proliferation in both SMs and RMs (Fig. 2f), but induced a 2–3-fold increase of the fraction of CD4+CCR5+ T-cells only in RMs (Fig. 2g). The IL-7-induced increase in CD4+CCR5+ T-cells observed in RMs was statistically significant at 72, 96, and 120 hours post-stimulation (p=0.0312; p=0.0156; p=0.0156, respectively) as compared to baseline. As previously reported in humans and macaques[24,25], treatment with anti-CD3/CD28 resulted in reduced fraction of CD4+ T-cells expressing CCR5 in both species (data not shown).

To determine whether this low fraction of SM CD4+CCR5+ T-cells following *in vitro* activation results from reduced CCR5 transcription vs. abnormal trafficking to the cell surface, we longitudinally assessed the levels of CCR5 mRNA on purified CD4+ T-cells at 0, 24, 72, and 120 hours post-stimulation. We found that the levels of CCR5 mRNA were significantly lower (p=0.0159) in CD4+ T-cells isolated from SMs when compared to RMs at 72 and 120 hours post-stimulation (Fig. 2h). Interestingly, at 120 hours post-stimulation the levels of CCR5 mRNA correlated directly with the fraction of CD4+CCR5+ T-cells (data not shown). In all, these data indicate that SMs CD4+ T-cells are relatively resistant to up-regulate CCR5 expression upon *in vitro* activation, and suggest that reduced transcription of *CCR5* contributes to this phenomenon.

Limited CCR5 expression upon *in vivo* activation in CD4+ T-cells of SMs

We next investigated how *in vivo* activation affects the fraction of CD4+CCR5+ T-cells in SMs and RMs by using two experimental conditions associated with CD4+ T-cell activation, i.e., acute SIV-infection and Ab-mediated CD4+ T-cell depletion. We first measured the levels of circulating CD4+CCR5+ T-cells during the acute phase of (i) pathogenic SIV_{mac239} infection of five RMs, and (ii) non-progressive SIV_{smm} infection of four SMs. In both species, acute SIV infection was associated with a detectable increase in T-cell activation and proliferation that lasted for 4–6 weeks[4]. Of note, the SIV-induced CD4+ T-cell activation was associated with increased fractions of CD4+CCR5+ T-cells in RMs, but not in SMs, with a statistically significant difference in the area under the curve (AUC) (p=0.0159) (Fig. 3). In the second experiment, monoclonal anti-CD4 antibody was administered four times over a 10-day period (dotted lines in Fig. 3c,d) to three uninfected RMs and SMs. This treatment induced a severe depletion of CD4+ T-cells followed by an increase in CD4+ T-cell proliferation that we interpreted as a homeostatic response[26]. Despite similar kinetics of CD4+ T-cell proliferation (data not shown), the AUC of the percentage of CD4+CCR5+ T-cells, expressed as both fraction (p=0.0127) and fold change (p=0.0389) vs. pre-depletion, was higher in RMs than SMs. The fact that CD4+ T-cells from SMs transiently up-regulated CCR5 expression early after depletion indicates that these cells are not genetically unable to express this molecule, but rather control CCR5 expression at the transcriptional and/or translational and/or post-translational level. Together with the

results presented in Figure 2, these findings demonstrate that SM CD4+ T-cells are resistant to CCR5 up-regulation upon various types of *in vitro* and *in vivo* stimulation.

Low fraction of CCR5+ cells after *in vitro* activation of SM CD4+ T_{CM}

Progression to AIDS in SIV_{mac239}-infected RMs is predicted by depletion of CD4+ T_{CM}[27–29]. Here we hypothesized that reduced up-regulation of CCR5 expression on CD4+ T_{CM} upon activation promotes the preservation of CD4+ T-cell homeostasis and the non-pathogenic outcome of SIV infection in SMs. To test this hypothesis, we longitudinally determined the fraction of CCR5+ cells in sorted CD4+ T_{CM} and T_{EM} after *in vitro* activation. While sorted CD4+ T_{EM} from both species showed similar kinetics of CCR5 expression (Fig. 4a), only CD4+ T_{CM} from RMs consistently up-regulated the expression of CCR5, which was significantly higher in RM than SM CD4+ T_{CM} at 48 (p=0.0087), 72 (p=0.0043), 96 (p=0.0041), 120 (p=0.0007), and 144 (p=0.0047) hours post-stimulation (Fig. 4b). The kinetics of CCR5 expression on *in vitro* activated, sorted T_{CM} and T_{EM} of SMs and RMs for each individual monkey as measured at selected time points after activation is also presented (Fig. S2). In all, these results indicate that CD4+ T_{CM} of SMs show a significant reduction of the activation-induced CCR5 up-regulation.

SM CD4+ T_{CM} are relatively resistant to SIV infection

We next investigated whether the different pattern of CCR5 expression observed in CD4+ T_{CM} and T_{EM} of SMs and RMs is associated with different susceptibility to SIV infection. We first determined the fraction of SIV-infected cells (measured as fraction of cells expressing SIVgag DNA[27]) in 18 chronically SIV_{smm}-infected SMs and 7 chronically SIV_{mac239}-infected RMs. We found that the fraction of SIV-infected CD4+ T_{CM} of SMs was approximately one log lower than in RMs (p=0.0008) (Fig. 5). In contrast, no significant difference was observed between SMs and RMs in terms of SIV-infected CD4+ T_{EM} (Fig. 5). We next sought to confirm *in vitro* the finding that SM CD4+ T_{CM} are relatively resistant to SIV infection *in vivo*. We first purified CD4+ T_{CM} and T_{EM} of six uninfected SMs and infected them *in vitro* with a strain of SIVsmm (i.e., M949) that also replicates in primary CD4+ T-cells of RMs[30]. Consistent with the *in vivo* observation, the levels of SIV replication (i.e., p27 in the supernatant) were significantly lower in CD4+ T_{CM} than CD4+ T_{EM} at days 6 (p=0.0194), 9 (p=0.0304), 12 (p=0.0304), and 15 (p=0.0301) post-infection (Fig. 6a). As a control, highly purified CD4+ T_{CM} and T_{EM} of eleven RMs were infected *in vitro* using the macaque-adapted SIV_{mac} (6 RMs) or the same virus used for SMs (SIV_{smm} M949; 5 RMs). In contrast to SMs, RMs showed similar (when infected with SIV_{mac}, Fig. 6b) or even higher (when infected with SIV_{smm}, Fig. 6c) levels of SIV replication in CD4+ T_{CM} than CD4+ T_{EM}. As a result of this shift in cell targets, the T_{CM}:T_{EM} ratio of SIV replication was significantly higher in RMs than SMs at all time points (Fig. 6d). To further confirm the reduced susceptibility to SIV infection of SM CD4+ T_{CM}, we infected unfractionated PBMCs from eight SMs with eGFP-expressing SIV_{smm}, and measured the fraction of GFP+ cells within the CD4+ T_{CM} and T_{EM} subsets at day 4 post-infection. We found that the fraction of GFP+ cells was ~1 log lower in SM CD4+ T_{CM} than CD4+ T_{EM} (p=0.0006) (Fig. 6e,f). Collectively, these findings indicate that CD4+ T_{CM} of SMs are less susceptible, both *in vivo* and *in vitro*, to direct SIV infection as compared to both CD4+ T_{EM} of SMs and CD4+ T_{CM} of RMs.

Discussion

Understanding the reasons why natural SIV hosts, such as the SMs, do not progress to AIDS despite high viremia is a key unanswered question in AIDS research, with important ramifications for HIV pathogenesis, therapy, and vaccines[1,3]. Here, we describe and characterize a fundamental, species-specific difference in the regulation of CCR5 expression and susceptibility to SIV infection by CD4+ T_{CM} of SMs and RMs. We show reduced CCR5 expression in CD4+ T_{CM} of SMs after *in vitro* activation, which is associated with: (i) lower levels of *in vivo* SIV-DNA-positive CD4+ T_{CM} in SIV-infected SMs; and (ii) reduced permissivity to *in vitro* SIV infection in SM CD4+ T_{CM} as compared to both CD4+ T_{EM} of SMs and CD4+ T_{CM} of RMs. Based on these results we propose that, in SIV-infected SMs, low CCR5 up-regulation upon activation protects CD4+ T_{CM} from virus-mediated depletion, and thus favors the preservation of CD4+ T-cell homeostasis.

The association between depletion of CD4+ T_{CM} and progression to AIDS has been elegantly shown in SIV_{mac}-infected RMs[27–29]. In these studies, the Authors used a complex combination of *in vivo* BrdU labeling and longitudinal tissue sampling to demonstrate that while insufficient delivery of effector CD4+ T-cells to mucosal tissues is the hallmark of rapid SIV progression, depletion of CD4+ T_{CM} during chronic infection predicts progression to AIDS better than viral load[27–29]. Our current data are fully consistent with a model in which a key determinant of AIDS pathogenesis is the progressive loss of CD4+ T_{CM} homeostasis. Of note, mechanisms of CD4+ T_{CM} loss may include not only direct virus infection, as emphasized in this study, but also bystander cell death, proliferative senescence, diminished input of naïve cells, and loss of anatomic niche[14,31]. Specifically, our data suggest that a fine-tuned control of CCR5 expression in SM CD4+ T_{CM} helps protect this cell subset from SIV infection when these cells are recruited to proliferate in response to either antigenic stimulation or homeostatic stimuli.

A recent study in AGMs has shown that CD4+ T-cells of these animals down-modulate the expression of the CD4 molecule upon transition from naïve to memory cells[32]. In this context, our study suggests that two species of natural SIV hosts may have reached the same evolutionarily important goal, i.e., to protect CD4+ T_{CM} from SIV infection, by down-modulating either one of the two main co-receptors for virus entry, i.e., CD4 in AGMs and CCR5 in SMs. It is tempting to speculate that this choice was dictated, in evolutionary terms, by the ability (or lack thereof) of the species-specific SIVs to use additional co-receptors, whose expression may or may not be down-regulated in CD4+ T_{CM}. The ability of SMs to protect their CD4+ T_{CM} cells from SIV infection may occur not only at the entry level (via regulation of CCR5 expression), but also at the level of cellular restriction factors that interfere with virus replication at the post-entry level.

The complexity of the mechanisms involved in protecting SM CD4+ T_{CM} from SIV infection is emphasized by the observation that ~6% of SIV-infected SMs are homozygous for allelic variants of CCR5 that are not expressed on the cell surface, thus indicating that additional co-receptors are used by SIV_{smm} in these animals[33]. Whether and to what extent additional, non-CCR5-dependent mechanisms of SIV entry occur in SIV-infected SMs with wild-type CCR5 remains to be determined. In this context, it is conceivable that, in SIV-

infected SMs, resistance of CD4+ T_{CM} to viral infection and/or killing is achieved through "multiple imperfect gatekeepers" that reduce permissivity to SIV more effectively than a "single strong gatekeeper"[34]. A full elucidation of the molecular determinants of the species- and cell type-specific regulation of CCR5 expression on CD4+ T_{CM} of SMs as compared to CD4+ T_{CM} of RMs and humans is not within the scope of the current body of work. However, our data on CCR5 mRNA expression after *in vitro* mitogen stimulation of CD4+ T-cells of SMs and RMs suggest that this molecule is regulated, at least in part, at a transcriptional level. Whether this transcriptional regulation occurs as a result of epigenetic phenomena (i.e., DNA methylation) or is the result of different activation of specific transcription factor(s) will be object of future studies.

One of the most striking features of natural SIV infections is the presence of low levels of T-cell activation during the chronic phase of infection[1,2]. Based on the current set of results, we propose that the low levels of immune activation in chronically SIV-infected SMs may represent, at least in part, a consequence of the ability of these animals to reduce the level of virus infection in CD4+ T_{CM}. In this view, the depletion of CD4+ T_{CM} occurring during pathogenic HIV and SIV infections may trigger a vicious cycle of increased CD4+ T-cell proliferation, production of pro-inflammatory/pro-apoptotic cytokines, and virus replication. It is also conceivable that, in pathogenic infections, higher levels of virus replication in anatomic sites (i.e., lymph nodes, spleen, Peyer patches) that are both enriched in T_{CM} and involved in the generation of innate and adaptive immune responses may contribute to the observed higher levels of chronic immune activation by dramatically increasing the local antigenic load.

While much work remains, these results strongly suggest that, in SIV-infected SMs, regulation of CCR5 expression on CD4+ T_{CM} and protection of these cells from direct virus infection are key factors that favor CD4+ T-cell homeostasis and limit immune activation, thus identifying a novel, and potentially critical, mechanism used by these animals to avoid AIDS despite continuous virus replication.

Methods

Animals

Sixty-nine (42 females and 27 males SIV-uninfected RMs and thirty-seven (24 females and 13 males) SIV-uninfected SMs, plus twelve (all males) SIV-infected RMs and twenty-two (8 females and 14 males) SIV-infected SMs were included in this study. Animals homozygous for CCR5-null alleles were excluded from this study. All animals were housed at the Yerkes National Primate Research Center of Emory University, Atlanta, GA, and maintained in accordance with NIH guidelines. Anesthesia was used for all blood collections. All studies were approved by the University of Pennsylvania and Emory University Institutional Animal Care and Usage Committees.

Cell stimulation

Peripheral blood mononuclear cells (PBMC), isolated by density gradient centrifugation, or purified central memory (T_{CM}) and effector memory (T_{EM}) CD4+ T-cells were cultured at

37°C in the presence of ConA (1 μg/ml) and IL-2 (30 U/ml) at the concentration of 1×10^6 cells/ml. In a subset of animals, PBMC were cultured in the presence of IL-7 (10ng/ml), or with anti-CD3 plus anti-CD28. The expression of CCR5 on CD4+ and CD8+ T-cells was assessed every 24 hours by flow cytometry. To determine *in vitro* T-cell proliferation RM and SM PBMC were labeled with CFSE (3μM) and stimulated as described above. The fraction of CD4+ T lymphocytes that dilute CFSE and express CCR5 was measured by flow cytometry at 120 hours post-activation.

Flow cytometry

The staining for flow cytometry studies was performed according to standard procedures using monoclonal antibodies that are cross-reactive with SMs and RMs[4,23]. Fourteen-parameter flow cytometric analysis was performed using a LSR II flow cytometer driven by the DiVA software package (Becton Dickinson, San Jose, CA). Analysis of the acquired data was performed using FlowJo software (Tree Star, Inc., Ashland, OR).

CCR5 mRNA levels

PBMC from RMs and SMs were cultured in the presence of ConA and IL-2. CD4+ T-cells were sorted from PBMCs at 0, 24, 72, and 120 hours post-stimulation using "CD4 MicroBeads for non human primate" (Miltenyi Biotec). Total RNA was isolated from purified CD4+ T-cells using the RNAeasy extraction kit (Qiagen, Valencia, CA). The quantification of mRNAs levels for CCR5 was performed using RT-PCR on the 7900HT Real-Time PCR System (Applied Biosystems) using both SYBR-Green and TaqMan assays. Levels of CCR5 mRNAs were normalized to the expression of the housekeeping gene GAPDH. Primers that detect CCR5 expression were designed based on the available sequences in RMs and SMs. SYBR: CCR5_F 5′-AGGGCTGTGAGGCTTATCTTC-3′; CCR5_R 5′-CACCTGCATGGCTTGGTCCA-3′. TaqMan: CCR5_F 5′-GAGATTTTCAGACGTCACCA-3′; reverse: 5′-ACCCCAGATACAATCTATCCG-3′; probe: 5′-TGTACAGTCATATCAAGCTCTCTTGG-3′.

Cell sorting

Sorting of CD4+ T_{EM} and T_{CM} cells from RMs and SMs, both infected and uninfected, was performed on a FACSVantage SE flow cytometer (Becton Dickinson, San Jose, CA). Cells were initially gated based on light scatter, followed by positive staining for CD3 and CD4. CD4+ T_{EM} and T_{CM} cell subsets were gated based upon characteristic expression patterns of CD28, CD95, and CD62L (see Fig. 1a for a representative gating strategy).

Quantitative PCR for SIV gag DNA

Quantification of SIV_{smm} or SIV_{mac} gag DNA was performed as previously described[27]. For cell number quantification, qPCR was performed simultaneously for monkey albumin gene copy number. The sequence of the forward primer for SIV_{smm} is 5′-GGCAGGAAAATCCCTAGCAG-3′. The reverse primer sequence is 5′-GCCCTTACTGCCTTCACTCA-3′. The probe sequence is 5′-AGTCCCTGTTCRGGCGCCAA-3′. The sequence of the forward primer for SIV_{mac} is 5′-GTCTGCGTCAT(T/C)TGGTGCATTC-3′. The reverse primer sequence is 5′-CACTAG(C/

T)TGTCTCTGCACTAT(A/G)TGTTTTG-3′. The probe sequence is 5′-CTTC(A/G)TCAGT(C/T)TGTTTCACTTTCTCTTCTGCG-3′.

In vitro SIV infections

Susceptibility of SM and RM CD4+ T-cells to *in vitro* SIV infection was assessed using total PBMC (in SMs) and purified CD4+ T_{CM} and T_{EM} (in both species). Unfractionated PBMCs from eight SMs were stimulated with ConA/IL-2 for 3 days, and then infected with a replication competent eGFP-expressing clones of SIV_{smm} FGb containing a IRES-eGFP fragment downstream of *nef*. The fraction of SM CD4+ T_{CM} and T_{EM} that express GFP was determined by flow cytometry at day 4 post-infection. For the experiments involving purified cell subsets, CD4+ T_{CM} and T_{EM} were sorted from six SMs and eleven RMs and stimulated with ConA/IL-2 for 3 days. Cells were then infected with SIV_{smm} M949, a strain that replicates in primary CD4+ T-cells of both species[30], or SIV_{mac}, by spin inoculation. Cells were then washed in PBS and maintained in media containing IL-2 (50 U/ml). Cell supernatants were collected before infection (day 0) and at days 3, 6, 9, 12 and 15 after infection, and replication measured by SIV Gag p27 ELISA (Advanced BioScience Laboratories).

Statistical analyses

The performed analyses for comparisons between groups include the one-way ANOVA and two-tailed t test or Mann-Whitney test. In addition, the area under the curve (AUC) has been determined in the longitudinal studies of experimental SIV infection and CD4+ T-cell depletion (Fig. 3). Significance was assessed at $p<0.05$ levels. All analyses were performed using the Prism 5.0 software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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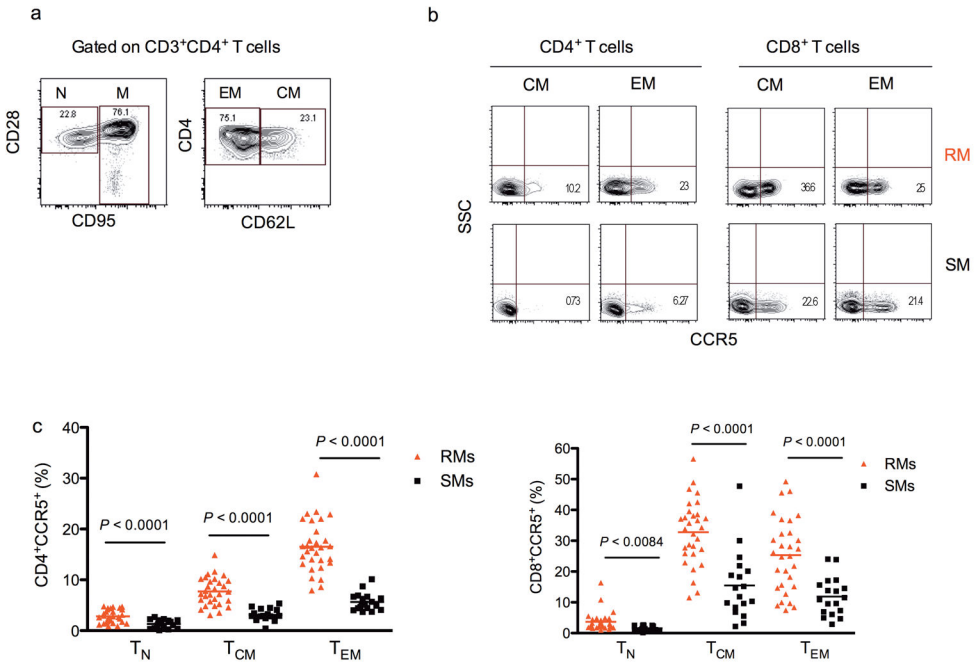

Figure 1. The fraction of CCR5+ cells *ex vivo* is significantly lower in all subsets of CD4+ T-cells of SMs as compared to RMs

The fraction of CCR5+ cells was determined in the naive (T_N), central memory (T_{CM}) and effector memory (T_{EM}) subsets of CD4+ and CD8+ T-cells of 18 SIV uninfected sooty mangabeys (SMs) and 30 SIV uninfected rhesus macaques (RMs). (**a**) T_N, T_{CM}, and T_{EM} cells were defined based on the expression of the surface markers CD28, CD95, and CD62L, as showed for CD4+ T-cells in a representative SM. (**b**) Staining of CCR5 on T_{CM} and T_{EM} CD4+ and CD8+ cells in a representative SM and a representative RM. (**c**) Fraction of CD4+ (left graph) or CD8+ (right graph) T-cells that express CCR5 in 18 uninfected SMs (■) and 30 uninfected RMs (▲). Statistical analyses were performed to compare, in SMs versus RMs, the fraction of CCR5+ cells within each CD4+ and CD8+ T-cell subset.

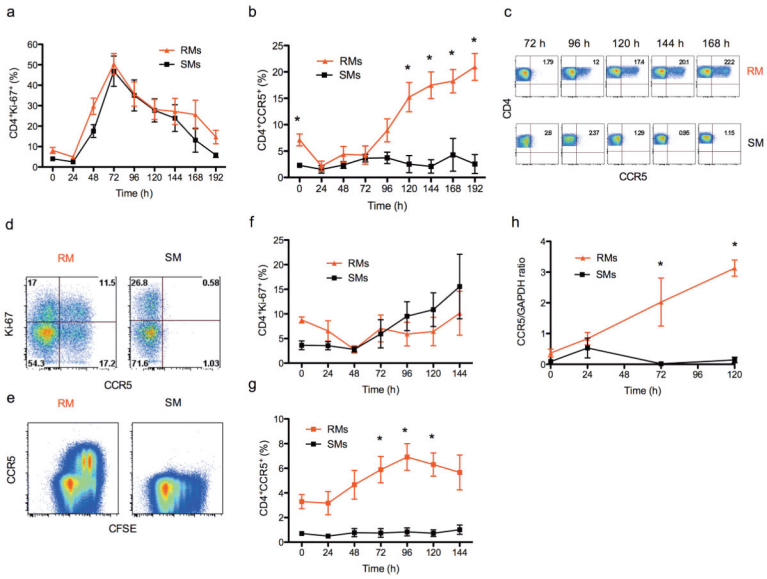

Figure 2. CCR5 expression upon *in vitro* activation and proliferation is significantly lower in CD4+ T-cells of SMs than RMs

Fractions of CD4+CCR5+ T-cells were determined in PBMCs from uninfected SMs (■) and RMs (▲) after *in vitro* stimulations. Fraction of CD4+Ki-67+ (**a**) and CD4+CCR5+ (**b**) T-cells following stimulation with ConA/IL-2. Asterisks indicate time points when the fraction of CD4+CCR5+ T-cells was significantly lower in SMs than RMs (p values are detailed in the Result section). (**c**) Representative dot plots showing the fraction of CD4+CCR5+ T-cells post-stimulation in RMs (top) and SMs (bottom). (**d**) Flow cytometry dot plots showing Ki-67/CCR5 double staining in a representative RM and SM at 120 hours post stimulation with ConA/IL-2. (**e**) PBMCs isolated from RMs and SMs were labeled with CFSE prior to mitogen stimulation; levels of CCR5 were analyzed on cells expressing various levels of CFSE dilution at 120 hours post-stimulation. (**f**) and (**g**) PBMCs isolated from SMs and RMs were stimulated with recombinant IL-7, and the fraction of CD4+Ki-67+ (**f**) and CD4+CCR5+ (**g**) T-cells determined following stimulation. Asterisks indicate time points where, in RMs, the IL-7-induced increase in CD4+CCR5+ T-cells (as compared to baseline) was statistically significant (p values are detailed in the Result section). (**h**) In a subset of animals, levels of CCR5 mRNA (expressed as CCR5/GAPDH ratio) were determined on purified CD4+ T-cells at 0, 24, 72 and 120 hours post-stimulation with ConA/IL-2. Statistical analyses were performed to compare CCR5/GAPDH ratio between SMs and RMs.

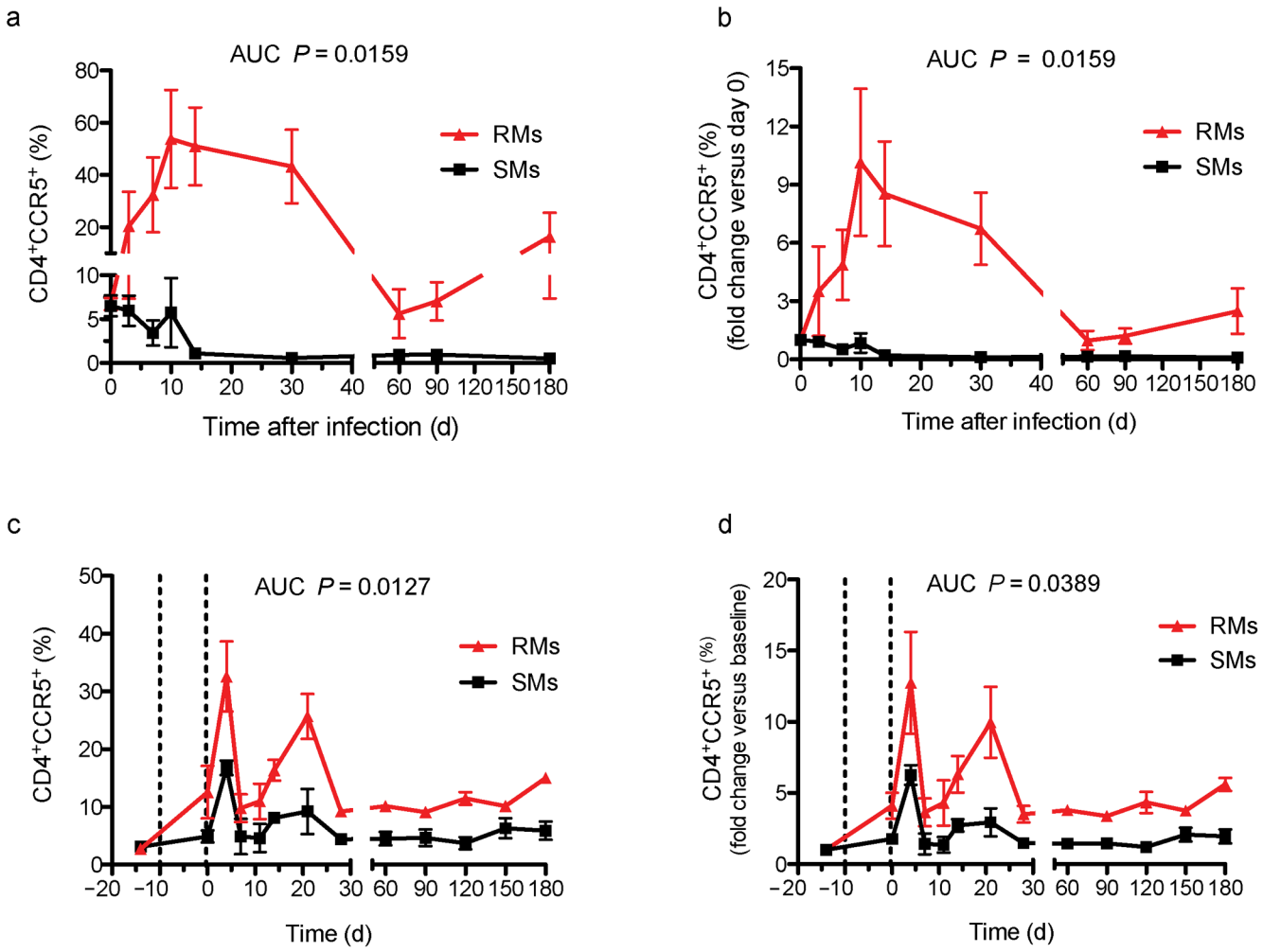

Figure 3. The fraction of CD4+CCR5+ T-cells upon *in vivo* activation is significantly lower in SMs as compared to RMs

We determined the fraction of CD4+CCR5+ T-cells from SMs (■) and RMs (▲) in two *in vivo* experimental conditions associated with activation of the CD4+ T-cell compartment, i.e., acute SIV-infection and Ab-mediated CD4+ T-cell depletion. Fraction (**a**) and fold change vs pre-infection, i.e. day 0 (**b**) of CD4+CCR5+ T-cells at different time points during (i) pathogenic SIV_{mac239} infection of five RMs and (ii) non-progressive experimental SIV_{smm} infection of four SMs. SIV-induced CD4+ T-cell activation is associated with an increased fraction of CD4+CCR5+ T-cells in RMs, but not in SMs, with a statistically significant difference in the area under the curve (AUC). Fraction (**c**) and fold change vs pre-depletion, i.e. day -14 (**d**) of CD4+CCR5+ T-cells at different time points following Ab-mediated CD4+ T-cell depletion in three uninfected RMs and three uninfected SMs. The AUC of the fraction of CD4+CCR5+ T-cells is significantly higher in RMs than SMs. The dotted lines in (**c,d**) indicate the 10-day period in which the anti-CD4 antibody was administered.

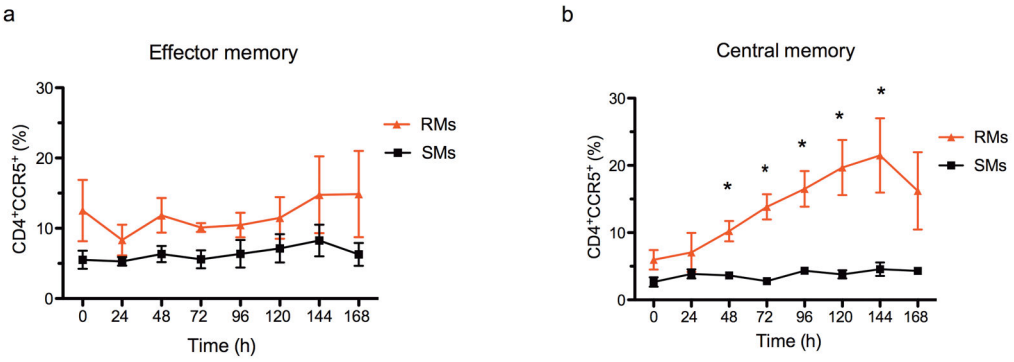

Figure 4. Lower fraction of CCR5+ cells after *in vitro* activation of sorted CD4+ T_{CM} of SMs as compared to RMs

The fractions of sorted CD4+ T_{CM} and T_{EM} that express CCR5 were longitudinally determined after *in vitro* mitogen activation in eight SIV-uninfected SMs (■) and eight SIV-uninfected RMs (▲) T_{CM} (CD95+CD62L+) and T_{EM} (CD95+CD62L−) cells that were sorted and stimulated with ConA/IL-2. The graphs show the fraction of CD4+CCR5+ T_{EM} (**a**) and T_{CM} (**b**) cells at different time points following stimulation. Asterisks indicate time points where values are significantly higher in RMs as compared to SMs.

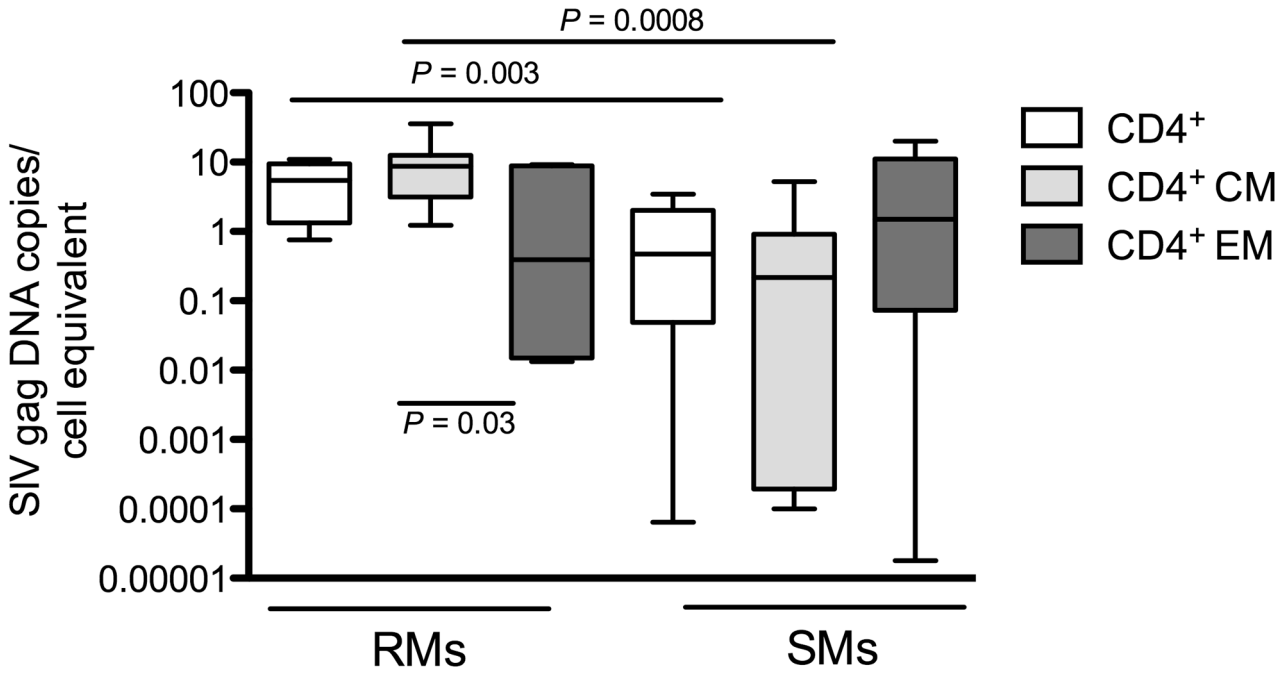

Figure 5. CD4+ T_{CM} of SMs are relatively resistant to SIV infection *in vivo*

The fraction of SIV-infected CD4+ (white boxes), CD4+ T_{CM} (light gray) and T_{EM} (dark gray) cells, determined measuring by q-PCR the number of SIVgag DNA copies for cell equivalent, was determined in 18 naturally SIV_{smm} infected SMs and 7 experimentally SIV_{mac239} infected RMs. As showed in the graph, the fraction of SIV-infected CD4+ T_{CM} was significantly lower in SMs than in RMs (p=0.0008), while no significant difference was observed between the two species with respect to the level of SIV-infected CD4+ T_{EM}. Of note, in RMs the fraction of SIV-infected cells was significantly higher (p=0.03) in CD4+ T_{CM} than T_{EM}.

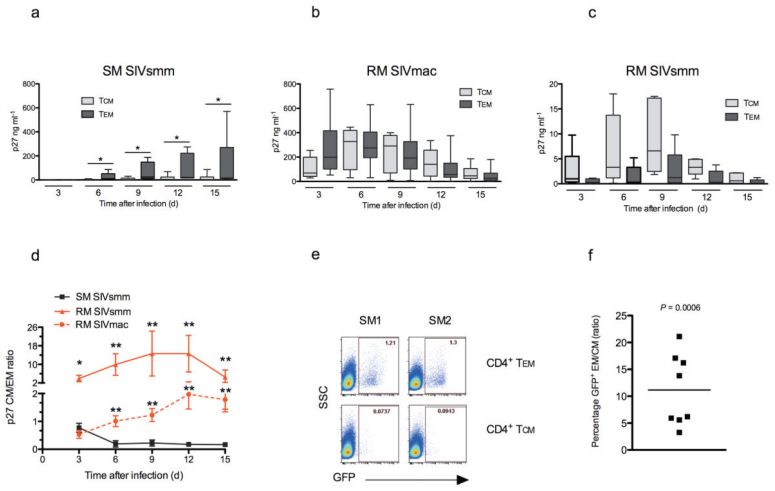

Figure 6. CD4+ T_{CM} of SMs are relatively resistant to SIV infection *in vitro*.

(**a–d**) CD4+ T_{CM} and T_{EM} purified from six SMs and eleven RMs were infected *in vitro* with SIVsmm M949 (six SMs and five RMs), or with SIV_{mac} (six RMs). The levels of SIV replication were determined by measuring p27 in the supernatants. In SMs (**a**) the levels of SIV replication were significantly lower in CD4+ T_{CM} than CD4+ T_{EM} at days 6, 9, 12, and 15 post-infection (as indicated by asterisks; p values are detailed in the Result section), while in RMs the levels of SIV replication in CD4+ T_{CM} where similar to those in CD4+ T_{EM} following infection with SIVmac (**b**) or higher than CD4+ T_{EM} following infection with SIVsmm (**c**). As a result, the T_{CM} vs T_{EM} ratios of levels of SIV replication were significantly higher (as indicated by asterisks; p values are detailed in the Result section) in RMs than SMs at all tested time points (**d**). (**e, f**) Unfractionated PBMCs from eight SMs were infected with replication competent eGFP-expressing clones of SIV_{smm}, and the fraction of infected SM CD4+ T_{CM} and T_{EM} that express GFP was determined by multiparametric flow cytometry at day 4 post-infection. Figure 6e shows flow plots of GFP expression in CD4+ T_{CM} and T_{EM} in two representative animals. In SMs the fraction of GFP+ cells (**f**) is approximately 1 log lower in CD4+ T_{CM} than CD4+ T_{EM} (p=0.0006).