

# Increased Stability and Limited Proliferation of CD4<sup>+</sup> Central Memory T Cells Differentiate Nonprogressive Simian Immunodeficiency Virus (SIV) Infection of Sooty Mangabeys from Progressive SIV Infection of Rhesus Macaques

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## ABSTRACT

Depletion of CD4<sup>+</sup> central memory T (TCM) cells dictates the tempo of progression to AIDS in simian immunodeficiency virus (SIV)-infected rhesus macaques (RMs) both in the natural history of infection and in the context of vaccination. CD4<sup>+</sup> TCM cells of sooty mangabeys (SMs), a natural host for SIV in which infection is nonpathogenic, are less susceptible to SIV infection than CD4<sup>+</sup> TCM cells of RMs. Whether this relative protection from infection translates into increased stability of CD4<sup>+</sup> TCM cells in natural versus nonnatural hosts has not yet been determined. Here we compared, both cross-sectionally and longitudinally, the levels of CD4<sup>+</sup> TCM cells in a large cohort of SMs and RMs and the association between CD4<sup>+</sup> TCM levels and the main virologic and immunologic markers of disease progression. Consistent with their lower susceptibility to infection, CD4<sup>+</sup> TCM cells of SIV-infected SMs are lost with kinetics 20 times slower than those of SIV-infected RMs. Remarkably, the estimated length of time of SIV infection needed for CD4<sup>+</sup> TCM cells to fall to half of their initial levels is <16 months for RMs but >17 years for SMs. Furthermore, the fraction of proliferating CD4<sup>+</sup> TCM cells is significantly lower in SIV-infected SMs than in SIV-infected RMs, and the extent of CD4<sup>+</sup> TCM cell proliferation is associated positively with CD4<sup>+</sup> T cell levels in SIV-infected SMs but negatively with CD4<sup>+</sup> T cell levels in SIV-infected RMs. Collectively, these findings identify increased stability and maintenance of the prohomeostatic role of CD4<sup>+</sup> TCM cells as features distinguishing nonprogressive from progressive SIV infections and support the hypothesis of a direct mechanistic link between the loss of CD4<sup>+</sup> TCM cells and disease progression.

## IMPORTANCE

Comparison of the immunologic effects of simian immunodeficiency virus (SIV) infection on rhesus macaques (RMs), a species characterized by progression to AIDS, and natural host sooty mangabeys (SMs), a species which remains AIDS free, has become a useful tool for identifying mechanisms of human immunodeficiency virus (HIV) disease progression. One such distinguishing feature is that CD4<sup>+</sup> central memory T (TCM) cells in SIV-infected SMs are less infected than the same cells in RMs. Here we investigated whether lower levels of infection in SMs translate into a better-preserved CD4<sup>+</sup> TCM compartment. We found that the CD4<sup>+</sup> TCM compartment is significantly more stable in SIV-infected SMs. Likely to compensate for this cell loss, we also found that CD4<sup>+</sup> TCM cells increase their level of proliferation upon SIV infection in RMs but not in SMs, which mechanistically supports their preferential infectivity. Our study provides new insights into the importance of long-term maintenance of CD4<sup>+</sup> TCM homeostasis during HIV/SIV infection.

The precise factors determining the rate of CD4<sup>+</sup> T cell decline, and ultimately the rate of progression to AIDS, in human immunodeficiency virus (HIV)-infected humans remain poorly defined. An understanding of this complex interplay between CD4<sup>+</sup> T cell homeostasis and immune control of the virus has been complicated by the paradoxical nature of their relationship (1). CD4<sup>+</sup> T cells are critical in enhancing both cellular and humoral immune responses that can effectively suppress virus replication, yet their activation makes these cells more susceptible to infection by HIV, thus creating more targets for virus replication (2, 3). In marked contrast to HIV-infected humans, and despite similar viral loads, natural simian immunodeficiency virus (SIV) hosts, such as sooty mangabeys (SMs) and African green monkeys (AGMs), generally maintain healthy CD4<sup>+</sup> T cell levels and avoid chronic immune activation, thus remaining AIDS free (4–10). Comparing and contrasting the mechanisms of CD4<sup>+</sup> T cell

homeostasis in natural hosts for SIV to those in experimentally SIV-infected rhesus macaques (RMs), which progress to AIDS, may provide important insights into the mechanisms of disease progression in HIV-infected humans.

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The ability of natural hosts of SIV to maintain low levels of immune activation despite high-level viremia represents a key difference between these infections and the typical pathogenic course of infection observed for HIV-infected humans and SIV-infected RMs. However, the mechanisms responsible for the benign nature of SIV infection in SMs and other natural hosts remain poorly understood. Several non-mutually exclusive mechanisms have been proposed to contribute to this phenomenon (7), including (i) preserved physical and immunological integrity of the mucosal barrier, with healthy levels of Th17 cells and an absence of microbial translocation into systemic circulation (11–13); (ii) timely resolution of the innate immune response initiated during the acute phase of infection (14–16); (iii) the preserved ability of the SIVsmm and SIVagm *nef* genes to downmodulate CD3/T cell receptor (TCR) expression (17); (iv) reduced expression of the dominant SIV coreceptor CCR5 on CD4<sup>+</sup> T cells (18); and (v) the ability of CD4<sup>+</sup> T cells to downmodulate the surface expression of CD4 during their differentiation into memory cells (in AGM), thus protecting this critical cell subset from SIV infection (19).

CD4<sup>+</sup> T cells are composed of several subsets that differ by phenotype, function, and anatomical localization. CD4<sup>+</sup> central memory T (TCM) cells express CD62L and CCR7, reside in lymph node (LN) and other inductive lymphoid tissues, and show limited effector functions but strong proliferation in response to antigenic restimulation (20). CD4<sup>+</sup> TCM cells are of particular importance for immune function since they are longer-lived, self-renewing cells that maintain CD4<sup>+</sup> T cell homeostasis by replenishing the pool of shorter-lived, non-self-renewing CD4<sup>+</sup> effector memory (TEM) cells (3). The importance of preserving CD4<sup>+</sup> TCM cell homeostasis is clearly highlighted by *in vivo* studies showing that depletion of CD4<sup>+</sup> TCM cells is the key factor dictating the tempo of progression to AIDS in SIV-infected RMs both in the natural history of infection and in the context of vaccination (21–24). Of note, circulating CD4<sup>+</sup> TCM cells are infected at high frequencies in the majority of HIV-infected humans both *in vivo* and *in vitro* and represent the largest reservoir of latently infected CD4<sup>+</sup> T cells in individuals treated with antiretroviral therapy (ART) (25–27). In a recent study, we showed that the infection frequencies of CD4<sup>+</sup> TCM cells of SMs are relatively lower than those of CD4<sup>+</sup> TCM cells of RMs (28). However, this difference was not found in CD4<sup>+</sup> TEM cells. This observation, originally made in blood (28), was also recently confirmed at the lymph node level (29). It is still unclear, however, whether and to what extent this protection from infection translates into increased stability and differential kinetics of depletion of CD4<sup>+</sup> TCM cells in natural versus nonnatural hosts.

To answer this question, we measured the number and proportion of CD4<sup>+</sup> TCM cells in a large cohort of SMs naturally infected with SIV that are housed at the Yerkes National Primate Research Center (YNPRC) as well as their association with the main virologic and immunologic markers of disease progression. These data were compared to those obtained for a cohort of experimentally SIV-infected RMs. We found that the levels of total and proliferating CD4<sup>+</sup> TCM cells are not significantly affected by SIV infection in SMs and that, in these animals, CD4<sup>+</sup> TCM cells are lost at a rate 20 times lower than in SIV-infected RMs. In addition, our results revealed that the extent of CD4<sup>+</sup> TCM cell proliferation is associated positively with the CD4<sup>+</sup> T cell levels in SIV-infected SMs but negatively with the levels of total CD4<sup>+</sup> T cells and CD4<sup>+</sup> TEM cells in RMs. These data support the hypothesis of a direct

mechanistic link between the loss of CD4<sup>+</sup> TCM cell homeostasis and disease progression as well as suggest that proliferation of CD4<sup>+</sup> TCM cells maintains its prohomeostatic role in SIV-infected SMs but becomes inefficient, if not deleterious, in maintaining CD4<sup>+</sup> T cell homeostasis in SIV-infected RMs.

## MATERIALS AND METHODS

**Animals.** Forty-six SIV-negative ( $11.2 \pm 0.7$  years old) and 94 naturally SIV-infected ( $16.9 \pm 0.4$  years old) SMs and 17 SIV-negative ( $9.1 \pm 0.6$  years old) and 23 experimentally SIVmac239-infected ( $7.4 \pm 0.7$  years old) RMs were included in the study. In the SIV-infected animals, the average durations of infection were  $12.0 \pm 0.5$  years for SMs, as estimated by the date of the first SIV-positive test, and  $0.8 \pm 0.1$  years for RMs. All animals were housed at the YNPRC, Atlanta, GA. Peripheral blood (PB) was collected from all animals at a single time point by using EDTA-containing tubes. Blood samples were used for a complete blood count and flow cytometry analyses, and plasma was separated by centrifugation within 1 h of phlebotomy. Due to the known downregulation of CD62L expression during the freeze-thaw process, all flow cytometry staining was performed on fresh blood.

**Study approval.** All animal experimentations were conducted according to guidelines established by the Animal Welfare Act and the NIH for housing and care of laboratory animals and were performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Usage Committee at the YNPRC.

**Flow cytometric analysis.** Twelve-parameter flow cytometric analysis was performed on whole-blood-derived cells according to standard procedures, using a panel of monoclonal antibodies that we and others have shown to be cross-reactive with SMs and RMs (28, 30, 31). Predetermined optimal concentrations of the following antibodies were used: anti-CD3-Alexa 700 (clone SP34-2), anti-CD3-allophycocyanin (APC)-Cy7 (clone SP34-2), anti-CD4-peridinin chlorophyll protein (PerCP)-Cy5.5 (clone L200), anti-CD8-Pacific Blue (PacBlue) (clone RPA-T8), anti-CD95-phycoerythrin (PE)-Cy5 (clone DX2), anti-CD62L-fluorescein isothiocyanate (FITC) (clone SK11), anti-CD62L-PE (clone SK11), anti-Ki-67-FITC (clone B56), and anti-Ki-67-Alexa 700 (clone B56) (all from BD Bioscience); anti-CD28-PE-Cy7 (clone CD28.2) (from eBioscience); anti-CD28-PE Texas Red (clone CD28.2) (from Beckman Coulter); anti-CD4-PacBlue (clone OKT4) (from Biolegend); and anti-CD8-Qdot705 (clone 3B5) (from Invitrogen). Flow cytometric acquisition was performed on an LSRII cytometer driven by FACS DiVa software. Analysis of the acquired data was performed by using FlowJo software (TreeStar).

**Viral load.** SIVsmm and SIVmac loads were measured in plasma samples by real-time PCR as previously described (32, 33).

**Statistical analysis.** Based on sample distribution (normal or nonnormal), *t* tests or Mann-Whitney tests were used to compare the differences of each parameter between SIV-negative and SIV-positive SMs or between SMs and RMs. Statistical tests were two sided. Pearson product-moment correlation coefficients were utilized to estimate linear associations for normally distributed data, and Spearman rank correlation coefficients were used for skewed and other nonnormal distributions. A *P* value of  $\leq 0.05$  was considered statistically significant. The means  $\pm$  standard errors of the means (SEM) were used for descriptive statistics for each parameter. In order to estimate the rate of loss of CD4<sup>+</sup> TCM cells for each group of animals, we used linear regression on the time since infection versus percent TCM cells. The slopes for SMs and RMs shown in Fig. 2E were compared by using analysis of covariance (ANCOVA).

## RESULTS

**Virologic and immunologic features of the SIV-infected SMs and RMs.** Forty-six SIV-uninfected and 94 naturally SIV-infected SMs as well as 17 SIV-uninfected and 23 experimentally SIV-infected RMs were included in this study. Within natural host SMs, the average age of infected animals was higher than that of unin-

infected animals ( $16.9 \pm 0.4$  years for SIV-infected SMs versus  $11.2 \pm 0.7$  years for SIV-uninfected SMs;  $P < 0.0001$ ). On the other hand, the average age of infected RMs was slightly lower than that of uninfected RMs ( $7.4 \pm 0.7$  years for SIV-infected RMs versus  $9.1 \pm 0.6$  years for SIV-uninfected RMs;  $P = 0.1125$ ), reflecting the fact that many studies involving experimental SIV infection of RMs preferentially involved younger animals. The average duration of infection in the SIV-infected SMs was  $12.0 \pm 0.5$  years, as estimated by the date of the first SIV-positive test, while the average duration of infection for RMs was  $0.8 \pm 0.1$  years. A well-documented feature that distinguishes natural SIV infection of SMs from progressive infection of RMs is the sustained preservation of circulating CD4<sup>+</sup> T cell counts despite high levels of virus replication (9, 32, 34, 35). To validate this feature in our study, we first compared the relative (fraction of CD3<sup>+</sup> T cells) and absolute (number of cells per mm<sup>3</sup> of blood) levels of CD4<sup>+</sup> T cells between SIV-infected and uninfected animals. As expected, both the fraction ( $33.97\% \pm 2.64\%$  versus  $57.88\% \pm 1.69\%$ ;  $P < 0.0001$ ) (see Fig. S1A in the supplemental material) and absolute number ( $315.8 \pm 51.8$  versus  $619.8 \pm 58.1$  cells/mm<sup>3</sup>;  $P = 0.0004$ ) (see Fig. S1B in the supplemental material) of CD4<sup>+</sup> T cells were significantly lower in SIV-infected than uninfected RMs. However, consistent with previous reports (9, 32, 34, 35), the large majority of SIV-infected SMs maintained their fraction and number of CD4<sup>+</sup> T cells at levels similar to those found in uninfected animals (see Fig. S1C and S1D in the supplemental material). As previously described (36), plasma levels of viral RNA copies were significantly higher in chronically SIV-infected RMs than in SIV-infected SMs ( $3.0 \times 10^6 \pm 1.0 \times 10^6$  copies/ml versus  $1.2 \times 10^5 \pm 1.2 \times 10^4$  copies/ml;  $P < 0.0001$ ) (data not shown). However, a significant loss of CD4<sup>+</sup> T cells was still present in SIV-infected RMs when only animals with viral loads similar to the average viral loads of the SIV-infected SMs were included, despite these animals being infected for  $<8$  months ( $41.3\% \pm 4.6\%$  versus  $58.9\% \pm 2.9\%$ ;  $P = 0.0193$ ) (data not shown). Collectively, these data confirm for this study cohort the well-described phenomenon of preservation of CD4<sup>+</sup> T cells in the vast majority of SMs naturally infected with SIV.

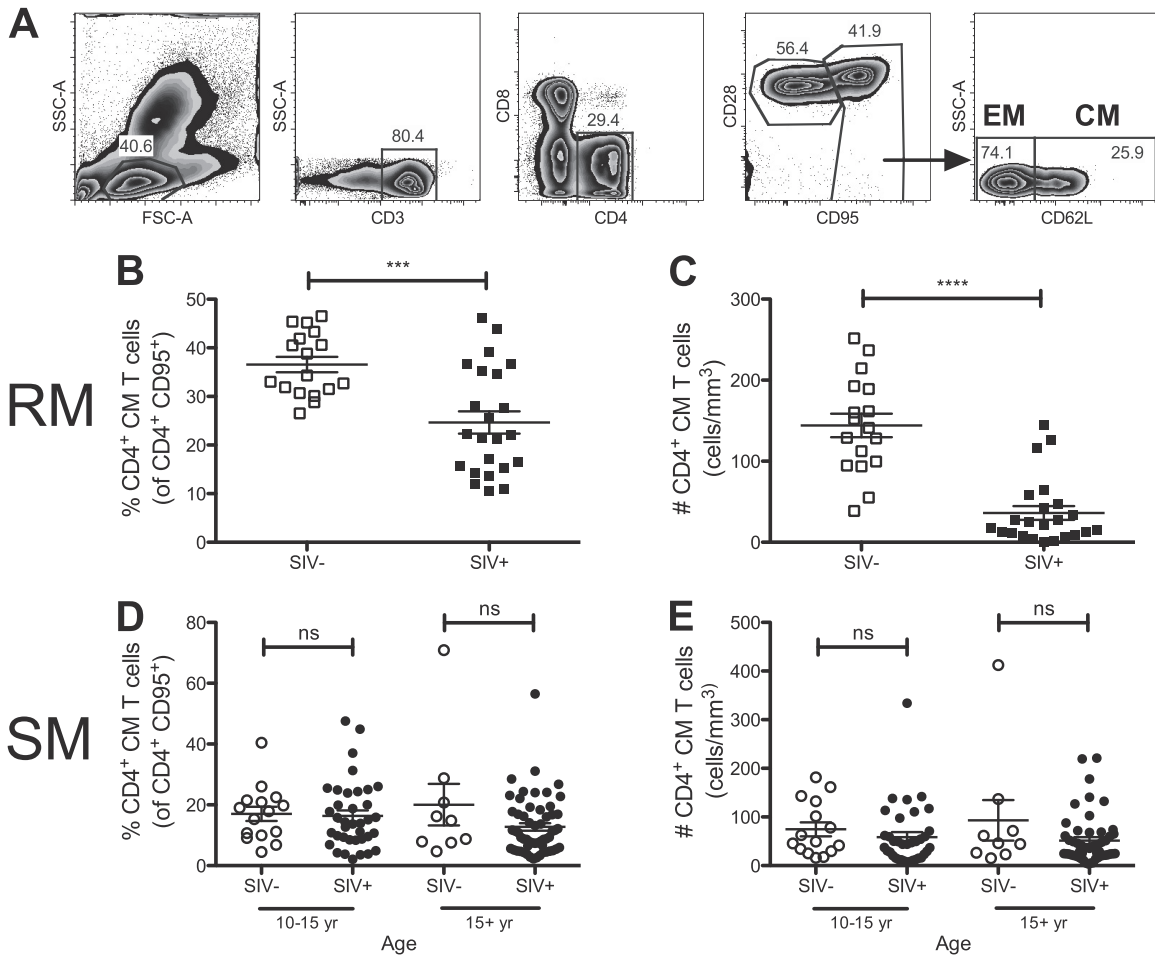
**CD4<sup>+</sup> TCM cells are preserved at healthy frequencies in SIV-infected SMs but are depleted in RMs.** The infection frequencies of CD4<sup>+</sup> TCM cells in natural host SMs are lower than those in RMs in both the blood and lymph node, and SM CD4<sup>+</sup> TCM cells are less permissive to SIV infection *in vitro* than are RM CD4<sup>+</sup> TCM cells (28, 29). Whether this lower level of infection translates into an increased stability of CD4<sup>+</sup> TCM cells in SIV-infected SMs compared to RMs has not yet been determined. To answer this question, we compared the relative (fraction of memory CD4<sup>+</sup> CD95<sup>+</sup> T cells) and absolute (number of cells per mm<sup>3</sup> of blood) levels of circulating CD4<sup>+</sup> T cells with a central memory (CD4<sup>+</sup> TCM) or effector memory (CD4<sup>+</sup> TEM) phenotype in SIV-infected and uninfected SMs and RMs. Specifically, cells pregated on lymphocytes, CD3<sup>+</sup>, and CD4<sup>+</sup> CD8<sup>-</sup> were defined as naive (CD28<sup>+</sup> CD95<sup>-</sup>) or memory (CD95<sup>+</sup>) CD4<sup>+</sup> T cells. CD4<sup>+</sup> CD95<sup>+</sup> memory T cells were then defined as TCM or TEM cells based on the expression, or lack thereof, of CD62L. Importantly, this combination of surface markers has been extensively characterized in nonhuman primates (28, 32, 37) and represents the same gating strategy used to determine the reduced frequency of infection of circulating CD4<sup>+</sup> TCM cells in SMs compared to the same cells in RMs (28). An example of the gating strategy used to

define CD4<sup>+</sup> TCM or TEM cells is shown for an SIV-infected SM in Fig. 1A.

Levels of CD4<sup>+</sup> TCM cells were compared between age-matched SIV-infected and uninfected SMs. This correction is particularly important due to the significant inverse correlation found between age and levels of CD4<sup>+</sup> TCM cells in SMs ( $P < 0.0001$ ) (see Fig. S2 in the supplemental material) and the fact that, within the YNPRC colony, the SIV-infected SMs are significantly older than SIV-uninfected animals ( $16.9 \pm 0.4$  versus  $11.2 \pm 0.7$  years of age;  $P < 0.0001$ ). However, because the age of SIV-infected RMs was not significantly different from that of SIV-uninfected RMs ( $7.4 \pm 0.7$  years versus  $9.1 \pm 0.6$  years of age;  $P = 0.1125$ ), and the age range in RMs was narrower, we did not stratify these animals by age. We found that the fraction of CD4<sup>+</sup> TCM cells was significantly lower in SIV-infected RMs than in SIV-uninfected RMs ( $24.6\% \pm 2.3\%$  versus  $36.6\% \pm 1.6\%$ ;  $P = 0.0003$ ) (Fig. 1B). As a consequence, the fraction of CD4<sup>+</sup> TEM cells was significantly higher in SIV-infected RMs than in uninfected RMs ( $75.4\% \pm 2.3\%$  versus  $63.4\% \pm 1.6\%$ ;  $P = 0.0003$ ) (see Fig. S3A in the supplemental material). The loss of CD4<sup>+</sup> TCM cells in SIV-infected RMs was particularly evident when expressed as an absolute count, with a severe decrease from  $144.2 \pm 14.6$  to  $36.2 \pm 8.5$  cells/mm<sup>3</sup> ( $P < 0.0001$ ) (Fig. 1C). Due to the overall depletion of CD4<sup>+</sup> T cells, the absolute number of CD4<sup>+</sup> TEM cells was also significantly lower in SIV-infected than in uninfected RMs ( $93.4 \pm 14.2$  versus  $250.1 \pm 25.4$  cells/mm<sup>3</sup>;  $P < 0.0001$ ) (see Fig. S3B in the supplemental material). In contrast to RMs, comparison of the levels of CD4<sup>+</sup> TCM cells in age-matched SMs revealed that the fraction and number of CD4<sup>+</sup> TCM cells were remarkably similar between SIV-infected and uninfected animals. For example, in animals 10 to 15 years old, the proportions of TCM cells in SIV-infected and uninfected animals were  $16.35\% \pm 1.82\%$  and  $17.04\% \pm 2.34\%$ , respectively, and the numbers of TCM cells were  $58.5 \pm 10.4$  and  $74.7 \pm 14.4$  cells/mm<sup>3</sup>, respectively. Likewise, for animals  $>15$  years of age, the proportion of TCM cells was  $12.74\% \pm 1.26\%$  for infected SMs versus  $20.05\% \pm 6.84\%$  for uninfected SMs (Fig. 1D), and the number of TCM cells was  $51.4 \pm 7.1$  cells/mm<sup>3</sup> versus  $93.0 \pm 41.7$  cells/mm<sup>3</sup> (Fig. 1E). Similarly, and supporting the model in which preservation of CD4<sup>+</sup> TCM cells results in maintenance of the whole CD4<sup>+</sup> T cell compartment, the fraction and absolute number of CD4<sup>+</sup> TEM cells were maintained in SIV-infected SMs compared to age-matched SIV-uninfected SMs (see Fig. S3C and S3D in the supplemental material). Taken together, these results suggest that, differently from RMs, SMs are able to preserve their memory CD4<sup>+</sup> T cell compartment despite SIV infection, a feature that is likely influenced by the reduced infectivity of CD4<sup>+</sup> TCM cells in SMs compared to RMs.

**Long-term maintenance of CD4<sup>+</sup> TCM cells in SIV-infected SMs compared to RMs.** To account for the different durations of infection in natural host SMs versus AIDS-susceptible RMs, we further investigated the rate of loss of CD4<sup>+</sup> TCM cells over time between the two species. We found that in RMs, a significant loss of CD4<sup>+</sup> TCM cells was already present in animals infected with SIV for  $<1.5$  years compared to uninfected animals ( $20.32\% \pm 2.41\%$  versus  $36.57\% \pm 1.58\%$ ;  $P < 0.0001$ ) (Fig. 2A). In contrast, SIV-infected SMs maintained a frequency of CD4<sup>+</sup> TCM cells comparable to those found in uninfected animals for up to 10 years postinfection. In fact, the fraction of CD4<sup>+</sup> TCM cells was significantly lower than that in uninfected SMs only for animals





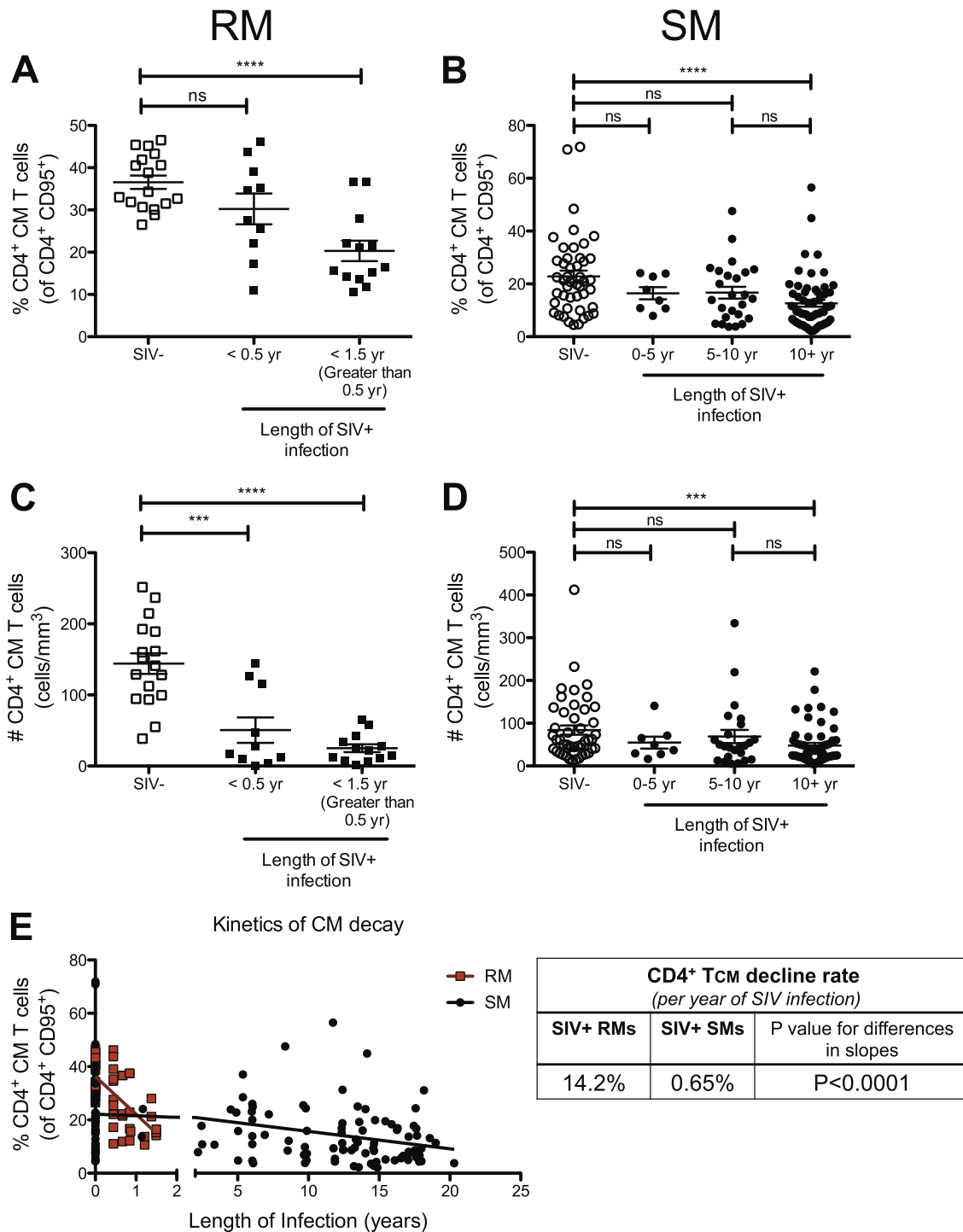
**FIG 1** Maintenance of CD4<sup>+</sup> central memory T cell levels in naturally SIV-infected SMs. (A) Representative plots of the flow cytometry gating strategy for measurement of CD4<sup>+</sup> TCM and TEM cells levels by CD62L staining for an SIV-infected SM. (B and C) Frequencies (of CD4<sup>+</sup> CD95<sup>+</sup> cells) (B) and counts (C) of CD4<sup>+</sup> TCM cells were quantified in the whole blood of uninfected (□) and SIV-infected (■) RMs (\*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  [determined by *t* tests]). (D) The percentages of CD4<sup>+</sup> TCM cells (of CD4<sup>+</sup> CD95<sup>+</sup> cells) in whole blood were compared between age-matched uninfected (○) and SIV-infected (●) SMs. (E) Numbers of CD4<sup>+</sup> TCM cells (cells/mm<sup>3</sup>) were measured in the same cohort of SMs (ns, *P* value was not significant, as determined by the Mann-Whitney U test).

infected for >10 years ( $12.66\% \pm 1.32\%$  versus  $22.86\% \pm 2.19\%$ ;  $P < 0.0001$ ) (Fig. 2B). The trend was upheld when absolute numbers of CD4<sup>+</sup> TCM cells of natural and nonnatural hosts were compared, as CD4<sup>+</sup> TCM cells were significantly depleted within half a year of infection in RMs ( $25.1 \pm 5.5$  versus  $144.2 \pm 14.6$  cells/mm<sup>3</sup>;  $P < 0.0001$ ) (Fig. 2C). This trend was not observed in natural host SMs, in which CD4<sup>+</sup> TCM cells were depleted at significant levels only after 10 or more years of SIV infection ( $47.9 \pm 6.2$  versus  $84.0 \pm 10.9$  cells/mm<sup>3</sup>;  $P = 0.0004$ ) (Fig. 2D).

We then used the data on the frequency of CD4<sup>+</sup> TCM cells in SIV-infected RMs and SMs with different durations of infection to model the kinetics of CD4<sup>+</sup> TCM cell loss in the two species. Levels of CD4<sup>+</sup> TCM cells declined at a rate of 14.2% per year in SIV-infected RMs; however, levels of CD4<sup>+</sup> TCM cells in SIV-infected SMs declined at a rate of only 0.65% per year (slopes estimated by linear regression, with a significant *P* value of <0.0001 for both RMs and SMs) (Fig. 2E). The slower loss of CD4<sup>+</sup> TCM cells in SMs was not due simply to a slowing of decay after prolonged infection, since when we analyzed the slopes for

animals infected for <10 years versus animals infected for >10 years, there was no significant difference ( $P = 0.74$  by ANCOVA). Therefore, it is estimated that CD4<sup>+</sup> TCM cells are lost at a rate approximately 20 times lower in SIV-infected SMs than that in RMs ( $P < 0.0001$  for differences in slopes, determined by ANCOVA). Consistent with the different susceptibilities to SIV infection in the two species, these data and those presented in Fig. 1 indicate that prolonged preservation of CD4<sup>+</sup> TCM cells is a critical feature distinguishing nonpathogenic SIV infection of SMs from pathogenic SIV infection of RMs.

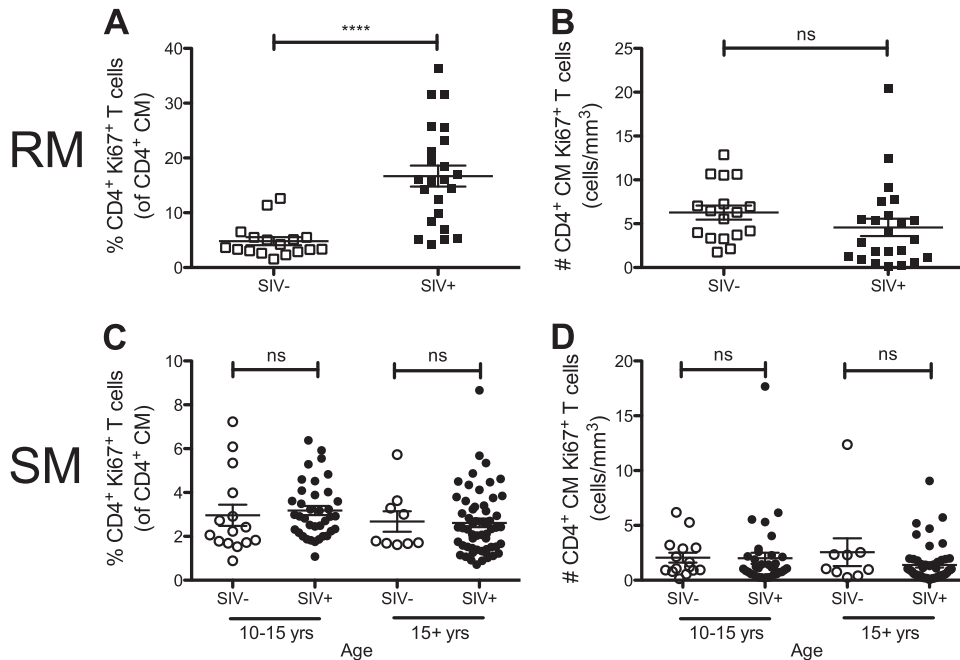
**Limited proliferation of CD4<sup>+</sup> TCM cells in SIV-infected SMs compared to RMs.** CD4<sup>+</sup> TCM cells are self-renewing cells that proliferate to maintain long-term stability of the CD4<sup>+</sup> T cell compartment (8). In the context of SIV infection of RMs, CD4<sup>+</sup> TCM cells undergo a striking increase in proliferation that is thought to represent, at least in part, an attempt to maintain CD4<sup>+</sup> T cell homeostasis by compensating for the cell loss due to both direct virus infection and chronic immune activation (21, 22). We next examined the proliferation levels of CD4<sup>+</sup> TCM cells in the SIV-infected and uninfected SMs and RMs included in our



**FIG 2** CD4<sup>+</sup> TCM cells have increased stability in naturally SIV-infected SMs compared to SIV-infected RMs. (A to D) The percentages (of CD4<sup>+</sup> CD95<sup>+</sup> cells) (A and B) and counts (cells/mm<sup>3</sup>) (C and D) of CD4<sup>+</sup> TCM cells were compared between uninfected and SIV-infected RMs (A and C) and SMs (B and D) based on their length of SIV infection (years) (\*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; ns,  $P$  value was not significant [as determined by  $t$  test {A and C} or Mann-Whitney U test {B and D}]). (E) Scatter plots depicting the relationship between the fraction of CD4<sup>+</sup> TCM cells and the duration of infection at the time of sampling for RMs and SMs in order to estimate a linear decay rate of central memory cells ( $P < 0.0001$  for differences in slopes, as determined by ANCOVA).

study by staining for the proliferative marker Ki-67. The fractions of proliferating CD4<sup>+</sup> TCM cells ( $16.69\% \pm 1.91\%$  versus  $4.82\% \pm 0.73\%$ ;  $P < 0.0001$ ) (Fig. 3A) and TEM cells ( $25.99 \pm 2.74\%$  versus  $9.54 \pm 1.09$ ;  $P < 0.0001$ ) (see Fig. S4A in the sup-

plemental material) were significantly higher in SIV-infected RMs than in uninfected RMs. Despite the significant increase in the fraction of these cells that were proliferating, the absolute number of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells remained similar between SIV-in-



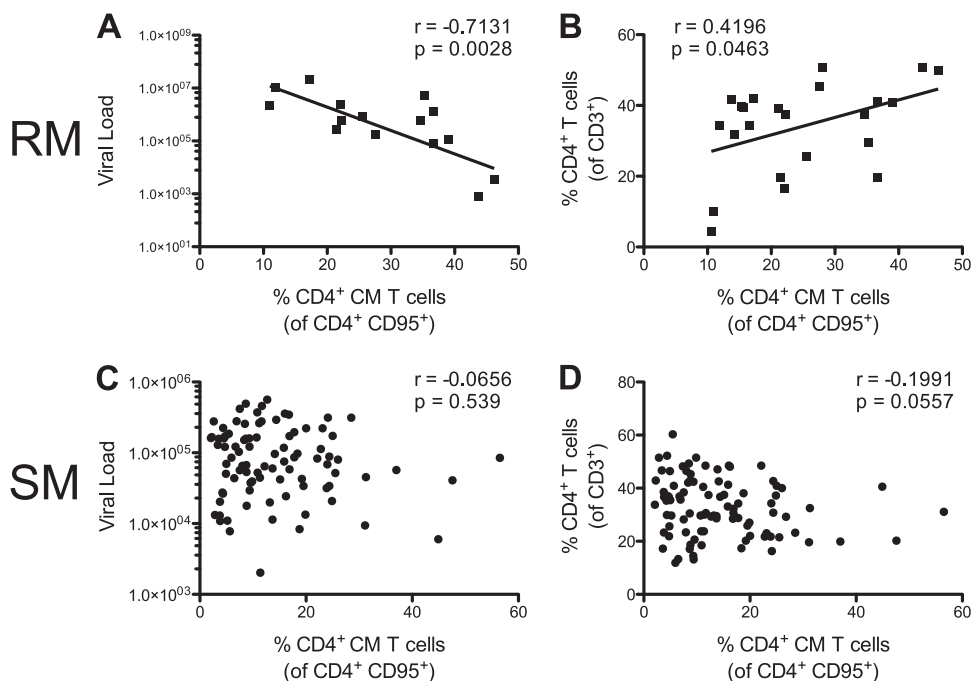
**FIG 3** SIV infection of RMs, but not of SMs, is associated with a significant increase in the level of proliferating CD4<sup>+</sup> TCM cells. (A and B) The percentages (A) and numbers (B) of proliferating (Ki-67<sup>+</sup>) CD4<sup>+</sup> TCM cells in uninfected (□) and SIV-infected (■) RMs were compared. (C and D) The levels of proliferating CD4<sup>+</sup> TCM cells were compared between age-matched uninfected (○) and SIV-infected (●) SMs by both frequencies (C) and cell counts (D) (\*\*\*\*,  $P < 0.0001$ ; ns, not significant [determined by Mann-Whitney U tests]).

ected and uninfected RMs due to the overall loss of CD4<sup>+</sup> TCM cells ( $4.58 \pm 0.99$  versus  $6.27 \pm 0.80$  cells/mm<sup>3</sup>) (Fig. 3B). This effect was also seen in CD4<sup>+</sup> Ki-67<sup>+</sup> TEM cells, in which SIV-infected RMs maintained numbers similar to those in uninfected RMs ( $22.07 \pm 4.20$  versus  $22.31 \pm 2.99$  cells/mm<sup>3</sup>) (see Fig. S4B in the supplemental material). These data clearly contrast with those found in SIV-infected SMs, in which SIV infection had little impact on the levels of proliferating CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells in age-matched SMs when measured as both fractions ( $3.18\% \pm 0.21\%$  versus  $2.96\% \pm 0.48\%$  for SMs 10 to 15 years old, and  $2.61\% \pm 0.20\%$  versus  $2.68\% \pm 0.46\%$  for SMs 15 or more years old) (Fig. 3C) and absolute numbers ( $2.00 \pm 0.51$  versus  $2.06 \pm 0.46$  cells/mm<sup>3</sup> for SMs 10 to 15 years old, and  $1.39 \pm 0.24$  versus  $2.56 \pm 1.26$  cells/mm<sup>3</sup> for SMs 15 or more years old) (Fig. 3D). Similarly, there were no differences in the fractions and numbers of CD4<sup>+</sup> Ki-67<sup>+</sup> TEM cells between age-matched SIV-infected and uninfected SMs (see Fig. S4C and S4D in the supplemental material). Overall, these data indicate that limited proliferation of the CD4<sup>+</sup> TCM compartment distinguishes nonprogressive SIV infection in SMs from progressive SIV infection in RMs. Furthermore, these data suggest that different levels of proliferation in chronic infection may account, at least in part, for the differential susceptibilities of CD4<sup>+</sup> TCM cells from SMs and RMs to SIV infection (28).

**Proliferation of CD4<sup>+</sup> TCM cells maintains its prohomeostatic role in SIV-infected SMs but becomes detrimental in SIV-infected RMs.** We then sought to determine whether the levels of total and proliferating CD4<sup>+</sup> TCM cells were correlated with the main virologic and immunologic markers of SIV infection. We first found that in SIV-infected RMs, the level of CD4<sup>+</sup> TCM cells correlated negatively with plasma viral load ( $r = -0.7131$ ;  $P = 0.0028$ ) (Fig. 4A) and positively with the fraction of CD4<sup>+</sup> T cells

( $r = 0.4196$ ;  $P = 0.0463$ ) (Fig. 4B). Thus, in SIV-infected RMs, loss of CD4<sup>+</sup> TCM cells is associated with the main critical features of pathogenic infection, including higher viral load and depletion of CD4<sup>+</sup> T cells. Unlike RMs, the levels of CD4<sup>+</sup> TCM cells did not correlate with viral load in SIV-infected SMs ( $r = -0.0656$ ;  $P = 0.539$ ) (Fig. 4C). Additionally, there was a weak negative correlation between the percentage of CD4<sup>+</sup> TCM cells and the fraction of CD4<sup>+</sup> T cells ( $r = -0.1991$ ;  $P = 0.0557$ ) (Fig. 4D), unlike the positive correlation found for RMs.

We then focused on the levels of proliferating CD4<sup>+</sup> TCM cells and found peculiar differences between progressive and nonprogressive SIV infection. Indeed, in SIV-infected RMs, the percentage of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells correlated negatively with the percentage ( $r = -0.5353$ ;  $P = 0.0085$ ) (Fig. 5A) and number ( $r = -0.5808$ ;  $P = 0.0037$ ) (Fig. 5B) of CD4<sup>+</sup> T cells as well as negatively with the number of CD4<sup>+</sup> TEM cells ( $P = 0.0174$ ) (data not shown). Intriguingly, the association was exactly the opposite for SIV-infected SMs, where the percentage of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells correlated positively with the percentage ( $r = 0.2916$ ;  $P = 0.0044$ ) (Fig. 5D) and number ( $r = 0.3168$ ;  $P = 0.0028$ ) (Fig. 5E) of CD4<sup>+</sup> T cells. Consistent with the different frequencies of CD4<sup>+</sup> TCM infection previously described between the two species described previously (28, 29), we found a positive correlation between the percentage of proliferating CD4<sup>+</sup> TCM cells and viral load in SIV-infected RMs ( $r = 0.6023$ ;  $P = 0.0175$ ) (Fig. 5C) but not in SIV-infected SMs ( $r = 0.0208$ ;  $P = 0.8456$ ) (Fig. 5F). Overall, these findings support the working hypothesis that proliferation of CD4<sup>+</sup> TCM cells maintains its beneficial, prohomeostatic role in SIV-infected SMs but becomes inefficient, if not deleterious, in maintaining CD4<sup>+</sup> T cell homeostasis in SIV-infected RMs.



**FIG 4** The level of central memory CD4<sup>+</sup> T cells correlates with viral load and CD4<sup>+</sup> T cell levels in SIV infection of RMs. Shown are the correlations between the percentages of CD4<sup>+</sup> TCM cells (of CD4<sup>+</sup> CD95<sup>+</sup> cells) and the viral load (A and C) and the percentages of CD4<sup>+</sup> T cells (of CD3<sup>+</sup> T cells) (B and D) for SIV-infected RMs (■) (A and B) and naturally SIV-infected SMs (●) (C and D). All statistical analyses were performed by using Spearman rank correlation tests.

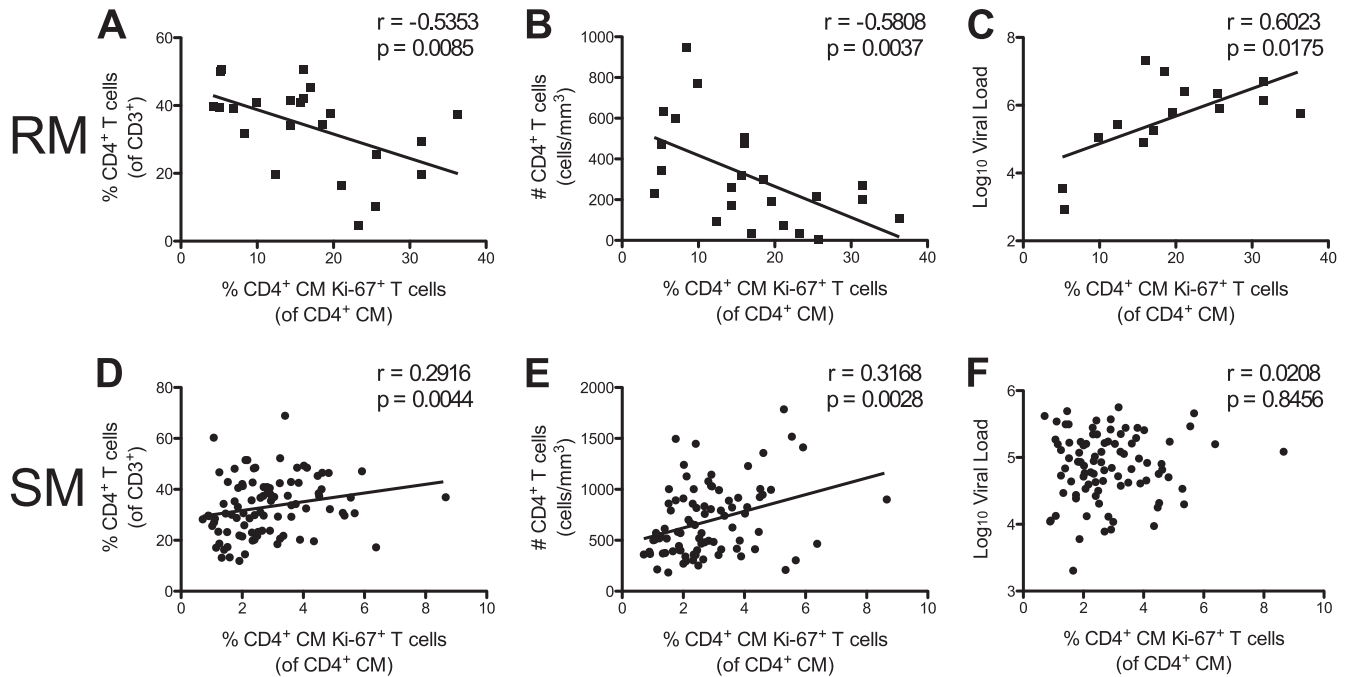
## DISCUSSION

We recently described that CD4<sup>+</sup> TCM cells are infected at lower levels in SIV-infected SMs than in SIV-infected RMs (28, 29). Based on these findings, we proposed that protection of CD4<sup>+</sup> TCM cells is a key factor used by SMs to remain AIDS free (7). In this view, we hypothesized that, in SIV-infected SMs, reduced infection of CD4<sup>+</sup> TCM cells will result in a better preservation of this important CD4<sup>+</sup> T cell compartment and that preservation of CD4<sup>+</sup> TCM cells is the proximal event that promotes the other key features of nonprogressive SIV infection, including preservation of overall CD4<sup>+</sup> T cell homeostasis and low levels of chronic immune activation. Indeed, CD4<sup>+</sup> TCM cells may contribute more than any other CD4<sup>+</sup> T cell subset to the long-term stability of the whole CD4<sup>+</sup> T cell compartment, given the elevated clonogenic potential and longer life span of these cells (20, 38). In turn, a preserved CD4<sup>+</sup> T cell compartment will reduce the homeostatic pressure on the immune system and contribute to limiting the chronic immune activation that is typical of pathogenic HIV and SIV infections in humans and RMs (7, 9, 39–41).

To test this hypothesis, in this study, we compared the levels of total and proliferating CD4<sup>+</sup> TCM cells between nonprogressive SIV infection of SMs and progressive SIV infection of RMs. Furthermore, we investigated the association between the levels of CD4<sup>+</sup> TCM cells, the maintenance of the overall CD4<sup>+</sup> T cell compartment, and the levels of T cell proliferation and viral replication. Importantly, these analyses were performed with a large cohort of SMs housed at the YNPRC, with 94 SIV-infected and 46 SIV-uninfected SMs being included in this study. The main findings generated here are the following: (i) the CD4<sup>+</sup> TCM cell compartment is remarkably more stable in SIV-infected SMs than in SIV-infected RMs; (ii) SIV infection of RMs, but not of SMs, is associated with a significant increase in the level of proliferating

CD4<sup>+</sup> TCM cells; (iii) the increased proliferation of CD4<sup>+</sup> TCM cells in RMs is not sufficient to maintain overall CD4<sup>+</sup> T cell homeostasis and in fact is associated with virologic and immunologic parameters of progression to AIDS; and (iv) proliferation of CD4<sup>+</sup> TCM cells in SMs positively contributes to CD4<sup>+</sup> T cell homeostasis and is maintained throughout SIV infection.

In contrast to SIV-infected RMs, SIV-infected SMs were able to maintain levels of CD4<sup>+</sup> TCM cells similar to those found in age-matched uninfected SMs. We also evaluated the stability of the central memory CD4<sup>+</sup> compartment in both species by determining the levels of CD4<sup>+</sup> TCM cells longitudinally in both experimentally SIV-infected RMs and naturally SIV-infected SMs. This approach allowed us, for the first time, to effectively model the kinetics of CD4<sup>+</sup> TCM cell loss as a result of SIV infection in both species. Consistent with our cross-sectional data, we found that the depletion of CD4<sup>+</sup> TCM cells is approximately 20 times slower in SIV-infected SMs than in SIV-infected RMs. In fact, this analysis revealed that it is estimated to take just over 15 months of infection for levels of CD4<sup>+</sup> TCM cells in RMs to fall to half of their initial percentage, whereas it would take SMs >17 years of SIV infection to reach half of their starting level of CD4<sup>+</sup> TCM cells. The loss of CD4<sup>+</sup> TCM cells that we did see in the SMs with >10 years of SIV infection is likely age related, since we found a significant inverse correlation between age and levels of CD4<sup>+</sup> TCM cells in SMs yet no significant correlation for RMs. These findings support the hypothesis that preservation of the CD4<sup>+</sup> TCM compartment is a critical feature that distinguishes nonprogressive SIV infection in SMs from progressive SIV infection in RMs. Furthermore, these data are consistent with our previous finding of reduced CD4<sup>+</sup> TCM cell infection in SMs (28, 29) as well as with the hypothesis that more differentiated, short-lived CD4<sup>+</sup> T cell subsets are the main cells supporting viral replication



**FIG 5** Increased levels of proliferating CD4<sup>+</sup> TCM cells are unable to maintain CD4<sup>+</sup> T cell levels in pathogenic SIV infection of RMs. The correlations between the percentages of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells (of CD4<sup>+</sup> TCM cells) and the percentages of CD4<sup>+</sup> T cells (of CD3<sup>+</sup> T cells) (A and D) and CD4<sup>+</sup> T cell counts (B and E) are shown for SIV-infected RMs (■) (A and B) and naturally SIV-infected SMs (●) (D and E). The fractions of proliferating CD4<sup>+</sup> TCM cells were positively correlated with the viral load for SIV-infected RMs (C) but not for naturally SIV-infected SMs (F). Viral load measurements were log transformed prior to graphically plotting them against frequencies of proliferating CD4<sup>+</sup> TCM cells; linear regression was then used to model the line of best fit between the two variables. All statistical analyses were performed by using Spearman rank correlation tests.

in SMs (7). An alternative explanation is that non-CD4<sup>+</sup> T cells, for example, myeloid cells, critically contribute to viral replication in SIV-infected SMs. However, an important contribution of non-CD4<sup>+</sup> T cells is not supported by our previous data showing that experimental depletion of CD4<sup>+</sup> T cells in chronically SIV-infected SMs is associated with a rapid and significant decline in viral load as well as the existence of a strong association between the availability of activated CD4<sup>+</sup> T cells and the level of viremia (42).

A second key finding is that the percentage of proliferating CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells was significantly increased in SIV-infected RMs but not in SIV-infected SMs. Proliferation of CD4<sup>+</sup> TCM cells is critical for their self-renewal capacity and the generation of more differentiated CD4<sup>+</sup> TEM cells (43–46). This CD4<sup>+</sup> TCM cell proliferative response is particularly important when the homeostasis of the CD4<sup>+</sup> T cell compartment is compromised, as it is during pathogenic HIV/SIV infection. On the other hand, HIV and SIV preferentially infect proliferating, antigen-experienced memory CD4<sup>+</sup> T cells (2, 47–49). Hence, in the context of HIV/SIV infection, the “prohomeostatic” role of CD4<sup>+</sup> TCM cell proliferation may be overruled by its effect on the expansion of the major target cell population for the virus (activated CD4<sup>+</sup> T cells). Remarkably, our data show that in chronically SIV-infected RMs, the percentage of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells positively correlates with viral load and negatively correlates with the number of total CD4<sup>+</sup> and CD4<sup>+</sup> TEM cells. These data thus suggest that in pathogenic SIV infection, increased proliferation of CD4<sup>+</sup> TCM cells fails to maintain the homeostasis of the overall CD4<sup>+</sup> T cell compartment. Of note, this finding lends support to a previously described model that a heightened level of CD4<sup>+</sup> T cell proliferation

can result in decreased numbers of uninfected CD4<sup>+</sup> T cells (35). The situation is exactly the opposite in SIV-infected SMs, in which the fraction of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells is not increased compared to that in uninfected animals and, in fact, correlates positively with the level of CD4<sup>+</sup> T cells. These data suggest that the limited proliferation of CD4<sup>+</sup> TCM cells in SMs contributes to the preservation of CD4<sup>+</sup> T cell homeostasis during this model of nonpathogenic SIV infection. Collectively, these data identify proliferation of CD4<sup>+</sup> TCM cells as a feature distinguishing progressive from nonprogressive SIV infection in nonhuman primates and indicate that during pathogenic SIV infection of RMs, increased proliferation of CD4<sup>+</sup> TCM cells is inefficient, if not deleterious, in maintaining the homeostasis of the overall CD4<sup>+</sup> T cell compartment.

A series of previous studies identified the progressive demise of CD4<sup>+</sup> TCM cells as a key determinant of the timing of disease progression in SIV-infected RMs (21, 22). Our current set of data adds to those previous studies by suggesting that in SIV-infected RMs, a failure in the regenerative capacity of CD4<sup>+</sup> TCM cells can also arise in the presence of CD4<sup>+</sup> TCM cells that proliferate at high levels due to the increased susceptibility of these activated and proliferating cells to virus-induced and/or activation-induced cell death. Consistent with this model, the absolute number of CD4<sup>+</sup> Ki-67<sup>+</sup> TCM cells remained similar between SIV-infected and uninfected RMs, despite the significant increase in the level of proliferating CD4<sup>+</sup> TCM cells when measured as a fraction of the total number of CD4<sup>+</sup> T cells.

Unfortunately, tissues were not collected during this survey of the SMs housed at the YNPRC; thus, we were unable to determine, and compare with RMs, the levels of CD4<sup>+</sup> TCM cells in key



anatomical sites such as lymph nodes and intestinal tissues. A second possible limitation of this study is that the SIV-infected SMs were, on average, significantly older than the SIV-infected RMs. This is important since we found a significant inverse correlation between the fraction of CD4<sup>+</sup> TCM cells and age in SMs. Interestingly, it seems that the slow decay of CD4<sup>+</sup> TCM cells seen in SIV-infected SMs may be more attributable to aging than to SIV infection, since the decay of CD4<sup>+</sup> TCM cells with age was not significantly different between SIV-infected and uninfected animals ( $P = 0.81$  by ANCOVA). By evaluating the levels of CD4<sup>+</sup> TCM cells between age-matched SMs, we believe that we compensated for any additive effects of aging on the percentages of CD4<sup>+</sup> TCM cells, although it may be possible that we are underestimating the stability of the CD4<sup>+</sup> TCM compartment in SIV-infected SMs compared to SIV-infected RMs.

Overall, these findings identify increased stability of CD4<sup>+</sup> TCM cells as a key feature distinguishing nonprogressive from progressive SIV infections and support the working hypothesis that proliferation of CD4<sup>+</sup> TCM cells maintains its beneficial, prohomeostatic role in SIV-infected SMs but becomes inefficient, if not deleterious, in maintaining CD4<sup>+</sup> T cell homeostasis in SIV-infected RMs. Therefore, the results of this study highlight the importance of investigating immunomodulatory interventions that are able to improve the homeostasis of CD4<sup>+</sup> TCM cells in HIV-infected humans.

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