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Initiation of Antiretroviral Therapy Restores CD4+ T Memory Stem Cell Homeostasis in Simian Immunodeficiency Virus-Infected Macaques

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ABSTRACT

Treatment of human immunodeficiency virus (HIV) infection with antiretroviral therapy (ART) has significantly improved prognosis. Unfortunately, interruption of ART almost invariably results in viral rebound, attributed to a pool of long-lived, latently infected cells. Based on their longevity and proliferative potential, CD4+ T memory stem cells (TSCM) have been proposed as an important site of HIV persistence. In a previous study, we found that in simian immunodeficiency virus (SIV)-infected rhesus macaques (RM), CD4+ TSCM are preserved in number but show (i) a decrease in the frequency of CCR5+ cells, (ii) an expansion of the fraction of proliferating Ki-67+ cells, and (iii) high levels of SIV DNA. To understand the impact of ART on both CD4+ TSCM homeostasis and virus persistence, we conducted a longitudinal analysis of these cells in the blood and lymph nodes of 25 SIV-infected RM. We found that ART induced a significant restoration of CD4+ CCR5+ TSCM both in blood and in lymph nodes and a reduction in the fraction of proliferating CD4+ Ki-67+ TSCM in blood (but not lymph nodes). Importantly, we found that the level of SIV DNA in CD4+ transitional memory (T_Tm) and effector memory (T_Em) T cells declined ~100-fold after ART in both blood and lymph nodes, while the level of SIV DNA in CD4+ TSCM and central memory T cells (T_Cm) did not significantly change. These data suggest that ART is effective at partially restoring CD4+ TSCM homeostasis, and the observed stable level of virus in TSCM supports the hypothesis that these cells are a critical contributor to SIV persistence.

IMPORTANCE

Understanding the roles of various CD4+ T cell memory subsets in immune homeostasis and HIV/SIV persistence during antiretroviral therapy (ART) is critical to effectively treat and cure HIV infection. T memory stem cells (TSCM) are a unique memory T cell subset with enhanced self-renewal capacity and the ability to differentiate into other memory T cell subsets, such as central and transitional memory T cells (T_Cm and T_Tm, respectively). CD4+ TSCM are disrupted but not depleted during pathogenic SIV infection. We find that ART is partially effective at restoring CD4+ TSCM homeostasis and that SIV DNA harbored within this subset contracts more slowly than virus harbored in shorter-lived subsets, such as T_Tm and effector memory (T_Em). Because of their ability to persist long-term in an individual, understanding the dynamics of virally infected CD4+ TSCM during suppressive ART is important for future therapeutic interventions aimed at modulating immune activation and purging the HIV reservoir.

Infection with pathogenic lentiviruses such as human and simian immunodeficiency viruses (HIV and SIV, respectively) significantly perturbs the homeostasis of the CD4+ T cell compartment through preferential infection and depletion of memory CD4+ T cells, which is the hallmark of progression to AIDS. While the availability of antiretroviral therapy (ART) has significantly reduced the mortality and morbidity of HIV infection, this treatment cannot eradicate the virus, and even after suppression of viremia for many years, interruption of ART results in rapid viral rebound (1, 2). This rebound is most often attributed to the presence of a small pool of long-lived, largely resting, latently infected cells that harbor replication-competent proviral DNA integrated in their genome (3–5). In addition, ART does not fully reverse the immunological abnormalities that are associated with HIV infection, including but not limited to CD4+ T cell depletion, chronic immune activation, premature immunological ageing, and mucosal immune dysfunction (6). It is now widely accepted that to reach a full understanding of the mechanisms responsible for virus persistence and residual immune dysfunction under ART, it is essential to conduct studies in which various subsets of memory CD4+ T cells are investigated.

Numerous studies have shown that the memory CD4+ T cell compartment is highly heterogeneous and includes at least four specific cell subsets that exhibit a characteristic anatomic distribution and can be defined based on their differentiation status as follows: CD4+ memory stem cells (TSCM, defined as CD45RA+ CCR7+ CD28+ CD95+ CD62L+), CD4+ central memory cells (T_Cm, defined as CD45RA− CCR95+ CCR7+ CD62L+), CD4+ transitional memory cells (T_Tm, defined as CD45RA− CCR95− CCR7− CD62L+), and CD4+ effector memory cells (T_Em, defined as CD95+ CCR7− CD62L−) (7). In particular, TSCM are a rela-
tively small (~2 to 4%) subset of circulating memory T cells that represent the “stem cells” of the memory T cell compartment, as they are uniquely able to both self-renew and differentiate into all other memory T cell subsets (i.e., Tcm, Ttm, and Tdm) (8, 9). Additional properties of Tcm include a long in vivo life span, greater proliferative potential than other T cell memory subsets, and preferential homing to secondary lymphoid tissues (8, 10). In the context of HIV and SIV infection, CD8+ Tcm are thought to be involved in the long-term maintenance of virus-specific cellular immune responses (11–13), while CD4+ Tcm were shown to be important targets of HIV infection both in untreated and in ART-treated individuals (14, 15). The observation of high levels of HIV DNA in CD4+ Tcm from HIV-infected individuals under ART, together with particular biological properties of these cells, such as high in vivo longevity, relative quiescence, and marked proliferative potential, led to the hypothesis that these cells represent a crucial contributor to virus persistence despite their relative low frequency (15). Indeed, two recent studies demonstrated that the duration of ART is directly correlated with the relative contribution of CD4+ Tcm to the HIV DNA reservoir in HIV-infected individuals (15, 16).

In a previous study, we investigated CD4+ Tcm during the natural history of experimental, pathogenic SIV infection of the nonnatural host rhesus macaques (RM) and the nonpathogenic natural history of experimental, pathogenic SIV infection of the RM (but not the nonpathogenic SIV infection of vervet monkeys (SM)) (17). We found that while the overall number of CD4+ Tcm was preserved in both species during infection, SIV-infected RM showed a significant decrease in the percentage of CD4+ Tcm expressing the SIV coreceptor CCR5 and a significant expansion of the percentage of proliferating cells (based on the expression of the marker Ki-67). This expansion of proliferating Tcm correlated inversely with the frequency of CD4+ Tcm (17). In addition, SIV-infected RM, but not SIV-infected SM, showed robust levels of direct virus infection (based on the measurement of the total cell-associated SIV DNA) (17). Given these results, we concluded that pathogenic SIV infection of RM (but not the nonpathogenic SIV infection of SM) is associated with a perturbation of the immunological homeostasis of CD4+ Tcm and robust levels of virus infection in these cells. Of note, our previous study did not include any ART-treated SIV-infected RM or SM and therefore did not address the potential role of these cells in contributing to the residual virological and immunological abnormalities that persist under ART.

To assess the impact of ART initiation on both CD4+ Tcm homeostasis and virus persistence during pathogenic SIV infection of RM, we conducted a longitudinal analysis of these cells in the blood and lymph nodes of 25 animals that were treated with ART for a period ranging between 2 and 6 months. The main findings of this study are that (i) ART induced a significant restoration of CD4+ CCR5+ Tcm in both blood and lymph nodes, (ii) ART resulted in a significant reduction in the fraction of proliferating CD4+ Ki-67+ Tcm in blood (but not lymph nodes), and (iii) ART did not induce a decline in the level of total cell-associated SIV DNA in CD4+ Tcm. The last finding is in stark contrast with the observation that the levels of SIV DNA declined ~100-fold after ART in both CD4+ Ttm and Tdm. Overall these data (i) indicate that ART is effective at partially restoring CD4+ Tcm homeostasis disrupted during SIV infection in RM and (ii) support the hypothesis that Tcm are a critical contributor to SIV persistence in this model of primate lentiviral infection.

**Materials and Methods**

**Animals.** This study was conducted using a total of 36 Indian-origin rhesus macaques (RM). Of these, 25 were SIV infected (13 infected intravenously with 3,000 tissue culture infectious doses [TCID50] of SIVmac239; 12 infected intravenously with 300 TCID50 of SIVmac239 and the remaining 11 were SIV uninfected (used for SIV-uninfected lymph node time points). All animals were housed at the Yerkes National Primate Research Center of Emory University and maintained in accordance with U.S. National Institutes of Health guidelines. Anesthesia was used for all blood and tissue collections. All studies were approved by the Emory University Institutional Animal Care and Usage Committee (IACUC).

**Antiretroviral therapy.** Twelve RM were put on a 4-drug ART regimen at 6 weeks following SIV infection that included 20 mg/kg of body weight tenofovir (PMPA), 40 mg/kg emtricitabine (FTC), and 2.5 mg/kg dolutegravir administered once a day by subcutaneous (s.c.) injection along with 400 mg darunavir orally twice daily. Thirteen additional RM were put on a 4-drug ART regimen at 8 weeks postinfection: 20 mg/kg PMPA and 30 mg/kg FTC administered once a day by s.c. injection along with 100 to 150 mg raltegravir and 400 to 800 mg darunavir orally twice daily. Oral antiretroviral treatment (raltegravir and darunavir) was given via orogastric tube on days where anesthesia was also administered, due to fasting and nausea.

**Sample collection and tissue processing.** Blood was collected in EDTA tubes. Plasma was obtained throughout the study by centrifugation. Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation using lymphocyte separation medium (LSM) from Lonza. Lymph node biopsy specimens were taken at 2 time points throughout the study (day 56 pre-ART initiation and between 8 and 29 weeks post-ART initiation). Axillary and inguinal lymph node biopsy specimens were taken at indicated time points, though a distinction was not made between them. Lymph node mononuclear cells (LMNC) were obtained by removing excess fat and connective tissue and grinding over a 70-μm cell strainer.

**Immunophenotype by flow cytometry.** Multiparametric flow cytometry was performed according to a standard protocol on PBMC and LMNC using fluorescently labeled monoclonal antibodies cross-reactive in RM. The following antibodies were used for immunophenotyping of CD4+ memory T cell subsets: CD3 PE-CF594 (L200); CD8 BV711 (RPA-T8); CCR7-FTTC (150503); CD45RA-PE/Cy7 (L48); CD95 PE/Cy5 (DX2); Ki-67-AlexaFluor700 (B56); CD45RA-APC (5H9); and CCR5-APC (3A9) from BD Biosciences; CD28-EC DNA (CD28-2) from Beckman Coulter; and CD4-BV650 (OK-T4) from Biolegend. All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star). The gating strategies for CD4+ Tcm in PBMC and lymph node specimens are shown in Fig. 1A and B, respectively.

**Cell sorting.** After isolation, cells were resuspended in 30 to 50 ml phosphate-buffered saline (PBS) containing 2 mM EDTA and spun at 200 × g for 15 to 20 min (depending on cell volume) to remove contaminating platelets. Prior to sorting, CD4+ T cells in PBMC and LMNC were enriched using magnetic beads and column purification (Miltenyi Biotec). Enriched cells were then stained with previously determined volumes of CD3-APC/Cy7 (SP34-2), CD4-PE-CF594 (L200); CD8 BV711 (RPA-T8); CCR7-FTTC (150503); CD45RA-PE/Cy7 (L48); CD95 PE/Cy5 (DX2); Ki-67-AlexaFluor700 (B56); CD45RA-APC (5H9); and CCR5-APC (3A9) from BD Biosciences; CD28-EC DNA (CD28-2) from Beckman Coulter; and CD4-BV650 (OK-T4) from Biolegend. All flow cytometry specimens were acquired on an LSR II (BD Biosciences) equipped with fluorescence-activated cell sorter software (FACS Diva), and analysis of the acquired data was performed using FlowJo software (Tree Star). The gating strategies for CD4+ Tcm in PBMC and lymph node specimens are shown in Fig. 1A and B, respectively.

**Plasma viral load and cell-associated SIV gag DNA.** Plasma viral quantification was performed as described previously (18). DNA was extracted from sorted peripheral and lymph node CD4+ T cells and CD4+ T-cell memory subsets using the Blood DNA minikit (Qiagen). Quantifi-
cation of SIV<sub>mac</sub> gag DNA was performed as previously described on the extracted cell-associated DNA by quantitative PCR using the 5'-nuclease (TaqMan) assay with an ABI7500 system (PerkinElmer Life Sciences) (19). The sequence of the forward primer for SIV<sub>mac</sub> gag was 5'-GCAGAGGAGGAAATTACCCAGTAC-3'; the reverse primer sequence was 5'-CACAATTTTACCCAGGCATTTAATGTT-3'; and the probe sequence was 5’-6-FAM-TGTCCACCTGCCATTAAGCCCGA-TAMRA-3', where 6-FAM is 6-carboxyfluorescein. For cell quantification, quantitative PCR was performed for monkey albumin gene copy number.

Statistical analysis. Comparisons between three groups (see Fig. 2 to 5) were done using two-tailed Kruskal-Wallis. Correlations were determined using the non-Gaussian Spearman correlation. Comparison between pre-ART and on-ART cell-associated DNA in Fig. 5 was done using the two-tailed non-Gaussian Mann-Whitney U test. Significance was attributed at P values of <0.05. Analysis was done using GraphPad Prism 6.0.

RESULTS

ART administration does not change the frequency or absolute number of CD4<sup>+</sup> T<sub>SCM</sub> in SIV-infected RM. As shown in Fig. 2A, initiation of ART suppressed viremia below the limit of detection of our standard SIV RNA assay (60 copies/ml of plasma) in 18 of 25 SIV-infected RM. Animals that were still viremic at the “on ART” time point were excluded from “on ART” analysis. Of note, time to reach SIV viremia below 60 copies/ml of plasma varied among this group of SIV-infected RM (range, between 2 and 23 weeks to achieve the first result below the limit of detection; data not shown), similar to what was published in previous reports (20, 21). As expected based on many studies conducted in HIV-infected individuals, we found that ART induces a significant increase in the absolute count of CD4<sup>+</sup> T cells (SIV-infected pre-ART median = 275 cells/µl, SIV-infected on-ART median = 578.5 cells/µl, P < 0.05), which reached levels comparable to those observed prior to SIV infection (Fig. 2B). To then investigate the role of ART in restoring CD4<sup>+</sup> T<sub>SCM</sub> homeostasis, we examined the absolute count of circulating CD4<sup>+</sup> T<sub>SCM</sub> (Fig. 2C), as well as the frequency of CD4<sup>+</sup> T<sub>SCM</sub> (as a percentage of total CD3<sup>+</sup> CD4<sup>+</sup> T cells) in peripheral blood mononuclear cells (PBMC) and lymph node mononuclear cells (LNMC) (Fig. 2D and E) from RM that were sampled before SIV infection (SIV<sup>-</sup>), after 6 to 8 weeks of SIV-infection (SIV<sup>+</sup>, i.e., pre-ART), and during ART (“on ART”). Consistent with previous observations (17), we found that SIV infection does not change the absolute number or frequency of CD4<sup>+</sup> T<sub>SCM</sub> or their frequency in PBMC or LNMC. We also found that the administration of ART did not induce a significant change in the absolute number or frequency of CD4<sup>+</sup> T<sub>SCM</sub> in either PBMC or LNMC (Fig. 2C to E). As expected, we found that initiation of ART results in a significant increase in the numbers of
Taken together, these data confirm that SIV infection of RM does not result in a major depletion of CD4\(^+\) TSCM and show that the overall levels of these cells are not changed after ART administration. ART induces a significant reconstitution of the pool of CD4\(^+\) CCR5\(^+\) TSCM in blood and lymph nodes of SIV-infected RM. In a previous study, we found that SIV infection of RM is associated with a significant decline of the percentage of CD4\(^+\) TSCM expressing the SIV coreceptor CCR5 (17). To determine whether administration of ART corrects this perturbation, we measured the levels of CCR5 on total CD4\(^+\) T cells and CD4\(^+\) memory T cell subsets in our cohort of SIV-infected RM before and after therapy. As shown in Fig. 3A and B, we found that ART induced a significant increase in the fraction of CD4\(^+\) CCR5\(^+\) T cells (measured as a percentage of total CD3\(^+\) CD4\(^+\) T cells) in both blood and lymph nodes. In addition, we found that ART administration was followed by a significant increase in the fraction of CD4\(^+\) CCR5\(^+\) TSCM (measured as a percentage of the total CD4\(^+\) TSCM population) in both blood (Fig. 3C) and lymph nodes (Fig. 3D). It should be noted, however, that the levels of CCR5\(^+\) TSCM remained lower than what was observed in these animals prior to SIV infection in PBMC. We also observed that ART administration significantly increased the fraction of CD4\(^+\) TCM and TTM (but not TEM) expressing CCR5 in blood and the fraction of CD4\(^+\) TCM and TTM (but not TEM) expressing CCR5 in lymph nodes (Fig. 3E and F). Overall, these data indicate that suppression of virus replication through ART administration induced a significant but partial re-

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**FIG 2** ART effectively reduces viremia and restores CD4\(^+\) T cells in PBMC. (A) Plasma viral load of 25 rhesus macaques prior to ART initiation (left side) and at the “on ART” time point (8 to 28 weeks following treatment initiation). Purple squares on the left side represent 12 animals infected with 300 TCID\(_{50}\) and put on 4-drug ART at 6 weeks following SIV infection. Red circles on the left side represent 13 animals infected with 3,000 TCID\(_{50}\) and put on 4-drug ART at 8 weeks following SIV infection. Animals with detectable viral loads were excluded from the “on ART” time point in subsequent analyses. The horizontal dashed line represents the limit of detection of plasma viral load assay (60 copies/ml). (B) Absolute number of CD4\(^+\) T cells in PBMC. (C) Absolute number of CD4\(^+\) TSCM in PBMC. (D and E) Fraction of CD4\(^+\) TSCM in PBMC (D) and lymph node (E). Bars are drawn at the median. Kruskal-Wallis, two-tailed test: *, \(P < 0.05\).
ART significantly decreases the fraction of proliferating T_{SCM} in blood but not lymph nodes of SIV-infected RM. We have previously shown that the fraction of proliferating CD4^{+} T_{SCM} (measured as a percentage of cells expressing the marker Ki-67) is increased in SIV-infected RM and that this increase is directly correlated with the severity of CD4^{+} T_{CM} depletion, thus suggesting the presence of a compensatory homeostatic mechanism (17).

To assess the impact of ART on CD4^{+} T_{SCM} proliferation in SIV-infected RM, we measured the level of Ki-67 in total CD4^{+} T cells and CD4^{+} T_{SCM} and T_{CM} in our cohort of SIV-infected RM at three time points (before SIV infection, before ART, and after ART initiation). As shown in Fig. 4A, we found that ART administration was followed by a decrease in the median Ki-67 expression in circulating CD4^{+} T cells (from 9.7% to ~3.96%). However, this decline was not statistically significant. In contrast, the frequency of CD4^{+} Ki-67^{+} T cells in lymph nodes remained stable after ART administration in this group of SIV-infected RM (Fig. 4B). When we next measured the fraction of CD4^{+} T_{SCM} expressing Ki-67, we found that ART induced a significant decline of CD4^{+} Ki-67^{+} T_{SCM} in the blood of SIV-infected RM, with levels similar to those observed before SIV infection (Fig. 4C). As shown in Fig. 4D, ART administration resulted in a nonsignificant trend toward lower levels of CD4^{+} Ki-67^{+} T_{SCM} in lymph nodes. In comparison, ART induces a significant reduction in proliferating CD4^{+} T_{CM} in both PBMC and lymph nodes (Fig. 4E and F, respectively). Overall, these data indicate that ART administration was partially effective in reducing CD4^{+} T cell proliferation, thereby improving T_{SCM} homeostasis, in our cohort of SIV-infected RM.

The inverse correlation between the frequency of proliferating CD4^{+} T_{SCM} and the level of circulating T_{CM} is maintained under ART in SIV-infected RM. Our previous work suggested that during SIV infection of RM, CD4^{+} T_{SCM} proliferation might act as a compensatory homeostatic mechanism to maintain the number of circulating CD4^{+} T_{CM} (and, ultimately, total CD4^{+} T cells as well) (17). To further investigate the relationship between CD4^{+} T_{SCM} proliferation and the maintenance of CD4^{+} T_{CM}, we performed a correlation analysis between these two measurements in our group of ART-treated SIV-infected RM at three time points (before SIV infection, before ART, and during ART). As shown in Fig. 5, we found a consistent inverse relationship between the fraction of proliferating (Ki-67^{+}) CD4^{+} T_{SCM} (measured as a percentage of total CD4^{+} T_{SCM}) and the level of circulating CD4^{+} T_{CM} (measured as a percentage of total CD4^{+} T cells) at all time points in our study, including before SIV infection (P = 0.0214) (Fig. 5, top), after SIV infection but before ART (P = 0.0197) (Fig. 5, middle), and after ART-mediated suppression of viremia (P = 0.0232) (Fig. 5, bottom). The observation that circulating CD4^{+} Ki-67^{+} T_{SCM} correlate inversely with the frequency of circulating CD4^{+} T_{CM} under all examined conditions is consistent with the hypothesis that the level of in vivo T_{SCM} proliferation is governed, at least in part, by the level of CD4^{+} T_{CM}. This regulation of CD4^{+} T cell homeostasis appears to be present in PBMC of healthy RM and is preserved in the setting of SIV infection. Though we see this relationship consistently throughout the three time points examined, the frequency with which CD4^{+} T_{SCM} differentiate into T_{CM} remains an open question.

Interestingly, the same correlation between proliferating CD4^{+} T_{SCM} and T_{CM} is not present in the lymph node (data not shown), perhaps suggesting a more complex regulation of T_{SCM}/T_{CM} homeostasis in the lymph node microenvironment.

ART administration does not induce a decline in the fraction of SIV DNA T_{SCM} in the blood or lymph nodes of SIV-infected RM. Previous studies have shown that CD4^{+} T_{SCM} are robustly infected with HIV and SIV in vivo and that in HIV-infected individuals, their contribution to the persistent reservoir of latently
infected cells under ART increases over time (15–17). To investigate the potential role of CD4+ TSCM as a source of virus persistence in SIV-infected RM, we measured the level of total cell-associated SIV DNA in the four main memory CD4+ T cell subsets (TSCM, TCM, TTM, and TEM) that were flow sorted from both peripheral blood and lymph nodes. The fraction of SIV DNA+ cells was measured at two time points, i.e., prior to ART initiation and after 8 to 29 weeks of therapy (when SIV viremia was below the limit of detection). As shown in Fig. 6, top left, we found that the average frequency of SIV DNA+ CD4+ TSCM decreased by approximately 1.5 log in blood. However, this difference was not statistically significant, possibly due to the relatively low number of SIV-infected RM in which we were able to sort a sufficient number of CD4+ TSCM to conduct this analysis. We found no difference in the frequency of SIV DNA+ CD4+ TSCM in the lymph nodes before and after ART (Fig. 6, top right). It should be noted that despite the relatively low number of animals included in this analysis, we observed a significant decline in the frequency of SIV DNA+ cells in the more differentiated memory subsets, specifically CD4+ TTM (P < 0.05) and CD4+ TEM (P < 0.05) in both peripheral blood and lymph nodes (Fig. 6). Overall, these data indicate that the kinetics of decay in the level of total cell-associated SIV DNA is different among memory CD4+ T cell subsets, with more rapid decline in TTM and TEM and slower decline in TSCM and TCM. As such, the current observation supports the hypothesis that CD4+ TSCM may represent an important contributor to HIV/SIV persistence during ART.

**DISCUSSION**

The current study sought to understand the role of ART in restoring the homeostasis of the CD4+ memory T cell compartment that is disrupted during chronic SIV infection, as well as to define how ART impacts the pattern of infected cells in blood and lymph nodes, with a focus on CD4+ TSCM. To our knowledge, this is the first study examining CD4+ TSCM in SIV-infected ART-suppressed RM.

The main findings of this study are that administration of ART to SIV-infected RM induced a marked improvement of CD4+ TSCM homeostasis, with a significant increase in the levels of CD4+ CCR5+ TSCM both in blood and in lymph nodes and a significant reduction in the fraction of proliferating CD4+ Ki-67+ TSCM in blood. While the signs of perturbed CD4+ TSCM homeostasis that are present in untreated SIV-infected RM (17) are only partially corrected by ART, one should consider that the duration of treatment was in the range of 2 to 6 months and that it is possible that longer treatment with ART may fully restore the levels of CD4+ CCR5+ TSCM and CD4+ Ki-67+ TSCM to the baseline frequencies observed prior to SIV infection. Importantly, we also found that the level of total cell-associated SIV DNA in CD4+ TSCM remains stable after ART initiation (which is in contrast to the decline of SIV DNA levels in CD4+ TTM and TEM), thus supporting the hypothesis that CD4+ TSCM are key contributors to the reservoir of latently SIV-infected cells that persists under ART in RM.

TSCM have been characterized over the past ~5 years by a series of studies that defined their key features, which include (i) their anatomic distribution in blood and lymphoid tissues but not mucosal sites, (ii) the expression of a specific set of phenotypic markers that allows their identification and sorting by standard flow cytometry, (iii) a characteristic gene expression pattern that clearly distinguishes them from both naive T cells and TCM, and (iv) the ability to sustain the homeostasis of memory T cells through both self renewal and differentiation into the more-mature memory subsets (TCM, TTM, and TEM).
In the setting of HV/SIV infection, CD4+ TSCM may play a role in both the preservation (or lack thereof) of CD4+ T cell homeostasis, which is severely affected during progression to AIDS, and in maintaining virus persistence under ART. A previous study from our group showed that SIV infection of RM is associated with a consistent set of changes in CD4+ TSCM homeostasis, including depletion of CD4+ CCR5+ TSCM and an increased frequency of CD4+ Ki-67+ TSCM. The current observations that administration of ART corrects, at least in part, these abnormalities and that this effect is associated with an improvement of CD4+ TSCM homeostasis (which is a hallmark of immunologic health in SIV infection [22]) suggest that the study of CD4+ TSCM before and after ART may provide useful information when predicting the overall immunological response to treatment. In this regard, further studies in which the dynamics of CD4+ TSCM under ART are examined in the setting of longer-term ART (i.e., 5 to 10 years) and correlated to the correction of immunological abnormalities in HIV-infected individuals appear to be warranted.

The role of CD4+ TSCM in maintaining HIV persistence under ART has been proposed in two independent studies published in the past 2 years (15, 16), and follow-up work from the group of Mathias Lichterfeld established the importance of the CD4+ TSCM reservoir even in early-treated individuals (23). Our current observation that the level of SIV DNA in CD4+ TSCM is stable under ART in SIV-infected RM is complementary to results obtained from longitudinal measurement of total (15) and integrated (16) HIV-1 DNA in CD4+ TSCM in ART-treated subjects. This reservoir stability contrasts with the decline in cell-associated viral DNA seen in CD4+ TFM and TCM in this study and in TFM and terminally differentiated CD4+ T cells in published work (15, 16), suggesting a link between the average in vivo life span of different memory CD4+ T cell subsets with the stability of their contribution to the persistent reservoir. Although the RM in this study received ART for only 2 to 6 months, data from HIV-1-infected individuals show that HIV-1 DNA in CD4+ T cells decays most rapidly in the first year of ART (24), suggesting that this is the most dynamic window of time in which we would be likely to observe ART-induced changes in SIV DNA. As such, this work supports the hypothesis that the relative contribution of CD4+ TSCM increases over time in the setting of ART and that these cells may represent the most difficult obstacle for HIV/SIV eradication efforts (7). Further studies in this area should include (i) a full functional investigation of the CD4+ TSCM-associated provirus with an assessment of its replication competence, a task that will likely require dedicated samples, given the technical challenge of conducting such studies on a relative small subset of CD4+ T cells, (ii) in vitro investigation of how latency can be specifically reversed in CD4+ TSCM by compounds that act specifically as latency reversing agents (LRAs), i.e., histone deacetylase inhibitors, bromodomain domain inhibitors, protein kinase C modulators, histone methyltransferase inhibitors, etc., and (iii) in vivo studies of ART-treated SIV-infected RM in which the CD4+ TSCM-based reservoir is specifically targeted by LRAs and/or drugs that promote CD4+ TSCM differentiation (i.e., beta-catenin inhibitors or NOTCH inhibitors) into shorter-lived memory CD4+ T cell subsets (7).

Taken together, the results of the current study of the immunological and virological responses to ART by CD4+ TSCM in SIV-infected RM provide further evidence that this subset of CD4+ T cells manifests a number of characteristic features that may impact both HIV/SIV pathogenesis and virus persistence under ART. Based on these data, we believe that future studies are warranted in which CD4+ TSCM are further investigated in SIV-infected RM to determine the best strategy to reduce their contribution to virus persistence, and ultimately this approach should be tested in HIV-infected humans to advance progress toward an HIV cure.

**FIG 5** The inverse relationship between proliferating CD4+ TSCM and the frequency of CD4+ TSCM is maintained during SIV infection and ART treatment. Correlation of CD4+ Ki-67+ TSCM and fraction of CD4+ TCM in PBMC of RM that are SIV uninfected (top), SIV infected but untreated (middle), and SIV infected and on ART (bottom). Spearman’s rank correlation, two-tailed test P values are shown.
Fraction of SIV-infected CD4\(^+\) T\(_{SCM}\) and T\(_{CM}\) is stable after ART initiation. Fraction of SIV-infected CD4\(^+\) T\(_{SCM}\), T\(_{CM}\), T\(_{TM}\), and T\(_{EM}\) cells as determined by quantitative PCR for the number of SIV gag DNA copies/10\(^6\) cells in PBMC (left panels) and lymph node (right panels). Data are shown for animals from which >10,000 cells were sorted. PBMC pre-ART: n = 7 T\(_{SCM}\), 7 T\(_{CM}\), 10 T\(_{TM}\), and 10 T\(_{EM}\); PBMC on-ART: n = 9 T\(_{SCM}\), 10 T\(_{CM}\), 10 T\(_{TM}\), and 10 T\(_{EM}\). Lymph node pre-ART: n = 4 T\(_{SCM}\), 4 T\(_{CM}\), 6 T\(_{TM}\), and 10 T\(_{EM}\); Lymph node on-ART: n = 6 T\(_{SCM}\), 6 T\(_{CM}\), 6 T\(_{TM}\), and 10 T\(_{EM}\). Mann-Whitney U test, two-tailed; **, P < 0.01; NS, not significant. Cell number was determined by using simultaneous PCR for albumin gene copy number. The dashed line represents the limit of detection (100 copies/million cells).
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