Antiretroviral Therapy in Simian Immunodeficiency Virus-Infected Sooty Mangabeys: Implications for AIDS Pathogenesis

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ABSTRACT

Simian immunodeficiency virus (SIV)-infected sooty mangabeys (SMs) do not develop AIDS despite high levels of viremia. Key factors involved in the benign course of SIV infection in SMs are the absence of chronic immune activation and low levels of infection of CD4+ central memory (T_{CM}) and stem cell memory (T_{SCM}) T cells. To better understand the role of virus replication in determining the main features of SIV infection in SMs, we treated 12 SMs with a potent antiretroviral therapy (ART) regimen for 2 to 12 months. We observed that ART suppressed viremia to <60 copies/mL of plasma in 10 of 12 animals and induced a variable decrease in the level of cell-associated SIV DNA in peripheral blood (average changes of 0.9-, 1.1-, 1.5-, and 3.7-fold for CD4+ transitional memory [T_{TM}], T_{CM}, effector memory [T_{EM}], and T_{SCM} cells, respectively). ART-treated SIV-infected SMs showed (i) increased percentages of circulating CD4+ T_{CM} cells, (ii) increased levels of CD4+ T cells in the rectal mucosa, and (iii) significant declines in the frequencies of HLA-DR+ CD8+ T cells in the blood and rectal mucosa. In addition, we observed that ART interruption resulted in rapid viral rebound in all SIV-infected SMs, indicating that the virus reservoir persists for at least a year under ART despite lower infection levels of CD4+ T_{CM} and T_{SCM} cells than those seen in pathogenic SIV infections of macaques. Overall, these data indicate that ART induces specific immunological changes in SIV-infected SMs, thus suggesting that virus replication affects immune function even in the context of this clinically benign infection.

IMPORTANCE

Studies of natural, nonpathogenic simian immunodeficiency virus (SIV) infection of African monkeys have provided important insights into the mechanisms responsible for the progression to AIDS during pathogenic human immunodeficiency virus (HIV) infection of humans and SIV infection of Asian macaques. In this study, for the first time, we treated SIV-infected sooty mangabeys, a natural host for the infection, with a potent antiretroviral therapy (ART) regimen for periods ranging from 2 to 12 months and monitored in detail how suppression of virus replication affected the main virological and immunological features of this nonpathogenic infection. The observed findings provide novel information on both the pathogenesis of residual immunological disease under ART during pathogenic infection and the mechanisms involved in virus persistence during primate lentiviral infections.

In stark contrast to pathogenic human immunodeficiency virus (HIV) infection of humans and experimental simian immunodeficiency virus (SIV) infection of Asian macaques, which are associated with progression to AIDS, SIV infections of African monkey species, such as sooty mangabeys (SMs), African green monkeys, and mandrills (often referred to as “natural hosts”), are typically nonpathogenic despite similarly high levels of virus replication (1). Key features of natural SIV infection of SMs include (i) preservation of peripheral CD4+ T cell counts (2), (ii) absence of chronic immune activation (2, 3), (iii) lower levels of CD4+ central memory (T_{CM}), stem cell memory (T_{SCM}), and follicular T helper cell infections than those in SIV-infected macaques (4–6), (iv) preservation of Th17 cells (7), and (v) absence of microbial translocation (8, 9). While SIV-infected SMs show an average life span that is comparable to that of SIV-uninfected animals (10), the infection is in fact associated with a number of immunological changes, including (i) high levels of immune activation during the acute phase of infection (11–13), (ii) early and persistent depletion of mucosal CD4+ T cells (9), (iii) a progressive increase in the expression of certain activation markers on T cells (3, 14), and (iv) severe depletion of CD4+ T cells in a small subset of animals (3, 15). As such, the typically nonpathogenic SIV infection of SMs cannot be considered immunologically silent, and the extent to which these immune abnormalities are directly related to virus replication remains incompletely understood.

Antiretroviral therapy (ART) represents one of the most im-

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portant successes in HIV/AIDS research, significantly reducing the mortality and morbidity of HIV infection. Importantly, ART results in a marked improvement of the complex immunological abnormalities that are associated with HIV infection, including a restoration of peripheral CD4+ T cell counts, the attenuation of HIV-associated generalized immune activation, and an improvement of the immune response against many opportunistic pathogens (16–18). While current antiretroviral drugs were designed, identified, and developed based on their ability to effectively inhibit specific enzymatic activities of HIV-1 gene products (i.e., reverse transcriptase [RT], protease, and integrase), most of these compounds are also able to effectively suppress the same enzymatic activities of SIV (19–22). In this regard, a number of studies involving ART administration in experimentally SIV- or simian-human immunodeficiency virus (SHIV)-infected rhesus macaques (RMs) were recently conducted by several groups, including ours (19, 22–25). In the setting of SIV infection of SMs, we previously conducted a study in which six animals were treated with short-term, two-drug ART (tenofovir and emtricitabine) and the decay of viremia was examined to estimate the average in vivo life span of productively SIV-infected cells, which was found to be similar to that described for HIV-infected humans (26). However, no studies have been performed involving longer, multidrug ART administration in SIV-infected SMs.

To directly assess the role of SIV replication in determining the main characteristics of chronic SIV infection of SMs, we treated a cohort of animals with a potent ART regimen consisting of tenofovir, emtricitabine, raltegravir, and darunavir for periods ranging from 2 to 12 months and conducted a number of virological and immunological analyses before, during, and after ART interruption. We observed that ART effectively suppressed viremia in 10 of 12 SIV-infected SMs and that these animals experienced (i) an increase of the percentage of circulating CD4+ TCM cells, (ii) an increase in the level of total CD4+ T cells in the rectal mucosa, and (iii) significant declines in the levels of activated HLA-DR+ CD8+ T cells in the blood and rectum. In addition, we observed that ART interruption resulted in rapid viral rebound in all treated animals, indicating that, in SIV-infected SMs, the virus reservoir persists for >1 year under ART despite relatively low levels of infection in CD4+ TCM and TSCM cells. Based on these data, we concluded that suppressive ART reverses some of the key immunological abnormalities observed during nonpathogenic SIV infection of SMs, thereby helping to define the direct effects of virus replication in SIV-infected natural hosts.

MATERIALS AND METHODS

Ethics statement. All animals were housed at the Yerkes National Primate Research Center (YNPRC), Atlanta, GA, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. SMs were maintained according to the Animal Welfare Act and NIH guidelines for housing and care of laboratory animals. Experimentation was performed in accordance with the institutional regulations and after approval by the Institutional Animal Care and Use Committee (IACUC) at the YNPRC (IACUC protocol YER-2002673-032017GA, entitled “Studies of the Natural SIV Infection of Sooty Mangabeys”). Commercial dry food supplemented with fruit was provided by the veterinary personnel, and water was available ad libitum. Adjustments were made as necessary depending on sex, age, and weight. Room temperature was maintained at 21°C, with a relative air humidity of 50% and a 12-h light-dark cycle. Appropriate procedures were performed to ensure limited distress, pain, discomfort, and/or injury. The sedatives ketamine and Telazol were applied by intramuscular injection at doses of 10 mg/kg of body weight and 4 to 5 mg/kg, respectively, for blood and tissue collections.

Animals and ART regimen. Twelve chronically SIV-infected SMs (3 females and 9 males; ranging in age from 14 to 25 years), not homozygous for CCR5-null alleles and with viral loads of >1,000 copies/ml, were included in this study. Starting at day 0, all animals were treated with a four-drug ART regimen comprising two nucleoside reverse transcriptase (RT) inhibitors (tenofovir [PMPA] and emtricitabine [FTC]), one integrase inhibitor (raltegravir [RAL]), and one protease inhibitor (darunavir [DRV]). PMPA and FTC were administered subcutaneously (s.c.) once a day at dosages of 20 mg/kg and 30 mg/kg, respectively. RAL and DRV were administered orally (mixed with food) twice a day at the following dosages: 150 mg twice a day (b.i.d.) and 400 mg b.i.d., respectively. The 12 SMs were divided into four groups of three animals each, and each group received ART for 2, 6, 9, or 12 months. ART was interrupted at day 60, 184, 274, or 365, and all animals were then monitored for an additional period of at least 2 months and up to 6 months. ART was administered to all 12 animals, but only 11 SMs completed treatment, as one animal was sacrificed due to complications unrelated to either SIV infection or ART.

Sample collection and processing. Peripheral blood (PB) and colorectal mucosa biopsy (RB) specimens were collected longitudinally at various experimental points prior to, during, and after ART administration. All animals were first sedated by intramuscular injection before any access. Blood collections of up to 40 ml and RB were performed every 6 weeks, while monitoring of viral load and immunophenotypic analysis were performed every 2 or 4 weeks on smaller blood volumes (3 to 5 ml). Collections and processing of PB and RB were performed as previously described (7, 27–29). Briefly, blood samples were used for complete blood counts and routine chemical analysis, and plasma was separated by centrifugation within 1 h of phlebotomy. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. For rectal biopsies, fecal material was removed from the rectum, and up to 10 colorectal pinches were collected with biopsy forceps under visual control via the use of an anoscope. RB-derived lymphocytes were isolated by digestion with 0.5 to 2 U/mg collagenase type II plus 10 U/μl DNase I recombinant, RNase-free solution for 2 h at 37°C and then passed through a 70-μm cell strainer to remove residual tissue fragments. All samples were processed to get a final concentration of 1 × 10⁶ cells/ml in complete RPMI 1640 medium and then were stained and fixed (1% paraformaldehyde). Flow cytometry analyses were performed within 24 to 48 h of collection.

Flow cytometric analysis. Fourteen-parameter flow cytometric analysis was performed on cells from PB and RB specimens by using panels of monoclonal antibodies that are cross-reactive to SMs (4, 5, 7, 27–30). Predetermined optimal concentrations of the following antibodies were used to determine variation in the concentrations of CD4+ T cells and specific CD4+ T cell subsets: anti-CD3–allophycocyanin (APC)–Cy7 (clone SP34-2), anti-CD95–phycocerythrin (PE)–Cy5 (clone DX2), anti-CD62L–PE (clone SK11), anti-CCR5–APC (clone 3A9), anti-CD45RA–fluorescein isothiocyanate (FITC) (clone L48), and anti-CCR7–PE–Cy7 (clone 3D12), all from BD Biosciences, San Jose, CA; anti-CD28–PE–Texas Red (clone CD28.2), from Beckman Coulter, Brea, CA; and anti-CD8–BV711 (clone RPA-T8), anti-CD4–BV650 (clone OKT4), anti-CD1–BV421 (clone EH12.2H7), and anti-CD27–BV605 (clone OX32), from BioLegend Inc., San Diego, CA. Activation and proliferation markers on CD4+ and CD8+ T cell populations were assessed using anti-CD69–PE-peridinin chlorophyll protein (PerCP)–Cy5.5 (clones G46-4 and Ki-67–Alexa 700 (clone B56) (both from BD Biosciences, San Jose, CA). The key CD4+ T cell subsets were defined as follows: naïve (T_N), CD45RA–CCR7+ CD27+ CD28+ CD95+; stem cell memory (T_SC), CD45RA–CCR7+ CD27+ CD28+ CD95–; central memory (T_CM), CD45RA–CD28+ CD95+ CCR7+ CD62L–; transitional memory (T_TM), CD45RA–CD28+ CD95– CCR7+ CD62L+; and effector memory (T_EM), CD45RA–CD28+ CD95+ CCR7+ CD62L+.

Flow cytometric acquisitions were performed on an LSRII flow cytometer.
driven by the FACS DiVa software package (BD Biosciences), and data were analyzed with FlowJo (Tree Star Inc., Ashland, OR).

Detection of plasma SIV RNA by standard RT-PCR assay. Plasma SIV loads were determined by standard quantitative RT-PCR as previously described (limit of detection, 60 copies/ml) (31). Briefly, 150 μl of plasma was used to extract viral SIVsamn RNA by using a QIAamp viral RNA minikit (Qiagen, Valencia, CA). Ten microliters of extracted RNA was subjected to RT-PCR by using random hexamers to prime reverse transcription (Invitrogen, Carlsbad, CA). Primer and probe sequences were targeted to the 5′-untranslated region of the SIVsmm genome (forward primer, 5′-GGCGAAGAATCTCCAGC-3′; reverse primer, 5′-GCTCTTACTGCTCTTACCTCA-3′; and probe, 5′-AGTCTCTGTTC RGGCGGCAA-3′) (4). The SIV RNA copy number was determined by comparison to an external standard curve created with virion-derived SIVsmm RNA. For graphical reasons, samples with SIV RNA levels below the detection limit of the assay were assigned a level of half the lower limit of detection.

SIVsamn sequence analysis for primer development. In order to generate a specific and sensitive quantitative SIVsamn real-time PCR assay, we sequenced the gag genes of the viruses present in all test animals prior to therapeutic intervention. The day −30 pre-ART plasma samples were utilized to assess overall sequence diversity in the cohort of naturally infected sooty mangabeys. Viral RNA was extracted from plasma by using a QIAamp viral RNA minikit (Qiagen). RNA was eluted and immediately subjected to cDNA synthesis. Reverse transcription of RNA to single-stranded cDNA was performed according to the manufacturer’s instructions (Invitrogen). Briefly, a 0.5 mM concentration of each deoxynucleoside triphosphate and 0.25 μM antisense primer SIV-Gag-R1 (5′-TGTGTGTTCCCATCTCT-3′) were incubated at 65°C for 5 min and then moved to ice for 1 min. Next, 1× RT buffer, 5 mM dithiothreitol, 2 μl RNaseOUT, and 10 U/μl SuperScript III reverse transcriptase were incubated at 50°C for 60 min, followed by an increase in temperature to 55°C for an additional 60 min. SuperScript III was then heat inactivated at 70°C for 15 min and treated with 2 U of RNase H at 37°C for 20 min. cDNA was used immediately for single genome amplification (SGA) PCR. Briefly, 1× High Fidelity Platinum PCR buffer, 2 mM MgSO4, 0.2 mM each deoxynucleoside triphosphates, 0.2 μM each primer, and 0.025 U/μl Platinum Taq high-fidelity polymerase were combined in a 20-μl reaction mixture. cDNA was serially diluted until a concentration was found where PCR-positive wells constituted fewer than 30% of the total number of reaction wells. First-round PCR primers included the sense primer SIV-U5-F1 (5′-AGCTAGTGTGTGTGTTCCCAT CTTCTCCTATACGGAAGG-3′) and the antisense primer SIV-Gag-R1. PCR was performed by denaturing at 94°C for 1 min followed by 35 cycles of 94°C for 20 s, 55°C for 30 s, and 68°C for 1 min, and the reaction was terminated with a single 10-min extension at 68°C. Next, 1 μl of each reaction mixture was transferred to a second-round reaction mixture that included the sense primer SIV-U5-F2 (5′-AGCTAGTGTGTGTGTTCCCAT CTTCTCCTATACGGAAGG-3′) and the antisense primer SIV-Gag-R2 (5′-GGCGGCAAAGAATTTGAGGTTGCAG-3′). The second-round PCR was performed under the same PCR conditions, but for 45 cycles. All PCR procedures were performed under PCR clean room conditions and using procedural safeguards against sample contamination, including prealiquoting of all reagents, use of dedicated equipment, and physical separation of sample processing from pre- and post-PCR amplification steps. Correctly sized amplicons, as determined by electrophoresis on an agarose gel, were directly sequenced by cycle sequencing using BigDye Terminator chemistry and protocols recommended by the manufacturer (Applied Biosystems). Individual sequence fragments for each amplicon were assembled and edited using Sequencher (Gene Codes).

qPCR assay design and validation. Based on the sequences determined as described above, the quantitative PCR (qPCR) assay primers and probe were designed to accommodate the SIVsamnvariation identified by this sequence analysis through the introduction of the nonstandard bases P and K (Gen Research) and degenerate bases (IDT). The newly designed assay consisted of the forward primer smGAGF (5′-GTCAGGG TACATGGGGATGTC-3′), the reverse primer smGAGr (5′-GACTAGA GTTCTCTGACTAT/GTTGTGTGG-3′), and the probe smGAGP (5′-6-carboxyfluorescein [FAM]-CTCTPCTAGTGTGTTCCTTCTTC TGGCG-BHQTM1-3′). Assay validation was performed on a mixture of in vitro transcripts generated individually from the 6 dominant variant templates, providing coverage of 96% of the 299 sequences obtained. The assay was optimized for a maximal sensitivity of specific target detection of eight copy equivalents per reaction mix (95% assurance).

Quantitative analysis of SIVsamn RNA samples by SM-specific RT-PCR assay. SIVsamn RNA isolation from cell-free sooty mangabey plasma samples was performed as previously described (32). Quantitative assessment of SIVsamn RNA was determined by a quantitative hybrid real-time/digital RT-PCR approach, which was modified from the plasma viral detection assay as described previously (33). The cDNA reaction mixtures were reduced to 15 μl, comprising 5 μl sample plus 10 μl concentrated reaction cocktail, and contained 5 μg/ml random hexamers, 5 U RNaseOUT RNase inhibitor (Invitrogen), and 10 U SuperScript III reverse transcriptase (Invitrogen). Twelve replicate reactions were performed per sample. Quantitative determinations for samples showing amplification in all replicates were derived by interpolation of the averaged threshold cycle (Ct) value for the reactions onto a standard curve of Ct versus input template copy number. Quantitative determinations for samples showing fewer than 12 positive amplifications in replicates were derived from the frequency of positive amplifications, corresponding to the presence of at least one target copy in a reaction mixture, according to a Poisson distribution of a given median copy number per reaction mixture. The assay yielded no positive reactions for a total of 72 total reaction mixtures for RNA samples derived from 6 uninfected animals.

Memory CD4+ T cell subset sorting. Enriched unlabeled CD4+ T cells were obtained by magnetic bead isolation and passage through a magnetically activated cell sorting (MACS) selection column placed in a MACS separator (Miltenyi, Auburn, CA). Cells in suspension were stained with the proper antibody mixture and then physically separated by fluorescence-activated cell sorting (FACS). Sorting of CD4+ Tcm, Tcm, and Tscm cells based on the expression of CD45RA, CCR7, CD28, CD95, and CD62L was performed on a FACS Aria II flow cytometer (BD Biosciences) for samples collected before and during ART. Cells were initially gated on the basis of light scatter, followed by positive staining for CD3 and CD4 and negative staining for the LIVE/DEAD fixable dead cell stain (Invitrogen, Carlsbad, CA).

Detection of cell-associated SIV DNA. Sorted CD4+ T cell subsets were lysed and homogenized in a highly denaturing guanidine isothiocyanate-containing buffer which inactivates DNases and RNase and disrupts cell membranes. Total DNA was then extracted by use of an AllPrep DNA/RNA minikit (Qiagen) according to the manufacturer’s recommendations. Quantitative assessments of cell-associated total SIV DNA within circulating memory CD4+ T cell subsets at day −30 pre-ART administration and days 45, 184, 274, and 365 on ART were performed by real-time PCR. Briefly, 45 ng of DNA was loaded into a 50-μl reaction mixture with an iVUtr primer/probe set. The same SIV-specific primers and probe were used to amplify and quantify total provirus copies of SIV as described before. Albumin was used as an internal control to quantify the cell number against an external standard curve. An albumin gene-specific probe (5′-VIC-TGCAGAGTCACAAATATGTGTCAGA-3′) and flanking primers (5′-TGCATGAGAAACGGCCTAATA-3′ and 5′-ATGGTGCC CGGTTCCTACCA-3′) (Applied Biosystems) were used to determine the proportion of SIV DNA+ cells by dividing the obtained SIV DNA copy number by the albumin gene copy number (5).

Statistical analysis. Comparisons between circulating CD4+ T cell, CD4+ T cell subset, and CD8+ T cell frequencies and absolute counts pre-ART and during ART were carried out using the nonparametric Wilcoxon matched-pair signed-rank test. The same analysis was carried out for mucosal populations as well. Comparisons between the levels of expression of different markers on CD4+ and CD8+ lymphocyte popula-
tions and their subsets were determined for points during ART versus previous time points and the baseline. Specifically, the unpaired t test, the unpaired t test with Welch’s correction, and the nonparametric Wilcoxon matched-pair signed-rank test were carried out. Variations between pre- and on-ART cell-associated virus (SIV DNA) levels in SM blood were monitored over time, and statistical significance was determined using repeated-measure analyses of each CD4 T cell subset (SAS MIXED Procedure, version 9.4). The means ± standard errors of the means (SEM) were used as descriptive statistics throughout the course of the study after ART initiation. In all cases, significance was attributed for P values of <0.05. All analyses were conducted using GraphPad Prism 5.0d and SAS MIXED Procedure, version 9.4.

RESULTS
Experimental design and baseline virological and immunological features of chronically SIV-infected SMs. To assess directly the effects of virus replication on the immunological parameters of interest during chronic SIV infection of SMs, we used a potent four-drug ART regimen that was shown to be very effective at suppressing SIV replication in rhesus macaques (19–22). Twelve SIV-infected SMs were included in this study and were divided into four groups, each with three animals receiving ART for 2, 6, 9, or 12 months. On day 0 of the study (Fig. 1A), all animals were treated with PMPA (20 mg/kg/day), FTC (30 mg/kg/day), DRV (400 mg b.i.d.), and RAL (150 mg b.i.d.). Blood and rectal biopsy (RB) specimens were collected longitudinally throughout the course of the study, as shown in Fig. 1A, to monitor a number of virological and immunological parameters prior to and during ART and after ART interruption. After ART interruption, all SIV-infected SMs were monitored for at least 2 months and up to 6 months (Fig. 1A). The 12 SIV-infected SMs included in this study were selected from the colony of animals housed at the YNPRC, and their basic immunological and virological features are shown in Fig. 1B, with viral loads ranging from 1,940 to 261,000 copies/ml of plasma and CD4+ T cell counts ranging from 167 to 1,336 cells/mm³ of blood. Note that, for this study, we selected animals that were not homozygous for the previously described CCR5-null genes (34).

ART is well tolerated and induces potent suppression of virus replication in chronically SIV-infected SMs. To investigate the effect of ART on the main virological and immunological pa-
parameters of SIV infection, the 12 SIV-infected SMs received ART for different periods (60, 184, 274, or 365 days). Overall, ART was safe and well tolerated by the majority of SIV-infected SMs that were included in this study. Two animals (FUv and FJy) exhibited a moderate weight loss that was reversed with diet supplementation, with a rapid return to baseline weight after ART interruption. The 21-year-old animal FWo, which was originally included in the 2-month treatment group, was euthanized on day 43 of therapy due to major cardiovascular symptoms that were attributed to aging by the YNPRC veterinary staff. As expected, all animals receiving ART experienced a rapid and significant decline in plasma viral load (Fig. 2A, gray boxed areas of the graphs). As shown in Fig. 2A, 6 of 11 SIV-infected SMs showed viremia suppression to below the level of detection of our standard SIVsmm viral load assay (i.e., 60 copies/ml) by day 30 after ART initiation, and the viral loads of four other animals became undetectable by day 45 of treatment. Only one animal (FSs) exhibited relative resistance to ART, with a viral load that declined but remained detectable after 184 days of ART. To further investigate the level of ART-induced virus suppression in SIV-infected SMs that had un-
detectable viral loads with our standard assay, we next measured viremia by using an ultrasensitive SIV<sub>smm</sub>-specific RT-PCR assay with a limit of detection of 10 copies/ml. The results of this ultra-sensitive assay indicated that low levels of virus production were still present in all ART-treated SIV-infected SMs, though it remains unclear whether this production was related to de novo cycles of virus replication versus persistent virus production in cells that were infected prior to ART (data not shown).

ART induces modest changes in the level of SIV DNA in memory CD4<sup>+</sup> T cell subsets. In previous studies, we showed that nonpathogenic SIV infection of SMs is associated with lower levels of infection (as measured by total cell-associated SIV DNA) in CD4<sup>+</sup> TCM and TSCM cells than those seen with pathogenic SIV infection of RMs (4–6). To investigate the effects of ART on the levels of SIV infection in different memory CD4<sup>+</sup> T cell subsets, we identified and sorted TSCM, TCM, TTM, and TEM cells based on the surface expression of CD45RA, CD62L, CCR7, CD28, and CD95 and quantified the levels of total SIV DNA by RT-PCR for samples collected at various time points during the study (i.e., day 30 pre-ART and days 60, 184, 274, and 365 on ART). As shown in Fig. 3A, a significant decrease in SIV DNA content was detected in the TEM CD4<sup>+</sup> T cell subset (P = 0.02) as a result of ART-mediated suppression of virus replication in this subset of short-lived cells. However, we found no statistically significant trends in infection of the other examined CD4<sup>+</sup> T cell subsets, i.e., TTM, TCM, and TSCM cells (average changes of 0.9-, 1.1-, and 3.7-fold for CD4<sup>+</sup> TTM, TCM, and TSCM cells, respectively). Note that one SIV-infected SM (FEz) exhibited undetectable levels of SIV gag copies in TSCM and TCM fractions both prior to ART treatment and during treatment. However, the apparent absence of SIV infection in these two long-lived memory CD4<sup>+</sup> T cell compartments was not sufficient to avoid rapid viral rebound once ART was stopped (Fig. 2B). Overall, these data indicate that administration of suppressive ART for 2 to 12 months induced a significant decline in the level of cell-associated SIV DNA in circulating CD4<sup>+</sup> TEM cells but had little impact on any of the other memory CD4<sup>+</sup> T cell subsets.

ART induces a significant repopulation of peripheral CD4<sup>+</sup> TCM cells and total intestinal CD4<sup>+</sup> T cells in SIV-infected SMs. In HIV-infected humans, initiation of ART induces a series of immunological changes, including a rapid increase in peripheral CD4<sup>+</sup> T cell counts as well as a slower and incomplete reconstitution of the mucosal CD4<sup>+</sup> T cell pool (13, 35–39). To evaluate the impact of ART-induced suppression of virus replication on CD4<sup>+</sup> T cell reconstitution, we next measured, for our cohort of ART-treated SIV-infected SMs, the levels of CD4<sup>+</sup> T cells in peripheral blood (PB) and rectal biopsy (RB) specimens taken at several time points during the study (i.e., day 30 pre-ART and days 60, 184, 274, and 365 on ART). As shown in Fig. 3A, a significant decrease in SIV DNA content was detected in the TEM CD4<sup>+</sup> T cell subset (P = 0.02) as a result of ART-mediated suppression of virus replication in this subset of short-lived cells. However, we found no statistically significant trends in infection of the other examined CD4<sup>+</sup> T cell subsets, i.e., TTM, TCM, and TSCM cells (average changes of 0.9-, 1.1-, and 3.7-fold for CD4<sup>+</sup> TTM, TCM, and TSCM cells, respectively). Note that one SIV-infected SM (FEz) exhibited undetectable levels of SIV gag copies in TSCM and TCM fractions both prior to ART treatment and during treatment. However, the apparent absence of SIV infection in these two long-lived memory CD4<sup>+</sup> T cell compartments was not sufficient to avoid rapid viral rebound once ART was stopped (Fig. 2B). Overall, these data indicate that administration of suppressive ART for 2 to 12 months induced a significant decline in the level of cell-associated SIV DNA in circulating CD4<sup>+</sup> TEM cells but had little impact on any of the other memory CD4<sup>+</sup> T cell subsets.
As shown in Fig. 4A, we found that the number of circulating CD4+ T cells remained stable during ART, with most animals experiencing only minor fluctuations. We next investigated how ART influenced the dynamics of the subsets of TN, TCM, TCM, TTM, and TEM CD4+ T cells in peripheral blood. As shown in Fig. 4B, we found that the percentages of CD4+ TN, TSCM, and TTM cells remained relatively constant after ART in our cohort of SIV-infected SMs, with only minor fluctuations observed in some animals. In contrast, ART-treated SIV-infected SMs showed a significant expansion of CD4+ TCM cells on days 45, 92, and 135 post-ART compared to baseline pre-ART levels (for day −30 versus day 45, P = 0.0039; for day −30 versus day 92, P = 0.0039; and for day −30 versus day 135, P = 0.0177) that was associated with a concomitant decline of CD4+ TEM cells on days 75 and 120 post-ART (for day −30 versus day 75, P = 0.0039; and for day −30 versus day 120, P = 0.0039). Note that the ART-induced increase in peripheral CD4+ TCM cells persisted on day 60 after ART interruption (Fig. 4B, day −30 versus day 60) (P = 0.0064). As shown in Fig. 4C, the analysis of CD4+ T cells in RB specimens (measured as the fraction of CD3+ T cells) from ART-treated SIV-infected SMs showed a significant increase compared to pre-ART levels (for day −30 versus day 92, P = 0.0391; and for day −30 versus day 135, P = 0.0117) (Fig. 4C). Interestingly, the observed increase of CD4+ T cells in RB specimens was not reversed once ART was interrupted, with the percentage of CD4+ T cells in the rectal mucosa at day 60 post-ART remain-

FIG 4 Longitudinal assessment of CD4 T cell levels in peripheral blood and rectal mucosa. (A) Levels of CD4 T cells in blood (expressed as numbers of cells per microliter) and mucosal tissue (RB; expressed as percentages of total CD3+ T cells) were calculated for each of the SMs before and during ART (gray shaded areas) and after ART cessation. (B) Levels (means ± SEM) of naive (yellow), TCM (light blue), TEM (purple), TTM (red), and TSCM (green) CD4+ T cell subsets in peripheral blood (PB) of SIV-infected SMs at the different experimental points, divided by group. The x axis shows the time post-ART initiation (day 0). In the bottom graphs, mean values, standard deviations, and statistically significant differences are shown (*, P < 0.05; ***, P < 0.01; and ***, P < 0.001). Statistical significance was determined by the Wilcoxon matched-pair signed-rank test. (C) Representative flow cytometric plots showing the fraction of CD4+ T cells (gated on live CD3+ T cells) in RB specimens before, during, and after ART administration for one representative SM. Comparisons of CD4+ T cell recovery were performed for different time points during ART (days 92 and 135) and after ART (day 60 post-ART). Statistically significant differences are shown (*, P < 0.05; ***, P < 0.01; and ***, P < 0.001). Statistical significance was determined by the Wilcoxon matched-pair signed-rank test.
ing higher than that at baseline (i.e., pre-ART) (Fig. 4C). Overall, these data indicate that ART induced a significant immune reconstitution in SIV-infected SMs, with increases in both peripheral CD4+ TCM and mucosal CD4+ T cells.

ART reduces the levels of activated CD8+ T cells in the peripheral blood and rectal mucosa of SIV-infected SMs. In HIV-infected humans, initiation of ART induces a reduction in the expression of activation and proliferation markers on CD4+ and CD8+ T cells in peripheral blood as well as on lymphoid and mucosal tissues (13,35–39). To characterize the impact of ART on the levels of immune activation of SIV-infected SMs, we next longitudinally assessed the expression of HLA-DR, PD-1, and Ki-67, i.e., three key markers of T cell activation (HLA-DR and PD-1) and proliferation (Ki-67), on CD4+ and CD8+ T cells from peripheral blood and rectal biopsy specimens. As expected based on previous studies, SIV-infected SMs showed relatively low levels of CD4+ and CD8+ T cell activation before ART. As shown in Fig. 5A, ART did not induce any change in the levels of CD4+ HLA-DR+ T cells in either the peripheral blood or the rectal mucosa. In contrast, we found that the percentage of CD8+ HLA-DR+ T cells declined significantly at day 135 of ART in peripheral blood (for day −30 versus day 135, P = 0.0039) and at day 92 of ART in RB specimens (for day −30 versus day 92, P = 0.0078) compared to the baseline (pre-ART) levels. Interestingly, ART interruption was followed by an increase of the percentage of CD8+ HLA-DR+ T cells in both blood and RB specimens (for PB at day −30 versus day 30, P = 0.0163; for PB at day 135 versus day 30, P = 0.0039; for PB at day −30 versus day 45, P = n.s. [not significant]; for PB at day 135 versus day 45, P = 0.0078; for RB specimens at day −30 versus day 106, P = n.s.; and for RB specimens at day 92 versus day 106, P = 0.0078) (Fig. 5B). No significant changes were found in the level of either PD-1+ or Ki-67+ T cells (either CD4+ or CD8+) in peripheral blood or rectal mucosa of ART-treated SIV-infected SMs (data not shown). Overall, these data suggest that ART is followed by a decline of CD8+ (but not CD4+) T cell activation that returns to pre-ART levels upon ART interruption.

ART interruption is followed by rapid virus rebound in SIV-infected SMs. Interruption of ART is followed by a rapid (i.e., within weeks) rebound of viremia in HIV-infected individuals, with numerous studies suggesting that virus persistence under ART is related to the latent infection of long-lived, resting memory CD4+ T cells, mainly belonging to the TCM and TSCM subsets (4, 5, 40–43). In our cohort of ART-treated SIV-infected SMs, we interrupted ART at various time points after initiation (see above for details) and measured the level of plasma viremia longitudinally. As shown in Fig. 2B, we found that regardless of the duration

FIG 5 Longitudinal assessment of HLA-DR-expressing CD4+ and CD8+ T cells in blood and RB specimens. (A) Mean values and standard deviations for percentages of HLA-DR+ CD4+ T cells in PB (red circles) and RB (blue circles) specimens at the different experimental time points are shown for the SM cohorts receiving ART for 2 (n = 2), 6 (n = 3), 9 (n = 3), and 12 (n = 3) months. Statistically significant differences are shown (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). (B) Longitudinal assessment of CD8+ T cells expressing HLA-DR in PB (upper panels) and RB (lower panels) specimens from all SMs.
of ART (ranging from 2 to 12 months), all SIV-infected SMs experienced a rapid rebound of virus replication. In most animals, the kinetics of virus rebound after ART interruption showed a peak of viremia at approximately day 30 post-interruption that then stabilized at slightly lower levels (decline of 0.5 to 1 log) in the following weeks (i.e., similar to the pre-ART set-point viremia). These results indicate that a reservoir of SIV-infected cells that harbor replication-competent virus clearly remain present in SMs under ART for up to 1 year, despite lower levels of infection in CD4⁺ T_CM and T_SCM cells.

DISCUSSION

Natural SIV infection of SMs is typically nonpathogenic despite high levels of virus replication and the short in vivo life span of productively infected cells (26). A series of studies indicated that the main mechanisms of protection from SIV disease progression in these animals are the lack of aberrant immune activation during the chronic phase of the infection, preservation of Th17 cells and mucosal integrity, and the preferential sparing of CD4⁺ T_CM and T_SCM cells from direct virus infection (4, 5, 41, 42). None of these published studies involved prolonged administration of potent, multidrug antiretroviral regimens, with only one study in which PMPA and FTC were used for a relatively short period as an experimental tool to assess the in vivo life span of productively infected cells (26). The main findings of the current study are that administration of ART in SIV-infected SMs was well tolerated overall and resulted in a rapid decline of viremia to levels below the limit of detection of our standard assay in all but one treated animal. The level of viremia decline observed in the current study is comparable overall to what we observed in similar studies conducted on SIV-infected RMs (19, 44). In addition, we found that ART initiation was followed by an increase in the percentage of CD4⁺ T_CM cells in peripheral blood, an increase of CD4⁺ T cells in the rectal mucosa, and a decline in the levels of activated CD8⁺ HLA-DR⁺ T cells in both blood and the rectum. Finally, we found that ART interruption resulted in rapid viral rebound in all treated animals.

The lower levels of infection in the long-lived CD4⁺ T_CM and T_CM cells of SIV-infected SMs suggested that the reservoir is possibly less stable under ART. While the current study does not directly support this hypothesis, there are several arguments against the conclusion that limited CD4⁺ T_CM and T_CM cell infection does not affect virus persistence in this model. These arguments include the following: (i) residual viremia was detected in all treated animals (using an ultrasensitive viral load assay that cannot distinguish, however, between rounds of de novo virus replication and residual virus production from cells infected prior to ART), (ii) the reservoir was measured only in peripheral blood, (iii) SIV DNA levels were low but still detectable in CD4⁺ T_CM and T_SCM cells in the majority of SIV-infected SMs, and (iv) ART was used for a period of 2 to 12 months only. An additional caveat is that this study included several SMs that were infected intravenously with SIV, while most of the naturally SIV-infected animals contracted the infection through sexual intercourse. All these caveats notwithstanding, we found it interesting that virus rebound was also observed in one SIV-infected SM with undetectable SIV DNA in both CD4⁺ T_CM and T_SCM cells (but still with detectable plasma viremia as measured by the ultrasensitive viral load assay). Future studies involving long-term ART (i.e., >2 years) and in which ART interruption is performed only when the treated SIV-infected SMs show undetectable viremia by the most sensitive assay (i.e., with quasispecies-specific probes and a limit of detection of 3 copies/ml of plasma) will be necessary to ascertain if, under those conditions, the virus reservoir may indeed prove to be less stable as a consequence of the lower infection levels of CD4⁺ T_CM and T_SCM cells.

An interesting and perhaps unexpected finding of this study is that ART had a positive impact on CD4⁺ T cell reconstitution in SIV-infected SMs, as revealed by significant increases of both total CD4⁺ T cells in the rectal mucosa and CD4⁺ T_CM cells in peripheral blood. While SIV infection of SMs is typically nonpathogenic, with infected animals experiencing a life span similar to that of uninfected animals (10), the infection is not devoid of some immunological abnormalities. In particular, we have shown that SIV-infected SMs experience a significant decline of CD4⁺ T cells in mucosal tissues (9), even though this decline is not progressive after the acute phase of infection, does not reach the nadirs often observed in pathogenic HIV and SIV infections, and is not associated with a loss of mucosal integrity or detectable microbial translocation (8, 9, 45). In this study, we observed, for the first time, that suppression of virus replication with ART induces a significant increase in the level of CD4⁺ T cells in the rectum and that this increase compared to pre-ART values persists even after ART interruption. This observation contrasts with the relatively slow and partial effect of ART in normalizing the levels of mucosal CD4⁺ T cells in humans. It is tempting to speculate that in SIV-infected SMs, the low levels of immune activation as well as the preservation of Th17 cells and mucosal immunity may favor the recovery of mucosal CD4⁺ T cells when virus replication is controlled by ART.

The observed impact of ART on the levels of circulating CD4⁺ T_CM cells as well as the levels of CD8⁺ T cells expressing the activation marker HLA-DR may also indicate that subtle immunological changes induced by persistent virus replication are present in SIV-infected SMs. The affected processes include CD4⁺ T cell homeostasis, which is largely dependent on the presence of healthy levels of T_CM cells in blood and tissues (46), and CD8⁺ T cell activation. The reasons for these changes not translating into a major immunological dysfunction with associated clinical signs of opportunistic infections remain unknown. However, in this regard, the current study opens up new avenues of research aimed at investigating what additional mechanisms may operate in SIV-infected SMs to limit the clinical impact of the immune abnormalities that are revealed and reversed by ART administration.

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REFERENCES


