Bone Marrow-Derived CD4⁺ T Cells Are Depleted in Simian Immunodeficiency Virus-Infected Macaques and Contribute to the Size of the Replication-Competent Reservoir

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ABSTRACT The bone marrow (BM) is the key anatomic site for hematopoiesis and plays a significant role in the homeostasis of mature T cells. However, very little is known on the phenotype of BM-derived CD4⁺ T cells, their fate during simian immunodeficiency virus (SIV) infection, and their contribution to viral persistence during antiretroviral therapy (ART). In this study, we characterized the immunologic and virologic status of BM-derived CD4⁺ T cells in rhesus macaques prior to SIV infection, during the early chronic phase of infection, and during ART. We found that BM memory CD4⁺ T cells are significantly depleted following SIV infection, at levels that are similar to those measured in the peripheral blood (PB). In addition, BM-derived memory CD4⁺ T cells include a high frequency of cells that express the coinhibitory receptors CTLA-4 and PD-1, two subsets previously shown to be enriched in the viral reservoir; these cells express Ki-67 at levels similar to or higher than the same cells in PB. Finally, when we analyzed SIV-infected RMs in which viral replication was effectively suppressed by 12 months of ART, we found that BM CD4⁺ T cells harbor SIV DNA and SIV RNA at levels comparable to those of PB CD4⁺ T cells, including replication-competent SIV. Thus, BM is a largely understudied anatomic site of the latent reservoir which contributes to viral persistence during ART and needs to be further characterized and targeted when designing therapies for a functional or sterilizing cure to HIV.

IMPORTANCE The latent viral reservoir is one of the major obstacles in purging the immune system of HIV. It is paramount that we elucidate which anatomic compartments harbor replication-competent virus, which upon ART interruption results in viral rebound and pathogenesis. In this study, using the rhesus macaque model of SIV infection and ART, we examined the immunologic status of the BM and its role as a potential sanctuary for latent virus. We found that the BM compartment undergoes a similar depletion of memory CD4⁺ T cells as PB, and during ART treatment the BM-derived memory CD4⁺ T cells contain high levels of cells expressing CTLA-4 and PD-1, as well as amounts of cell-associated SIV DNA, SIV RNA, and replication-competent virus comparable to those in PB. These results enrich our understanding of which anatomic compartments harbor replication virus and suggest that BM-derived CD4⁺ T cells need to be targeted by therapeutic strategies aimed at achieving an HIV cure.

KEYWORDS CTLA-4, HIV-1, PD-1, SIV, bone marrow, coinhibitory receptors, viral reservoir

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Recent advancements in antiretroviral therapy (ART) have effectively been able to suppress human immunodeficiency virus type 1 (HIV-1) replication and have reduced HIV-related morbidities and mortalities (1, 2). Despite these successes, individuals infected with HIV must remain on a lifelong ART regimen due to viral persistence of latently infected cells which contain intact and transcriptionally silent proviruses that are able to evade the host immune system (3). Within the latent population, a fraction of these integrated proviruses are replication competent, and upon ART interruption, this replication competence results in recrudescence of virus. Therefore, identifying the cellular and anatomic nature of the latent viral reservoir is paramount in achieving a functional or sterilizing cure for HIV.

In healthy individuals and mice, the bone marrow (BM) compartment contains mature T cells (4), with T cells comprising 3% to 8% of total nucleated BM cells, and has a reduced CD4/CD8 ratio compared with that of peripheral blood (PB) mononuclear cells (4–6). After priming of T cells in mice, contraction has been shown to be less pronounced in the BM compartment than in other lymphoid organs and blood, leading to persistence of BM antigen (Ag)-specific memory T cells (7–9). Within mouse BM, there resides a large proportion of memory T cells, as defined by expression of CD44^{hi}, that increases with age and Ag encounter (4, 10, 11). BM-derived memory T cells are primarily comprised of central memory (T_{CM}) and effector memory (T_{EM}), two subsets of circulating T cells found in the blood (12). Others have also shown that in human BM there exists a reservoir for CD4⁺ CD25⁺ T regulatory (T_{Reg}) cells (13). A large majority of human and mouse BM memory T cells are nonproliferative and in a quiescent state; however, there is a small fraction of memory T cells that proliferate under steady-state conditions (8, 14). It has been postulated that proliferation of T cells is stimulated by the BM microenvironment, where high levels of cytokines regulating T cell homeostasis, such as interleukin-7 (IL-7) and IL-15, are present (15–18). CD4⁺ T cells in the BM have also been shown to be enriched for CCR5, the necessary coreceptor for HIV infection (19). Previous studies with nonhuman primates (NHPs) showed early BM hematopoietic defect after simian immunodeficiency virus (SIV) or simian-human immunodeficiency virus (SHIV) infection, which resulted in an impaired T cell production (20, 21). A recent study looking at myeloid-derived suppressor cells (MDSC) revealed that depletion of these cells in BM could contribute to systemic immune activation and exacerbate SIV pathogenesis (22). In our paper, we focus on the CD4⁺ T cell contribution to viral persistence; however, others have shown that other cellular subsets in the bone marrow may harbor virus as well (23).

The above-listed immunologic features suggest that the BM compartment can be an important anatomic location targeted by HIV and in which HIV can persist. Indeed, resting memory CD4⁺ T cells are considered the main cellular reservoir for HIV, and homeostatic proliferation of CD4⁺ T cells is considered a central mechanism for viral persistence during ART. However, the BM remains largely understudied in regard to its potential contribution to HIV pathogenesis and viral persistence, both in humans and in NHPs, with most published work focusing on other anatomic locations such as the PB, lymph node, gut, spleen, and brain.

In this study, using the well-established model of SIV infection in rhesus macaques (RMs), we characterized the immunologic and virologic status of BM and compared it with that of PB before and after SIV infection as well as after 12 months of ART. In accordance with previous studies, we show that the BM compartment indeed has a lower CD4/CD8 ratio than PB. In contrast to previous studies, we show a paucity of T_{Reg} cells (CD25⁺ CD127[−] FoxP3⁺) in the BM. During the course of SIV infection, BM memory CD4⁺ T cells undergo a severe depletion, similar to what occurs in the periphery and lymphoid organs. Recently, we and others have shown that CTLA-4⁺ and PD-1⁺ memory CD4⁺ T cells are enriched in the viral reservoir (24–27); here we show that BM-derived CD4⁺ T cells contain high levels of cells expressing CTLA-4 and PD-1 during early chronic infection and after ART. Finally, after 12 months of ART, BM CD4⁺ T cells harbor SIV DNA and SIV RNA at levels comparable to those found in PB CD4⁺ T cells, including similar amounts of replication-competent SIV. In conclusion, the BM is

FIG 1 CD4 and CD8 T cell subset frequencies in BM and PB of healthy RMs. (A) Frequencies of CD4⁺ and CD8⁺ T cells within live CD3⁺ lymphocytes were measured from uninfected RMs. (B) Ratios of CD4 to CD8 were determined by calculating the ratio of paired CD4⁺ and CD8⁺ T cells. (C) Representative CD4-by-CD8 staining in BM and PB. (D and E) Frequencies of naive, central memory (CM), and effector memory (EM) CD4⁺ and CD8⁺ T cells were measured for uninfected RMs. (F) Representative staining in BM and defining subsets of CD4⁺ and CD8⁺ T cells ($n = 41$ RMs). *, $P < 0.0001$.

an additional, previously unappreciated site for the viral reservoir that needs to be further characterized and considered when designing therapies for a functional or sterilizing cure for HIV.

RESULTS

Immunologic characterization of BM-derived T cells in healthy RMs. For this study, we used bone marrow (BM) aspirate and peripheral blood (PB) longitudinally collected from 41 RMs before and after experimental intravenous (i.v.) infection with SIV_{mac239}. An ART regimen consisting of tenofovir (TDF), dolutegravir (DTG), and emtricitabine (FTC) was initiated at day 60 postinfection (p.i.) and was maintained for up to 12 months, with all animals achieving undetectable levels (<60 copies/ml) of plasma viremia. Microscopic examination after Wright-Giemsa staining was performed to exclude major blood contamination in the BM aspirate (see “Sample collection and processing” below).

Immunophenotypic analysis shows that in healthy uninfected RMs, the BM contained a mean of $46\% \pm 8.23\%$ CD4⁺ T cells and $35.6\% \pm 6.97\%$ CD8⁺ T cells among CD3⁺ lymphocytes, as opposed to $60.1\% \pm 6.91\%$ CD4⁺ ($P < 0.0001$) and $26.8\% \pm 6.19\%$ CD8⁺ ($P < 0.0001$) cells among CD3⁺ lymphocytes seen in PB (Fig. 1A), with a significant decrease in the CD4/CD8 ratio compared to that in PB (Fig. 1B; $P < 0.0001$). Representative CD4-by-CD8 staining in BM and PB is shown in Fig. 1C. We then analyzed the frequencies of CD4⁺ (Fig. 1D) and CD8⁺ (Fig. 1E) T cells with a naive (CD28⁺ CD95[−] CCR7⁺), central memory (CM; CD95⁺ CCR7⁺), or effector memory (EM; CD95⁺ CCR7[−]) phenotype; the gating strategy for the different T cell subsets is shown in Fig. 1F for BM. BM-derived CD4⁺ T cells had significantly lower levels of CM (BM,

FIG 2 Expression of coinhibitory receptors (co-IRs) and frequencies of T_H-like and T_{Reg} subsets in BM and PB of healthy RMs. (A to C) Frequencies of CD4⁺ T cell subsets with CTLA-4⁺ PD-1⁻ (A), CTLA-4⁻ PD-1⁺ (B), and CTLA-4⁺ PD-1⁺ (C) phenotypes. (D) Representative CTLA-4-by-PD-1 staining in BM and PB CM CD4⁺ T cells. (E) Expression of Ki-67 in CTLA-4⁺ PD-1⁻, CTLA-4⁻ PD-1⁺, and CTLA-4⁺ PD-1⁺ memory CD4⁺ T cell subsets. Ki-67 expression was measured in a different cohort of 22 RMs (see Materials and Methods). (F) Frequencies of memory CD4⁺ (CD95⁺) T cell subsets: T_H-like (CXCR5⁺ PD-1⁺), CD25⁺ CD127⁻, T_{Reg} (CD25⁺ CD127⁻ FoxP3⁺), and T_{Reg}-like (CXCR5⁺ PD-1⁺ CD25⁺ CD127⁻ FoxP3⁺). (G) Representative staining of subsets in PB (n = 41 RMs). *, $P < 0.0001$.

17.35% $\pm$ 5.51%; PB, 21.66% $\pm$ 6.37%; $P = 0.0010$) and higher levels of EM (BM, 14.55% $\pm$ 7.09%; PB, 9.15% $\pm$ 3.62%; $P < 0.0001$) cells than blood (Fig. 1D). Similar to the case with CD4⁺ T cells, the frequency of CM CD8⁺ T cells was also lower in BM than PB (BM, 4.29% $\pm$ 1.86%; PB, 7.09% $\pm$ 2.13%; $P < 0.0001$), with no significant difference for EM (BM, 43.82% $\pm$ 16.21%; PB, 39.5% $\pm$ 12.98%; $P = 0.2545$) or naive cells.

The expression of coinhibitory receptors (co-IRs), such as CTLA-4 and PD-1, on Ag-specific T cells defines an exhausted T cell population that has impaired effector function and diminished production of effector cytokines (28). Recently, it has been shown that PD-1⁺ as well as CTLA-4⁺ PD-1⁻ memory CD4⁺ T cells critically contribute to viral persistence during ART in humans and nonhuman primates (24–27). Thus, we looked at CTLA-4 and PD-1 expression in the BM and PB of healthy RMs. In the CTLA-4⁺ PD-1⁻ population, we saw similar expression patterns between BM-derived CD4⁺ T cells and PB-derived CD4⁺ T cells, except for BM having a higher frequency of CTLA-4⁺ PD-1⁻ CD4⁺ CM cells (BM, 4.95% $\pm$ 1.09%; PB, 4.03% $\pm$ 0.91%, $P < 0.0001$) (Fig. 2A). We also saw similar expression levels of CTLA-4⁻ PD-1⁺ and CTLA-4⁺ PD-1⁺ CD4⁺ T

cell subsets between BM- and PB-derived cells (Fig. 2B and C). Representative PD-1-by-CTLA-4 staining in BM and PB is shown in Fig. 2D. The levels of expression of Ki-67 within memory CD4⁺ T cells expressing CTLA-4 and/or PD-1 were comparable between BM and PB (Fig. 2E). Since PD-1 and CTLA-4 are highly expressed on T follicular helper (T_{FH}) and T_{Reg} CD4⁺ T cells, respectively, we measured and compared the frequencies of these two functional subsets between BM and PB. For both T_{FH}-like (CXCR5⁺ PD-1⁺; BM, 1.32% ± 1.42%; PB, 12.24% ± 3.94%; $P < 0.0001$) and T_{Reg} cells (CD25⁺ CD127[−] FoxP3⁺; BM, 0.13% ± 0.29%; PB, 5.15% ± 1.30%; $P < 0.0001$), we saw a lower frequency of each subset within memory CD4⁺ cells in the BM compartment (Fig. 2F); the gating strategy for the memory CD4⁺ T cell subsets is shown in Fig. 2G for PB. Of note, although BM harbored a population of memory CD4⁺ T cells with a CD25⁺ CD127[−] phenotype, the paucity of T_{Reg} cells in BM described above derived from the lack of expression of FoxP3, the master regulator of T_{Reg} cells (Fig. 2F). We also saw lower levels of T follicular regulatory (T_{FReg}) cells in the BM, but this can be attributed to the lack of FoxP3 expression as seen with conventional T_{Reg} cells.

Immunologic characterization of BM-derived T cells in SIV-infected and ART-treated RMs. SIV infection in RMs is normally pathogenic and results in a massive depletion of CD4⁺ T cells, particularly those with a memory phenotype, during the viremic phase of infection. Here, we showed that BM memory CD4⁺ T cells were depleted during the early chronic phase of infection (day 52 p.i.), similarly to the depletion seen in PB, with the frequency of BM memory CD4⁺ T cells going from 33.67% ± 10.19% before infection to 13.8% ± 8.61% at day 52 p.i. ($P < 0.0001$) (Fig. 3A). After 12 months of ART administration, memory CD4⁺ T cells were reconstituted in both BM and PB (Fig. 3A); however, in BM they remained at levels still significantly lower than those at preinfection (preinfection, 33.67% ± 10.19%; ART, 25.46% ± 8.45%; $P = 0.0008$). BM memory CD8⁺ T cells were increased in frequency during early chronic infection, comparable to what was seen in the PB, going from 48.65% ± 15.67% prior to infection to 58.48% ± 13.63% following infection ($P = 0.0052$) (Fig. 3A). Following ART, BM memory CD8⁺ T cells were slightly lower than preinfection levels (preinfection, 48.65% ± 15.67%, ART, 38.41% ± 15.21%; $P = 0.0062$), whereas in the PB no difference was seen between preinfection and on-ART levels ($P = 0.9279$). The CD4/CD8 ratio was decreased during the course of SIV infection and restored during ART, although at all phases of infection it remained lower in the BM than in the PB (Fig. 3B). During the course of infection, the frequency of BM CM and EM CD4⁺ T cells was diminished and was restored upon ART administration, but to a lower level than preinfection levels, whereas, in the PB, the levels returned to preinfection levels (Fig. 3C). BM and PB CM CD8⁺ T cells were increased during infection, but after ART, they returned to levels lower than baseline (Fig. 3D). In BM EM, CD8⁺ T cells were slightly elevated during infection, and similarly to CM cells, they returned to levels below baseline during ART, whereas, in PB, the levels remained constant throughout (Fig. 3D). Similar to what can be seen for uninfected animals (Fig. 2F), throughout the course of SIV infection and ART T_{FH}-like and T_{Reg} cells were present at lower frequencies in the BM than in PB (Fig. 3E). The combined ART regimen was very effective in suppressing plasma viremia in all RMs (Fig. 3F).

Next, we examined the expression of CTLA-4 and PD-1 throughout the course of infection (Fig. 4A to C). We saw comparable frequencies of CTLA-4⁺ PD-1[−] or CTLA-4⁺ PD-1⁺ cells among BM and PB CD4⁺ subsets during viremia and ART (Fig. 4A and C). The frequencies of CTLA-4[−] PD-1⁺ cells among BM and PB CD4⁺ subsets were equivalent throughout, except for the EM population during viremia (BM, 22.86% ± 9.68%; PB, 45.35% ± 11.37%; $P < 0.0001$) (Fig. 4B). Expression of Ki-67 was overall similar within CTLA-4⁺ and/or PD-1⁺ memory CD4⁺ T cells in the BM to that in PB, with the exception of a higher frequency of cycling CTLA-4⁺ PD-1⁺ cells at day 36 p.i. (Fig. 4D to F). To avoid quantification of Ki-67 on very few events, we opted to show cell cycling for memory CD4⁺ T cells expressing CTLA-4 and/or PD-1 and not for the CM or EM subsets.

FIG 3 Longitudinal characterization of BM- and PB-derived T cells following SIV infection and ART treatment. (A) Frequencies of total memory CD4⁺ and CD8⁺ T cells before SIV infection (SIV⁻), at day 52 after SIV infection (SIV⁺), and following ART (ART⁺) (days 248 to 358 p.i.). (B) Ratio (calculated in same manner as for Fig. 1B) of CD4⁺ to CD8⁺ T cells longitudinally. (C and D) Frequencies of CD4⁺ and CD8⁺ T cell subsets (naive, CM, and EM) longitudinally. (E) Frequencies of memory CD4⁺ (CD95⁺) T cell subsets: T_H1-like (CXCR5⁺ PD-1⁺), CD25⁺ CD127⁻, T_{Reg} (CD25⁺ CD127⁻ FoxP3⁺), and T_{Reg}-like (CXCR5⁺ PD-1⁺ CD25⁺ CD127⁻ FoxP3⁺). (F) Plasma viral loads (VL) are shown during preinfection, during early chronic infection (day 52 p.i.), and after ART (days 248 to 358 p.i.). VLs were quantified using qRT-PCR (limit of detection [LOD], 60 copies/mL of plasma; indicated by the dotted line) ($n = 40$ RMs). *, $P < 0.0001$.

Overall, these data indicate that BM-derived memory CD4⁺ T cells are depleted during SIV infection of RMs and reconstituted during ART to lower levels than those measured in blood; furthermore, BM contains a high frequency of CD4⁺ T cells expressing co-IRs, which have been previously shown to be enriched in the HIV reservoir (24–27).

BM CD4⁺ T cells harbor SIV DNA and SIV RNA as well as replication-competent virus at levels comparable to those in PB. The finding that BM contains subsets of memory CD4⁺ T cells previously shown to critically contribute to the viral reservoir,

FIG 4 Levels of BM- and PB-derived CD4⁺ T cells expressing co-IRs and expression of Ki-67 following SIV infection and ART. Frequencies of CD4⁺ T cell subsets with CTLA-4⁺ PD-1⁻ (A), CTLA-4⁻ PD-1⁺ (B), and CTLA-4⁺ PD-1⁺ (C) phenotypes at day 52 postinfection (SIV+) and following 12 months of ART (ART) ($n = 40$ RMs). Expression of Ki-67 in CTLA-4⁺ PD-1⁻ (D), CTLA-4⁻ PD-1⁺ (E), and CTLA-4⁺ PD-1⁺ (F) subsets measured in 22 RMs during SIV infection and 14 RMs during ART (see Materials and Methods). Data for 17 animals are shown in panel D due to the low numbers of CTLA-4⁺ PD-1⁻ memory CD4⁺ T cells in 5 of the 22 animals during SIV infection. *, $P < 0.0001$.

such as those expressing coinhibitory receptors, led us to hypothesize that the BM may significantly contribute to viral persistence during ART. To test this hypothesis, we sorted paired BM- and PB-derived CD4⁺ T cells from 6 SIV-infected RMs treated with suppressive ART for 12 months and performed quantitative PCR (qPCR) to detect cell-associated SIV DNA and SIV RNA levels. Consistent with our hypothesis, we found that in the BM the amount of SIV DNA was $2,162 \pm 1,234$ copies/ 1×10^6 CD4⁺ T cells, which was comparable to the levels seen in the PB, at $2,220 \pm 1,177$ copies/ 1×10^6 CD4⁺ T cells ($P = 0.8182$) (Fig. 5A). Similar results were obtained for SIV RNA in the BM and PB, with levels at 581.8 ± 340.5 copies/ 1×10^6 CD4⁺ T cells and 419 ± 187.6 copies/ 1×10^6 CD4⁺ T cells, respectively ($P = 0.3095$) (Fig. 5B). As a result, the ratios of SIV RNA to SIV DNA were also very similar in BM and PB ($P = 0.8182$) (Fig. 5C). Importantly, as determined by viral outgrowth assays (VOA) performed with BM and PB of two ART-treated, SIV-infected RMs, a subset of BM CD4⁺ T cells harbors replication-competent virus at levels that are at least comparable to those seen in the PB (Fig. 5D).

DISCUSSION

Reduction of the HIV reservoir is paramount for the development of a sterilizing or functional cure for HIV. However, this goal has been hampered by the inability to properly quantify and identify the anatomic location of latently infected cells. In humans, the bone marrow compartment contains a high proportion of memory T cells, with T cells representing 3% to 8% of total nucleated BM cells (4). The BM microenvironment has been shown to have high levels of IL-7 and IL-15, both shown to be important for T cell proliferation and maintenance of homeostasis (15–18). Expression

FIG 5 Cell-associated SIV DNA and SIV RNA and viral outgrowth assay (VOA) in BM- and PB-derived CD4⁺ T cells of ART-treated RMs. Copies of cell-associated SIV-DNA (A) and SIV-RNA (B) per 10^6 CD4⁺ T cells purified from BM and PB ($n = 6$ RMs). (C) Ratio of SIV RNA to SIV DNA in BM and PB. (D) CD4⁺ T cells were isolated from BM and PB ($n = 2$ RMs) and used in the viral outgrowth assay to confirm the presence of replication-competent virus. The graph shows SIV_{mac239} RNA copies/mL of supernatant at 2, 4, and 6 weeks of the VOA.

of CCR5, the coreceptor for HIV, has also been shown to be enriched in BM CD4⁺ T cells (19). These findings suggest that the BM compartment could serve as a potential anatomic location targeted and exploited by HIV and can serve as a sanctuary of latent infection. To test this hypothesis, we performed a comprehensive study of BM as an anatomic site of viral persistence, its role in maintaining the viral reservoir, and studied the phenotypal features of BM-derived CD4⁺ and CD8⁺ T cells during the course of SIV infection and ART treatment in 41 rhesus macaques. To the best of our knowledge, this is the first detailed, longitudinal characterization of the immunologic and virologic features of BM following SIV infection and ART.

In the healthy RMs, we found that BM was characterized by a lower ratio of CD4 to CD8 than in PB, aligning with what others have shown (4–6); lower levels of CM CD4⁺ and CD8⁺ T cells in BM, with higher levels of EM CD4⁺ T cells; and similar levels of expression of CTLA-4 and PD-1, except for higher levels of CTLA-4 expressed on CM CD4⁺ T cells. Furthermore, BM harbors significantly lower levels of T_{FH} and T_{Reg} cells than does blood. Of note, we saw very low expression of FoxP3 in the BM, even though the CD25⁺ CD127[−] population was present. Although a previous study with mice reported the BM being enriched in T_{Reg} cells, quantification of T_{Reg} cells in that study was limited to CD4⁺ CD25⁺ T cells and to mRNA levels of FoxP3 (13).

SIV infection in RMs normally leads to a severe depletion of memory CD4⁺ cells. In our study, we saw that the memory CD4⁺ cells in BM undergo a depletion similar to that in PB. Upon 12 months of ART, the levels of BM memory CD4⁺ T cells is restored, but not to the levels seen during preinfection and to a lesser extent than in PB. Although our study was not designed to formally prove it, one possible explanation for the lower reconstitution of CD4⁺ T cells seen in the BM during ART is cells trafficking

to the periphery. The CD4/CD8 ratio, an important immunologic marker of disease progression, remains significantly lower in BM than PB following SIV infection and ART (29–31).

Recent studies highlight CD4⁺ T cells expressing coinhibitory receptors, including PD-1 and/or CTLA-4, as the main cellular reservoirs in blood and lymphoid tissues of HIV- and SIV-infected subjects (24–27, 32, 33). Importantly, BM-derived CD4⁺ T cells express PD-1 or CTLA-4 at levels comparable to those found in blood CD4⁺ T cells during the course of infection and ART. Furthermore, BM CD4⁺ T cells that are CTLA-4⁺ PD-1⁺ express Ki-67 at levels significantly higher than in the same cells in the PB in untreated, SIV-infected RMs. Thus, not only does BM contain CD4⁺ T cell subsets that have been identified as critical to viral persistence, but also these cells seem to be of a phenotype which may favor viral infection and, potentially, viral persistence. Indeed, consistent with the immunologic data showing BM harboring subsets of CD4⁺ T cells in which HIV and SIV can persist during ART, we found comparable levels of cell-associated SIV DNA and SIV RNA among CD4⁺ T cells of the two anatomic sites after 12 months of suppressive ART. Unfortunately, due to low cell yield from a BM aspirate and the fact that BM CD4⁺ T cells are depleted during infection, we were unable to quantify the levels of cell-associated SIV DNA and SIV RNA during the pre-ART phase of the study. Finally, by performing viral outgrowth assays, we showed that the BM CD4⁺ T cells harbor replication-competent virus. Although we were unable to perform quantitative viral outgrowth assays (QVOA), as we were limited in cell numbers isolated from BM, our VOA results indicate that a fraction of BM-derived CD4⁺ T cells harbored replication-competent virus during ART, supporting the presence of latent HIV-infected cells in a previously understudied anatomic location.

In summary, our results highlight that SIV is able to establish and maintain viral persistence within the BM. Thus, the BM compartment represents an additional viral reservoir that needs to be targeted for a functional or sterilizing cure.

MATERIALS AND METHODS

Study approval. All animal experiments were conducted following the guidelines established by the Animal Welfare Act and the *Guide for the Care and Use of Laboratory Animals* (34) and performed in accordance with institutional regulations after review and approval by the Institutional Animal Care and Usage Committee (IACUC, 3000065, 2003297, 2003470, and PROTO201700665) at the Yerkes National Primate Research Center (YNPRC; Atlanta, GA). Anesthesia was administered prior to performing any procedure, and the proper steps were taken to minimize any suffering the animals may have experienced.

Animals. Forty-one male Indian rhesus macaques (RMs; *Macaca mulatta*) (aged 2 to 3.5 years at time of assignment), all housed at the YNPRC, were included in this study. All RMs were HLA*B07[−] and HLA*B17[−]. Prior to study assignment, all RMs were screened for SIV, cercopithecine herpesvirus 1 (B virus), simian T-lymphotropic virus (STLV), respiratory syncytial virus (RSV), and tuberculosis (TB) and dewormed. After experimental infection, animals were housed in isolation, in order to lower the risk of superinfection, in metal wire cages at an ambient temperature of 72°F. RMs were fed a diet consisting of jumbo biscuits supplemented with 15% protein, half an orange per day, and produce enrichment and foraging material (cereals, grains, seeds, etc.) five times per week. All procedures were approved by the Emory University IACUC, and animal care facilities are accredited by the U.S. Department of Agriculture (USDA) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

All RMs were intravenously (i.v.) infected with 300 50% tissue culture infective doses (TCID₅₀) of SIV_{mac239}. An ART regimen, consisting of tenofovir (TDF; 5.1 mg/kg of body weight/day), dolutegravir (DTG; 2.5 mg/kg/day), and emtricitabine (FTC; 40 mg/kg/day), was initiated at day 60 p.i. and was maintained for up to 12 months. Animals remained on ART until plasma viremia was undetectable (limit of detection 60 copies/ml of plasma) for at least 3 months. Ages of RMs pre-ART ranged from 2.1 to 3.6 years, and ages during ART measurement ranged from 2.7 to 4.2 years. The expression of Ki-67 among the CTLA-4 and/or PD-1 memory CD4⁺ T cell subsets was determined in a different cohort of animals, specifically in 22 Indian RMs at preinfection and at day 36 after SIV_{mac239} infection (i.v., 300 TCID₅₀), as well as in 14 of these 22 animals after 12 months of ART (day 399 p.i.). Ages of these 22 animals at preinfection ranged from 3 years and 10 months to 9 years and 2 months.

Sample collection and processing. BM and PB were collected preinfection, during early chronic infection, and during ART. Animals were anesthetized with either intramuscular (i.m.) ketamine (5 to 10 mg/kg) or tiletamine-zolazepam (Telazol; 3 to 5 mg/kg) prior to BM collection. The animals were placed in either dorsal or lateral recumbency. The area over the iliac crest was clipped and surgically scrubbed with 3 alternating applications of chlorhexidine or betadine scrub and alcohol before aseptic introduction of a 14- to 20-gauge needle connected to a syringe (with or without heparin coating) into the bone.

The desired volume was aspirated into the syringe. Suction was released before removing the bone marrow needle. Bone marrow aspirations were limited to a volume of 1 to 1.5 ml to avoid contamination with PB. The quality of our samples was assessed by performing a Wright-Giemsa stain on a glass slide smear. Samples were accepted and further processed only if the stained smear showed the cellular morphology typical of a BM aspirate, including a sufficient number of bone spicules and significant representation of all hematopoietic lineages with normal distribution of hematopoietic precursors. BM- and PB-derived cells were isolated by density gradient (Ficoll-Paque Premium; GE Healthcare) centrifugation.

Determination of viral load RNA. Quantitative real-time reverse transcription (RT)-PCR was performed to determine SIV plasma viral load as previously described (35).

Flow cytometric analysis. Eighteen-parameter flow cytometric analysis was performed on peripheral blood- and bone marrow-derived cells according to procedures using a panel of monoclonal antibodies that we and others have shown to be cross-reactive with RMs (25, 36). The following antibodies were used at predetermined optimal concentrations: anti-FoxP3-allophycocyanin (APC) (clone 150D), anti-CD4-APC-Cy7 (clone OKT4), anti-CD95-BV605 (clone DX2), anti-CD25-BV711 (clone BC96), and anti-PD-1-BV785 (clone EH12.2H7), all from Biolegend; anti-CXCR5-phycoerythrin (PE) (clone MU5UBEE) and anti-CD127-PE-Cy5 (clone eBioRDR5), both from eBioscience; anti-CCR7-PE-Cy7 (clone 3D12), anti-Ki-67-Alexa700 (clone B56), anti-CTLA-4-BV421 (clone BNI3), anti-CD3-BUV395 (clone SP34-2), anti-CD8-BUV496 (clone RPA-T8), and anti-CD28-BUV737 (clone CD28.2), all from BD Biosciences; and Aqua LIVE/DEAD amine dye-AmCyan from Invitrogen. To detect the expression of FoxP3 intracellularly, mononuclear cells were fixed and permeabilized with FoxP3 fixation/permeabilization solution (Tonbo) and subsequently stained intracellularly with FoxP3. Flow cytometric acquisition was performed on at least 100,000 CD3⁺ T cells on an LSRFortessa (BD Biosciences) cytometer driven by fluorescence-activated cell sorting (FACS) DIVa software. The data acquired were analyzed using FlowJo software (version 10.4.2; TreeStar).

Flow cytometry cell sorting. Mononuclear cells isolated from blood and bone marrow were sorted on a FACSARIA II (BD Biosciences) driven by FACS DIVa software. The following antibodies were used at predetermined optimal concentrations: anti-CD8-fluorescein isothiocyanate (FITC) (clone RPA-T8), anti-CD3-APC-Cy7 (clone SP34-2), anti-CD4-BV650 (clone OKT4), and Aqua LIVE/DEAD amine dye-AmCyan from Invitrogen. Sorted cells, with a purity higher than 95%, were used to determine the content of cell-associated SIV DNA and RNA or for viral outgrowth assay.

Quantitation of cell-associated SIV DNA and SIV RNA. Cellular DNA and RNA were extracted from at least 50,000 CD4⁺ T cells lysed in RLT Plus buffer (Qiagen) and isolated using the AllPrep DNA/RNA minikit (Qiagen) per the manufacturer's manual.

Viral outgrowth assay. BM- and PB-derived CD4⁺ T cells were sorted using a FACSARIA II using the protocol described above. Cells were stimulated with CD3 and CD28 and allowed to incubate for 12 h. Sorted CD4⁺ T cells were plated at a 1:1 ratio and supplemented with IL-2. At weeks 2, 4, and 6, supernatant was taken for RNA analysis.

Statistical analysis. All analyses were performed using GraphPad Prism 7 software. Prior to implementation of any specific statistical analysis for each outcome, assumptions were assessed (i.e., normality and homogeneity of variance). If the underlying assumptions were met, a two-sided two-sample equal-variance *t* test was performed to compare the differences. If the assumptions were violated, the two-sample Mann-Whitney *U* test was used. Error bars in figures represent standard deviations. A *P* value of ≤ 0.05 was considered significant.

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