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HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation

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HIV persists in a reservoir of latently infected CD4$^+$ T cells in individuals treated with highly active antiretroviral therapy (HAART). Here we identify central memory (T_{CM}) and transitional memory (T_{TM}) CD4$^+$ T cells as the major cellular reservoirs for HIV and find that viral persistence is ensured by two different mechanisms. HIV primarily persists in T_{CM} cells in subjects showing reconstitution of the CD4$^+$ compartment upon HAART. This reservoir is maintained through T cell survival and low-level antigen-driven proliferation and is slowly depleted with time. In contrast, proviral DNA is preferentially detected in T_{TM} cells from aviremic individuals with low CD4$^+$ counts and higher amounts of interleukin-7–mediated homeostatic proliferation, a mechanism that ensures the persistence of these cells. Our results suggest that viral eradication might be achieved through the combined use of strategic interventions targeting viral replication and, as in cancer, drugs that interfere with the self renewal and persistence of proliferating memory T cells.

Treatment of HIV infection has markedly reduced the death rate from AIDS and improved the quality of life of HIV-infected individuals[1]. However, complete eradication of HIV with antiretroviral drugs seems impossible, as the virus persists in cellular reservoirs[2,3]. The major HIV reservoir is a small pool of latently infected resting memory CD4$^+$ T cells carrying an integrated form of the viral genome[4,5] that lacks the ability to produce viral proteins[6]. CD4$^+$ T cell depletion is associated with a larger viral reservoir size[7], whereas early initiation of HAART is often associated with a reduced size of the HIV reservoir[8] and the normalization of the CD4/CD8 ratio[9]. These observations suggest that CD4$^+$ T cell depletion, which is directly associated with increased levels of CD4$^+$ T cell proliferation[10,11], may drive the size of the HIV reservoir by as yet unidentified mechanisms.

Two distinct mechanisms could contribute to the persistence of this reservoir. First, low levels of viral replication could lead to *de novo* infection of memory CD4$^+$ T cells, ensuring the continuous replenishment of the HIV reservoir[12,13]. However, the absence of genetic evolution in viral reservoirs does not support this possibility[13–17]. Moreover, plasma viral RNA sequences under HAART diverge from cell-associated viral DNA, providing further evidence that *de novo* infection of cells is unlikely to be implicated in the persistence of the reservoir[18]. The intrinsic stability of latently infected CD4$^+$ T cells constitutes an alternative explanation for the stability of the reservoir, as T_{CM} cells survive for years[19–21]. Indeed, we have shown that prosurvival pathways are specifically triggered in T_{CM} cells[21,22], indicating that T_{CM} cells may encompass the long-lived HIV reservoir. The differentiation of T_{CM} cells into T_{EM} cells is observed after T cell receptor (TCR) triggering[23,24] and, to a lesser extent, in response to homeostatic cytokines such as interleukin-7 (IL-7) and IL-15 (ref. 25). The contribution of antigen-induced or homeostatic proliferation to the size and maintenance of the reservoir has yet to be defined. This is particularly relevant in the context of HAART, where high levels of immune activation, increased plasma concentrations of IL-7 and an increased percentage of cycling CD4$^+$ T cells have been observed in individuals with low absolute CD4$^+$ T cell counts[26]. Here we show the presence of two distinct HIV reservoirs: one in T_{CM} cells, regulated by antigen-driven proliferation, and one in T_{TM} cells, regulated by homeostatic proliferation.

RESULTS

HIV proviral DNA is detected in T_{CM} and T_{TM} cells

To identify the major molecular forms of HIV associated with its persistence, we measured levels of total DNA, integrated DNA and 2-LTR circles, which are labile intermediates in the virus life cycle, in

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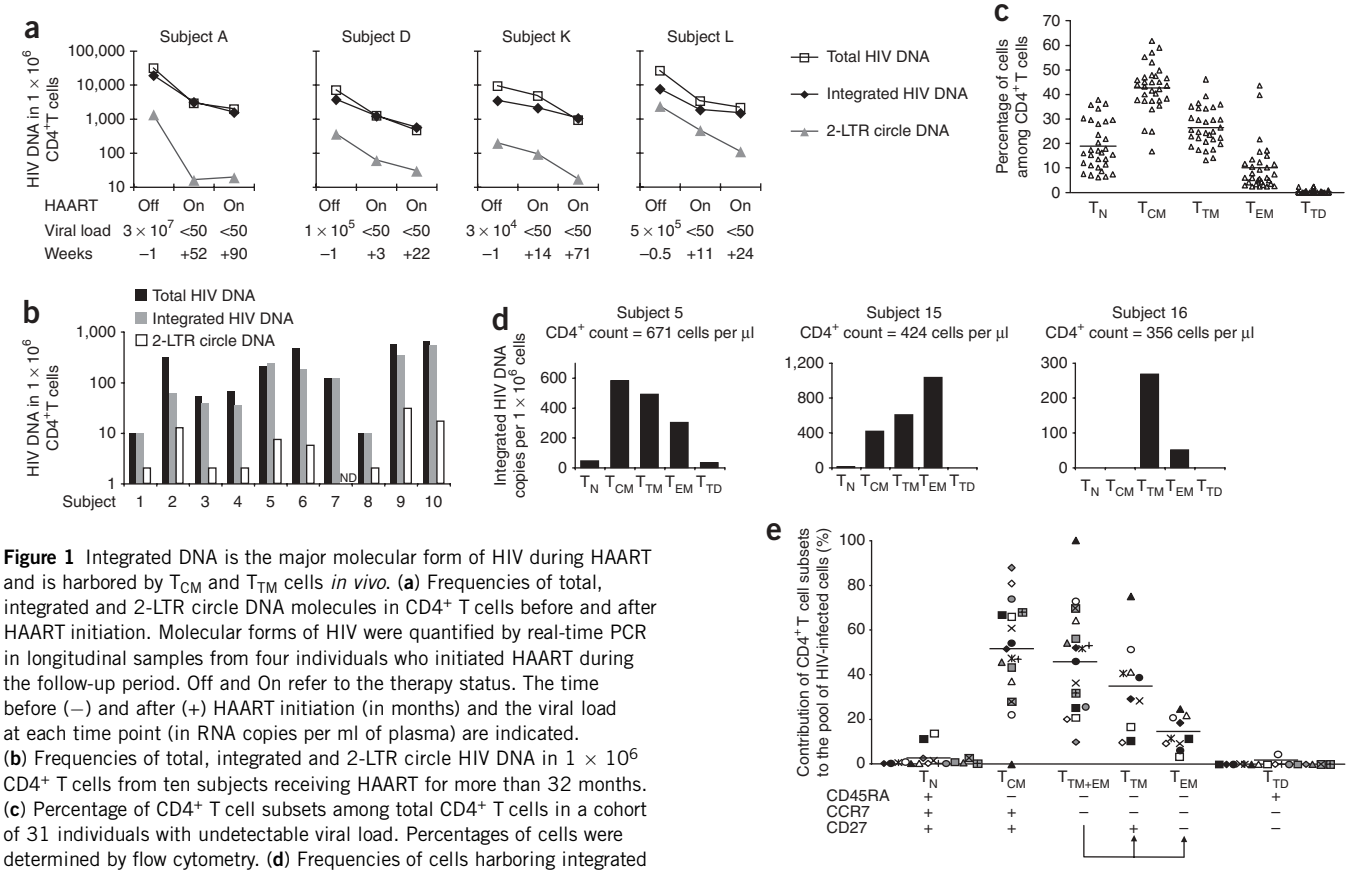

Figure 1 Integrated DNA is the major molecular form of HIV during HAART and is harbored by T_{CM} and T_{TM} cells *in vivo*. (**a**) Frequencies of total, integrated and 2-LTR circle DNA molecules in CD4$^+$ T cells before and after HAART initiation. Molecular forms of HIV were quantified by real-time PCR in longitudinal samples from four individuals who initiated HAART during the follow-up period. Off and On refer to the therapy status. The time before (−) and after (+) HAART initiation (in months) and the viral load at each time point (in RNA copies per ml of plasma) are indicated. (**b**) Frequencies of total, integrated and 2-LTR circle HIV DNA in 1 × 10^6 CD4$^+$ T cells from ten subjects receiving HAART for more than 32 months. (**c**) Percentage of CD4$^+$ T cell subsets among total CD4$^+$ T cells in a cohort of 31 individuals with undetectable viral load. Percentages of cells were determined by flow cytometry. (**d**) Frequencies of cells harboring integrated HIV DNA in CD4$^+$ T cell subsets from three representative aviremic subjects. Results are expressed as the HIV copy number in 1 × 10^6 cells of a given subset. (**e**) Contribution of T_N, T_{CM}, T_{TM+EM} and T_{TD} cell subsets to the pool of HIV-infected cells calculated in 17 aviremic HIV-infected subjects. HIV integrated DNA copy number was determined in sorted subsets by highly sensitive real-time PCR. Each symbol represents a different individual. In ten subjects, CD45RA$^-$CCR7$^-$ CD4$^+$ T cells were further sorted according to the expression of CD27 to distinguish between T_{TM} cells and T_{EM} cells. Horizontal lines indicate mean values.

CD4$^+$ T cells from 14 successfully treated individuals, including four subjects from whom we obtained peripheral blood mononuclear cells (PBMCs) before and after initiation of HAART. The results indicated a rapid and profound drop in levels of 2-LTR circles upon initiation of HAART (**Fig. 1a**), whereas HIV proviral DNA was more stable than and present in similar amounts to total HIV DNA after prolonged HAART (**Fig. 1b**), confirming that the total HIV DNA found in CD4$^+$ T cells after prolonged virus suppression largely represents integrated HIV genomes, as recently reported[27]. These results identify integrated viral DNA as the major and most stable molecular form of HIV in CD4$^+$ T cells from successfully treated subjects.

To determine whether the stability of integrated viral DNA can be attributed to the persistence of specific memory CD4$^+$ T cell compartments, we sorted CD4$^+$ T cell subsets from 17 aviremic subjects (**Supplementary Table 1**) on the basis of surface expression of CD45RA, CC chemokine receptor-7 (CCR7) and CD27 and performed highly sensitive quantification of integrated HIV proviral DNA on the sorted subsets. On the basis of CD45RA and CCR7 expression, we identified various CD4$^+$ subsets, including naive (T_N), central memory (T_{CM}) and terminally differentiated (T_{TD}) cells (**Supplementary Fig. 1a**). CD27 expression allowed us to distinguish effector memory (T_{EM}) cells from the transitional memory CD4$^+$ T cell subset (T_{TM} cells); the latter cells show functional and transcriptional characteristics that are intermediate between those of T_{CM} cells and T_{EM} cells[21].

We determined the percentage of each cellular subset within the pool of CD4$^+$ T cells from 31 aviremic individuals (**Fig. 1c**). The frequency of cells within each subset harboring HIV proviral DNA ranged from 0 to 10.2 × 10^3 copies of HIV provirus per 1 × 10^6 cells of a given subset (**Fig. 1d** and **Supplementary Fig. 1b**). Our results clearly indicate that the HIV reservoir constitutes cells harboring a memory phenotype (mean contributions of T_{CM} cells and T_{TM} and T_{EM} cells of 51.7% and 46.6%, respectively), whereas T_N cells and T_{TD} cells marginally contribute to the pool of cells harboring HIV proviral DNA (mean contributions of 1.9% and 0.3%, respectively, **Fig. 1e**). Overall, the contributions of T_{CM} cells and T_{TM} cells to the HIV reservoir were higher than that of T_{EM} cells (mean contributions of 51.7%, 34.3% and 13.9%, respectively). To confirm that CD4$^+$ T cells harbor replication-competent virus, we sorted cells from each subset of four aviremic subjects and measured viral production after co-culture and stimulation with allogeneic dendritic cells and phytohemagglutinin-activated CD4$^+$ T cells from HIV-negative donors (**Supplementary Fig. 2**). These results showed that CD4$^+$ T cells subsets harboring HIV proviral DNA are able to produce infectious virus after stimulation.

We also measured the frequency of cells harboring HIV proviral DNA in lymph nodes and gut biopsies from successfully treated individuals (**Supplementary Fig. 3a–f**). We found that lymph nodes were enriched (fold enrichment of 2.4) for memory CD4$^+$ T cells as compared to blood, but the frequencies of memory CD4$^+$ T cells harboring proviral DNA were comparable in both compartments

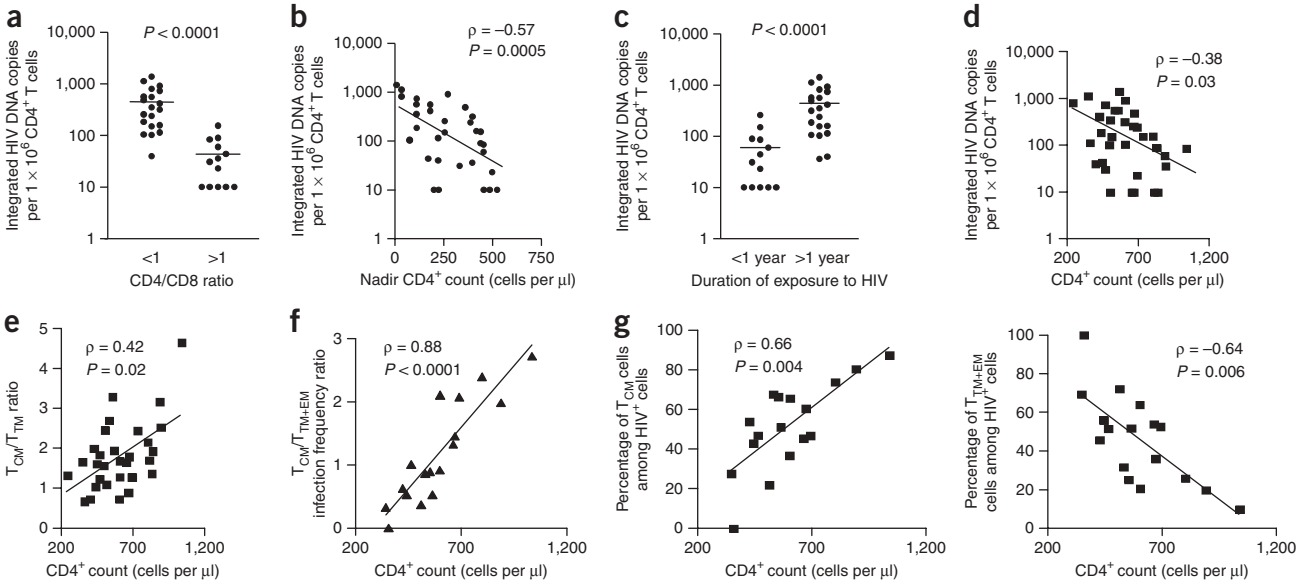

Figure 2 CD4+ T cell depletion drives the size and the localization of the HIV reservoir. (**a**–**d**) Integrated HIV DNA copy number, as determined in the CD4+ T cells from 33 virally suppressed HIV-infected subjects by *Alu* real-time PCR. (**a**) Comparison of the size of the HIV reservoir in subjects with a CD4/CD8 ratio <1 or >1. (**b**) Correlation between the size of the HIV reservoir and the nadir CD4+ count. (**c**) Comparison of the size of the HIV reservoir in subjects who have started HAART before or after the first year of infection. (**d**) Correlation between the size of the HIV reservoir and absolute CD4+ counts. (**e**) Correlation between the absolute CD4+ count and the T_{CM}/T_{TM} ratio in 33 subjects receiving HAART. The frequencies of T_{CM} and T_{TM} cells within total CD4+ T cells were measured by flow cytometry after staining for the CD4, CD45RA, CCR7 and CD27 markers. (**f**) Correlation between the T_{CM}/T_{TM+EM} infection frequency ratio and absolute CD4+ counts in the 17 individuals on whom HIV quantifications in CD4+ T cell subsets were performed. HIV integrated DNA copy number was determined in sorted subsets by highly sensitive real-time PCR. The ratio was calculated by dividing the number of copies of integrated HIV DNA in 1×10^6 T_{CM} by the number of copies of integrated HIV DNA in 1×10^6 T_{TM+EM} cells. (**g**) Correlations between the contribution of T_{CM} cells and T_{TM+EM} cells to the HIV reservoir and the absolute CD4+ count in the same individuals. To calculate the contribution of each CD4+ T cell subset to the global pool of HIV-infected cells, the percentage of each subset within the CD4+ compartment as well as the frequency of the cells in the subset harboring HIV integrated DNA were taken into account. Horizontal lines indicate mean values.

(means of 516 copies and 603 copies of HIV integrated DNA in 1×10^6 memory CD4+ T cells from PBMCs and lymph node mononuclear cells, respectively). We did the same quantifications in PBMCs and matched gut biopsies from eight subjects on HAART; the results showed that the frequencies of cells harboring HIV proviral DNA are similar and highly correlated between these two compartments (**Supplementary Fig. 3f**). Together, these experiments show that T_{CM}, T_{TM} and T_{EM} cells contribute to various degrees to the HIV reservoir and can produce infectious viral particles upon activation. Moreover, experiments with PBMCs of individuals on HAART, aimed at quantifying proviral DNA, did indeed reflect the contribution of the lymphoid tissue environment to the HIV reservoir.

Absolute CD4+ count identifies the HIV reservoir

Our results also showed that the relative contributions of T_{CM} and T_{TM} cells to the pool of HIV-infected cells are highly variable from one subject to another (**Fig. 1e**). We observed that CD4/CD8 ratios >1, high nadir CD4+ count and initiation of HAART within the first year of HIV infection are strongly associated with an HIV reservoir of limited size ($P < 0.0001$, $P = 0.0005$ and $P < 0.0001$, respectively; **Fig. 2a–c**). As CD4+ T cell depletion was also associated with an increased HIV reservoir size ($P = 0.03$, **Fig. 2d**), we examined whether absolute CD4+ counts affect the reservoir localization in T_{CM} and T_{TM} cells. We found that T_{CM} cells were underrepresented in subjects with low CD4+ counts, suggesting that T_{CM} cells are selectively depleted or differentiated into T_{TM} cells in these subjects ($P = 0.02$, **Fig. 2e**). Moreover, the frequency of infected T_{TM} cells was higher in subjects with low CD4+ counts, whereas T_{CM} cells were preferentially infected in subjects with high absolute CD4+ counts ($P < 0.0001$, **Fig. 2f**). As T_{CM} cells were overrepresented and preferentially infected in individuals with high CD4+ counts, they constituted the major reservoir for HIV in those subjects ($P = 0.004$, **Fig. 2g**). Conversely, the HIV reservoir was mainly localized in T_{TM} cells in individuals with low CD4+ counts ($P = 0.006$).

Immune activation identifies the HIV reservoir

Depletion of CD4+ T cells is associated with high levels of immune activation and increased proliferation of CD4+ T cells[10,11,26]. As we found that the absolute CD4+ count determines the size and the localization of the HIV reservoir, we examined the impact of the residual immune activation on these parameters. Absolute CD4+ counts were negatively correlated with the expression of the immune activation and proliferation marker Ki67 in CD4+ T cells ($P = 0.007$, **Fig. 3a**). Notably, high frequencies of $Ki67^+CD4^+$ T cells were associated with a larger size of the viral reservoir ($P = 0.01$, **Fig. 3b**), suggesting that T cell proliferation might provide a mechanism for the maintenance of the HIV reservoir. Moreover, we found that the levels of cellular proliferation could predict not only the size but also the localization of the HIV reservoir, as high levels of Ki67 protein expression were correlated with preferential infection of T_{TM} cells ($P = 0.008$, **Fig. 3c**) and with a higher contribution of this subset to the HIV reservoir ($P = 0.0006$, **Fig. 3d**). Conversely, T_{CM} cells were the main reservoir in individuals with limited levels of cellular proliferation ($P = 0.002$; **Fig. 3d**).

Together, these results indicate that low absolute CD4+ counts are associated with high levels of cellular proliferation and an increased

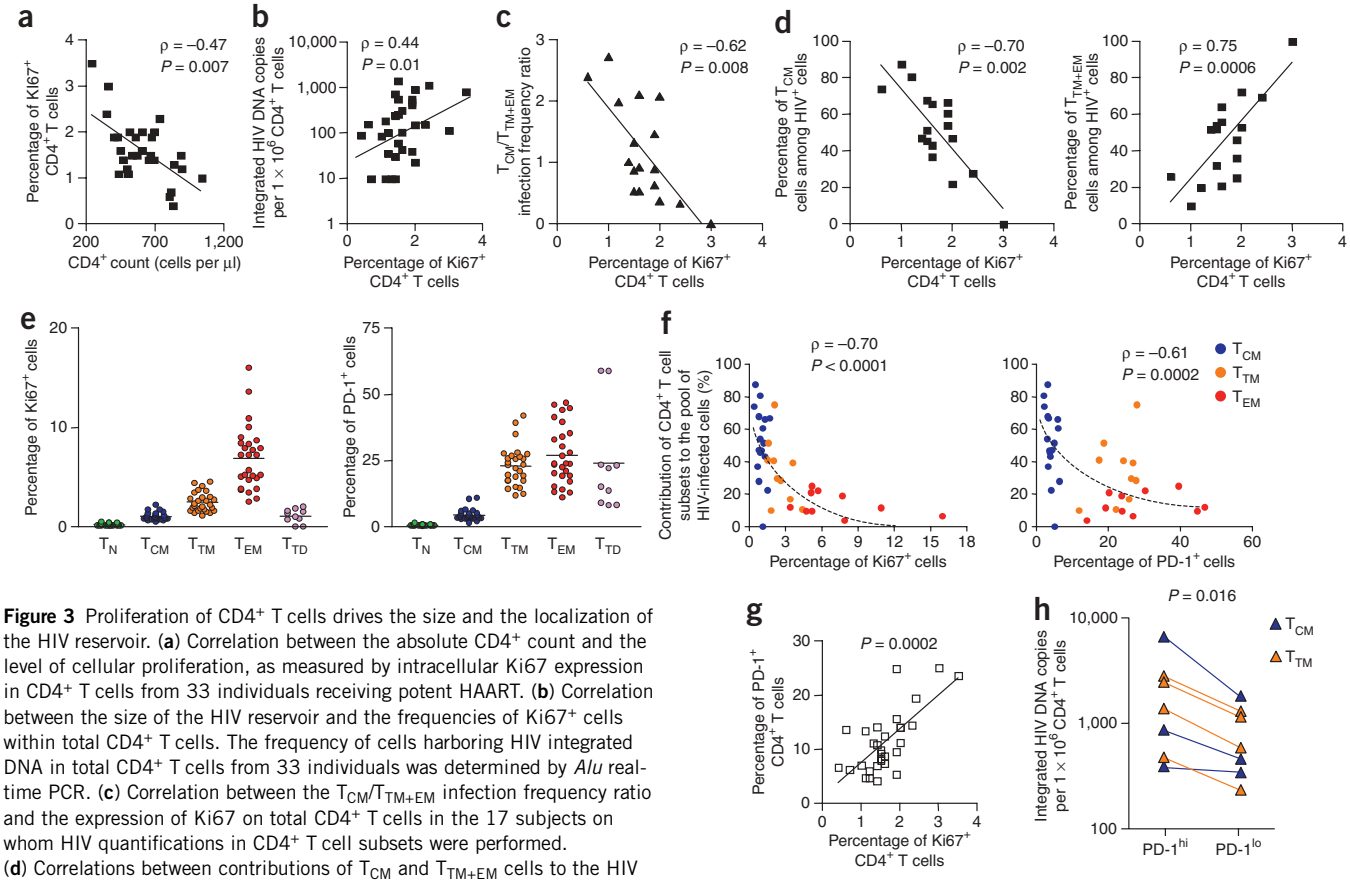

Figure 3 Proliferation of CD4+ T cells drives the size and the localization of the HIV reservoir. (**a**) Correlation between the absolute CD4+ count and the level of cellular proliferation, as measured by intracellular Ki67 expression in CD4+ T cells from 33 individuals receiving potent HAART. (**b**) Correlation between the size of the HIV reservoir and the frequencies of Ki67+ cells within total CD4+ T cells. The frequency of cells harboring HIV integrated DNA in total CD4+ T cells from 33 individuals was determined by *Alu* real-time PCR. (**c**) Correlation between the T_{CM}/T_{TM+EM} infection frequency ratio and the expression of Ki67 on total CD4+ T cells in the 17 subjects on whom HIV quantifications in CD4+ T cell subsets were performed. (**d**) Correlations between contributions of T_{CM} and T_{TM+EM} cells to the HIV reservoir and the expression of Ki67 in the same individuals as in **a**–**c**. (**e**) Percentage of CD4+ T cells from each cellular subset expressing Ki67 and PD-1. (**f**) Correlations between the contribution of a given CD4+ T cell subset to the HIV reservoir and the expression of Ki67 and PD-1. (**g**) Correlation between PD-1 and Ki67 expression in total CD4+ T cells. (**h**) T_{CM} cells and T_{TM} cells from three and four virally suppressed subjects were sorted according to their relative expression of PD-1. The frequency of cells harboring HIV proviral DNA was determined in both fractions by *Alu* real-time PCR.

size of an HIV reservoir mainly harbored by T_{TM} cells. Conversely, we observed a viral reservoir of limited size mainly harbored by T_{CM} cells in individuals with high absolute CD4+ counts and limited proliferation of CD4+ T cells.

T_{CM} and T_{TM} CD4+ T cells define distinct HIV reservoirs

We next characterized the phenotypes of T_{CM} cells and T_{TM} cells to determine whether their contribution to the HIV reservoir is a function of their proliferative status. T_{TM} cells included higher frequencies of Ki67+ and PD-1+ cells than T_{CM} cells but lower frequencies than T_{EM} cells (**Fig. 3e**). Moreover, and irrespective of the phenotype of the CD4+ subset, the HIV reservoir was mainly composed of cells endowed with limited proliferation indexes (Ki67, $P < 0.0001$ and PD-1, $P = 0.0002$), confirming the quiescent nonactivated state of cells that constitute the HIV reservoir in HAART-treated subjects[2] (**Fig. 3f**). Our results indicate that highly proliferating T cells such as T_{EM} cells (range of Ki67+ cells, 3.4–10.9%) do not constitute a stable reservoir for HIV, which is consistent with their limited contribution to the pool of latently HIV-infected cells. In contrast, T_{CM} cells and T_{TM} cells are characterized by low to intermediate levels of proliferation and constitute the main viral reservoir. These results suggest a model in which HIV can persist in T_{CM} and T_{TM} cells by continuous low-level proliferation, ensuring the persistence of integrated viral DNA through mitosis. We thus sorted T_{CM} cells and T_{TM} cells from four HAART-treated subjects according to their PD-1 expression, a marker of homeostatic[28,29] and antigen-induced[30] proliferation, as indicated by the strong correlation between PD-1 and Ki67 expression in CD4+ T cells from HAART-treated individuals ($P = 0.0002$, **Fig. 3g**). Quantification of HIV proviral DNA in each fraction showed that PD-1^{hi} cells were enriched for HIV proviral DNA when compared to the corresponding PD-1^{lo} fractions ($P = 0.016$), confirming that proliferating T_{CM} and T_{TM} cells constitute a privileged cellular reservoir for HIV (**Fig. 3h**). These results indicate that HIV preferentially persists in T_{CM} and T_{TM} cells that proliferate at low levels.

The T_{TM} cell reservoir is fueled by proliferating T_{CM} cells

We amplified *Env* sequences from proviral DNA obtained from T_{CM} cells and T_{TM} cells of ten HAART-treated subjects and performed phylogenetic analyses to assess the interdependence of each cellular reservoir by comparing HIV sequences obtained from both cellular reservoirs (**Fig. 4a**). With the exception of three clones obtained from one subject, all viruses ($n = 427$) were predicted to use CCR5; hence, co-receptor usage could not allow us to evidence the interdependence of the T_{CM} and T_{TM} reservoirs. However, sequences obtained from T_{CM} cells and T_{TM} cells of individuals that were good responders to HAART (see subject D as an example, $CD4^+Ki67^+ = 1.4\%$) did not cluster together, indicating that in those subjects these reservoirs are genetically independent (**Fig. 4a**). In contrast, in aviremic subjects characterized by higher levels of CD4+ T cell proliferation (for example, subject A, $CD4^+Ki67^+ = 3.5\%$), viral quasispecies were shared by T_{CM} cells and T_{TM} cells, as shown by the unique cluster

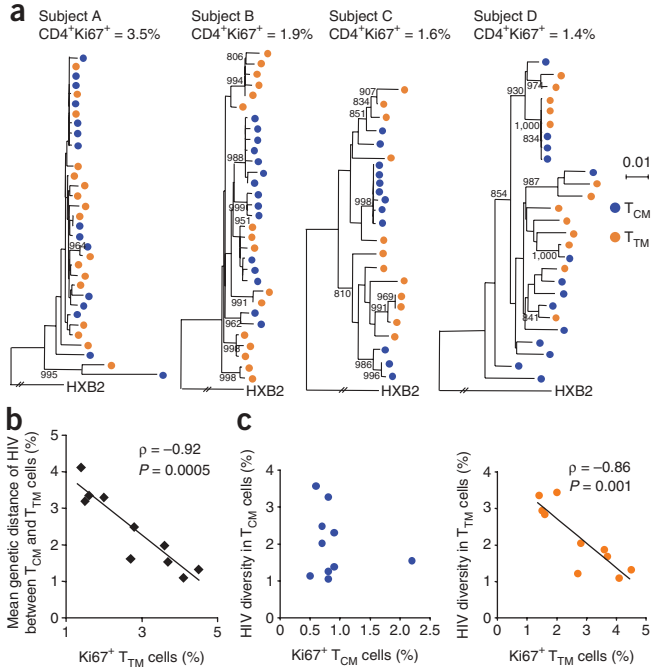

Figure 4 T_{CM} and T_{TM} cells define a distinct HIV reservoir. (**a**) Neighbor-joining trees derived from HIV sequences obtained from T_{CM} cells and T_{TM} cells of four representative HAART-treated HIV-infected subjects. T_{CM} and T_{TM} cells from ten aviremic subjects were sorted, and a minimum of 11 clones deriving from at least ten independent positive PCRs of the *Env* gene were sequenced in each case. The numbers near nodes indicate the percentage of bootstrap replicates (1,000 resampling). The scale refers to the distance between sequences. Vertical lines are for clarity only. (**b**) Correlation of the mean genetic distances of HIV *Env* sequences between T_{CM} and T_{TM} cells to the percentage of Ki67$^+$ T_{TM} cells in ten aviremic subjects. Each diamond represents the mean genetic distance existing between the clones obtained from T_{CM} cells and T_{TM} cells. (**c**) Correlations between the HIV genetic diversity in T_{CM} cells and T_{TM} cells and the expression of Ki67 in these subsets. HIV diversity was estimated by calculating the mean genetic distance between the clones within a given subset.

of variants found in the neighbor-joining tree (**Fig. 4a**). When performed in ten subjects, the phylogenetic analysis confirmed that increased turnover of T_{TM} cells is associated with a reduced HIV genetic distance measured between the two compartments ($P = 0.0005$, **Fig. 4b**), a consequence of the residual immune activation and cell proliferation leading to the differentiation of T_{CM} cells into T_{TM} cells[10,11].

Cell proliferation ensures HIV reservoir stability

We determined the impact of cellular proliferation on the genetic diversity of proviral DNA within both cellular reservoirs. We did not find any association between the genetic diversity of the T_{CM} reservoir and levels of T_{CM} cell proliferation (**Fig. 4c**), indicating that T_{CM} cells harboring archived proviral DNA constitute a stable reservoir with minimal proliferation levels, in agreement with their enhanced survival[21]. In contrast, proliferation of T_{TM} cells was associated with a reduced HIV diversity in this subset ($P = 0.001$, **Fig. 4c**). This result suggested a mechanism by which HIV-infected T_{TM} cells maintain the size and the lack of HIV genetic diversity of the viral reservoir by continuous self-renewal through the proliferation of a small number of cells. To verify this hypothesis, we performed a longitudinal analysis in five aviremic subjects from whom we obtained viral sequences at two time points separated by at least 14 months (**Supplementary Table 2** and **Supplementary Fig. 4**). We aligned sequences obtained from T_{CM} cells and T_{TM} cells at these two time points and drew phylogenetic trees reflecting the evolution of viral sequences (**Fig. 5a** and **Supplementary Fig. 5**). To measure the genetic evolution of the HIV reservoir, we quantified the divergence as the average genetic distance between sequences from the two time points within each T cell subset (**Fig. 5b**). Our results indicated that

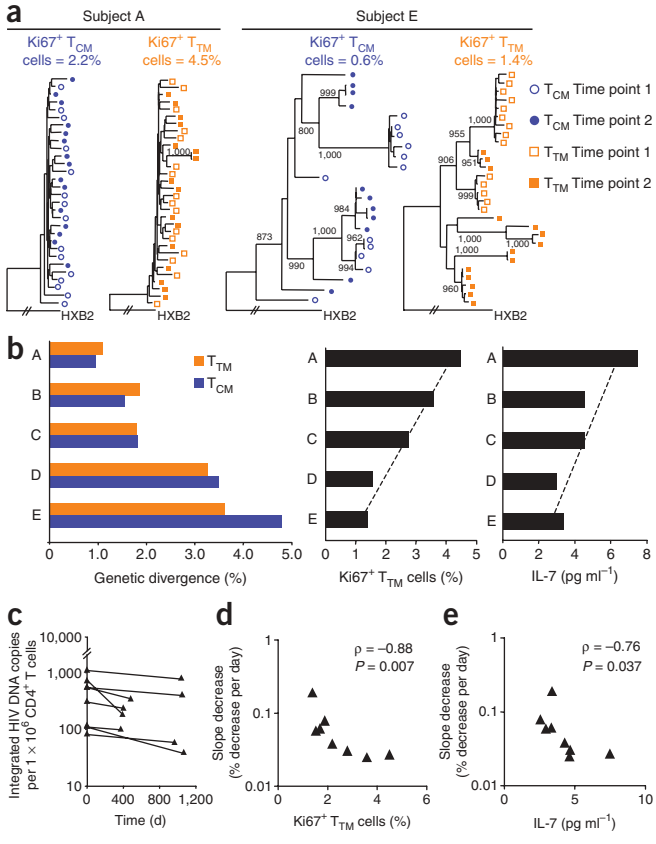

Figure 5 Proliferation of T_{TM} cells is associated with a genetic stability of the HIV reservoir over time. (**a**) Neighbor-joining trees derived from HIV sequences obtained from T_{CM} cells and T_{TM} cells of two representative aviremic individuals at first and second time points (closed and filled symbols, respectively). Percentages of T_{CM} cells and T_{TM} cells expressing the Ki67 proliferation marker are indicated. (**b**) Genetic evolution of the HIV reservoir in T_{CM} cells and T_{TM} cells from five aviremic individuals followed longitudinally. Results are expressed as the mean genetic distance existing between HIV sequences from both time points, corresponding to the estimated genetic divergences of the *Env* region (left). Percentage of Ki67$^+$ T_{TM} cells (middle) and plasma IL-7 concentrations (right) are also shown. (**c**) Evolution of the HIV reservoir size in eight aviremic individuals. The integrated HIV DNA copy number was measured by *Alu* real-time PCR in purified CD4$^+$ T cells at two time points. For each subject, the line between the two time points reflects the slope of the decrease in the frequency of CD4$^+$ T cells harboring HIV proviral DNA. (**d**) Correlation between the slope decrease in the number of HIV-infected CD4$^+$ T cells and the percentage of Ki67$^+$ T_{TM} cells. The decrease in the HIV reservoir size is expressed as a decrease of the percentage of cells harboring HIV proviral DNA per day. (**e**) Correlation between the slope decrease in the number of HIV-infected CD4$^+$ T cells and the plasma IL-7 level measured by ELISA.

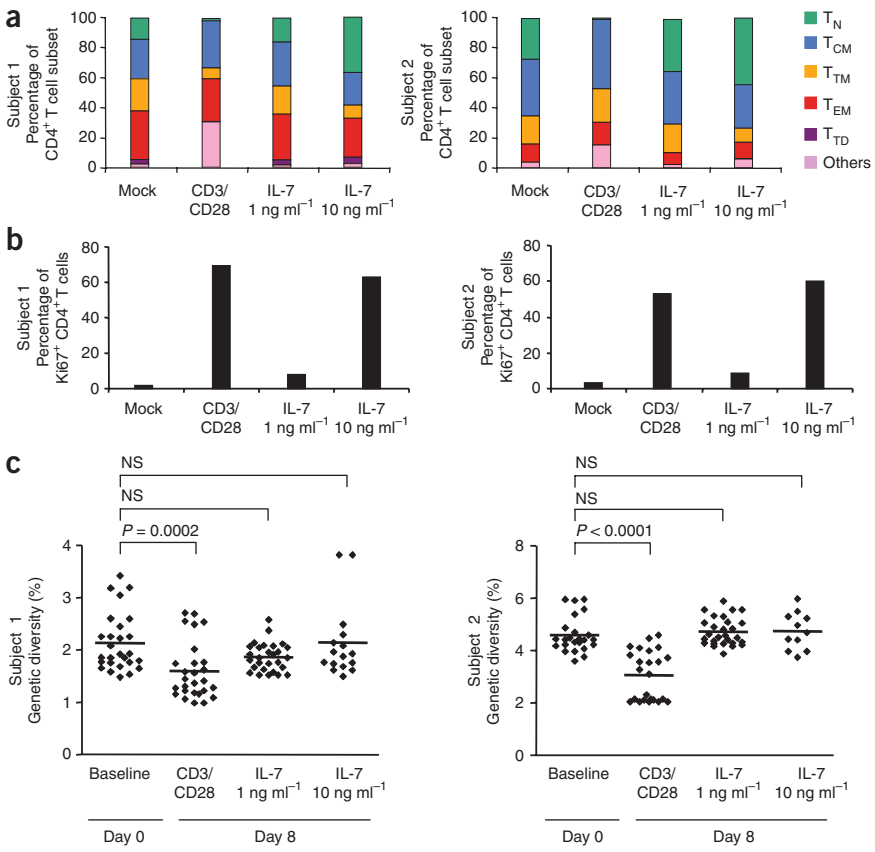

Figure 6 IL-7 induces homeostatic proliferation of CD4$^+$ T cells and ensures HIV reservoir stability through cellular proliferation at low levels. (**a**) Phenotype of CD4$^+$ T cells after TCR-induced (CD3/CD28) or IL-7 proliferation. Cells were harvested at day 8, and their phenotype was determined by flow cytometry. (**b**) Percentage of CD4$^+$ T cells expressing the proliferation marker Ki67 after 8 d of CD3/CD28 or IL-7 stimulation. (**c**) HIV genetic diversities at baseline and after 8 d of treatment. Each dot represents the mean genetic distance between one given clone and the entire population. The horizontal bar represents the mean of these values and is reflecting the genetic diversity of the viral population in the various conditions.

high proliferation levels in T_{TM} cells are associated with a limited genetic evolution of the reservoir over time, in agreement with a mechanism by which persistence of this reservoir is ensured by a small number of HIV-infected proliferating CD4$^+$ T cells (subject A, **Fig. 5a**,**b**).

To our surprise, in subjects with low frequencies of Ki67$^+$ cells, sequences obtained from the two time points did not cluster together (subject E, **Fig. 5a**,**b**). The apparent evolution of HIV sequences in individuals with high CD4$^+$ T cell counts probably reflects the sampling of different archived sequences rather than a genetic evolution of the proviral populations. These sequences may be present at variable frequencies as a consequence of the proliferation and death of distinct T cell clones in subjects who are still endowed with a functional CD4$^+$ compartment[31–33].

HIV reservoir is maintained by IL-7–mediated proliferation

CD4$^+$ T cell depletion is directly correlated with high levels of plasma IL-7 (refs. 26,34–36), a cytokine responsible for the survival and homeostatic proliferation of CD4$^+$ T cells[25,37,38]. We observed higher amounts of IL-7 in the plasma of individuals with a high frequency of proliferating cells and limited evolution of the viral reservoir over time (**Fig. 5b**). We then assessed the role of this cytokine in the maintenance of the size of the reservoir. We determined the frequency of CD4$^+$ T cells harboring proviral DNA in eight aviremic HAART-treated subjects at two different time points with at least 14 months between independent measurements (**Fig. 5c**). The size of the viral reservoir decreased very slowly with time in the eight aviremic subjects tested. We analyzed our data by using a previously described regression model[39], and we found a mean half-life of this reservoir of 39.5 months, indicating that an average of 65.7 years of treatment would be necessary to eradicate 1×10^6 infected cells. Of note, we observed a significant negative correlation between this decrease and the percentage of proliferating T_{TM} cells ($P = 0.007$, **Fig. 5d**). Moreover, we observed that plasma IL-7 concentrations inversely correlated with the decrease of the reservoir size over time ($P = 0.037$, **Fig. 5e**).

Altogether, these results indicate that IL-7 is responsible for the persistence of latently HIV-infected CD4$^+$ T cells by promoting homeostatic proliferation of memory CD4$^+$ T cells, resulting in the quantitative and qualitative stability of the HIV reservoir.

Cellular proliferation influences the HIV reservoir

We performed *in vitro* experiments to evaluate the relative impact of antigen-induced and homeostatic proliferation on the maintenance of the HIV reservoir. We stimulated CD4$^+$ T cells from two virally suppressed subjects through TCR triggering (CD3 and CD28) or incubate them with IL-7 in the presence of zidovudine and ritonavir to avoid possible *de novo* infection of cells with HIV. We observed that TCR triggering induced modifications in the distribution of CD4$^+$ T cell subset and was accompanied by high levels of proliferation (**Fig. 6a**,**b**). In contrast, IL-7 at 1 ng ml^{-1} and 10 ng ml^{-1} was able to induce proliferation of CD4$^+$ T cells with limited impact on the distribution of the various naive and memory T cell subsets, confirming the homeostatic nature of the proliferation induced by this cytokine. Analysis of HIV sequences after IL-7 treatment indicated that this cytokine induced the proliferation of CD4$^+$ T cells without modifying the diversity of the viral reservoir, as indicated by the absence of a significant difference in the genetic diversity of the proviral populations at baseline and after 8 d of culture (**Fig. 6c**). In contrast, CD4$^+$ T cells treated with CD3 and CD28 showed a significant reduction in the diversity of the proviral population (subjects 1 and 2, $P = 0.0002$ and $P < 0.0001$, respectively). Of note, we used the same number of positive PCR reactions for cloning of proviral quasispecies under each experimental condition ($n = 7$), indicating that the decrease in viral genetic diversity is unlikely to be attributable exclusively to cell death. Thus, the decrease in the viral genetic diversity observed after TCR triggering is most probably attributable to the specific expansion of a small number of HIV-infected memory CD4$^+$ T cell clones, which are undergoing substantial proliferation in our cultures at day 8, as indicated by increased frequencies of Ki67$^+$ cells (**Fig. 6b**). This observation supports our findings *in vivo* showing that TCR-induced proliferation is responsible

for the specific expansion of a restricted number of $CD4^+$ T cell clones harboring a limited set of divergent HIV sequences (**Fig. 5a**). Thus, these results indicate that IL-7 can mediate the survival of HIV-infected cells through homeostatic proliferation, thereby ensuring the persistence of a genetically stable HIV reservoir, whereas antigen-induced proliferation leads to the genetic evolution of the proviral population through the preferential proliferation and survival of a restricted number of $CD4^+$ T cell clones.

DISCUSSION

We have identified two viral reservoirs within memory $CD4^+$ T cell subsets of virally suppressed subjects. The T_{CM} reservoir is the major long-lasting reservoir in immune responders to HAART. As T_{CM} cells are characterized by their extremely low degree of cellular proliferation, and, because of their intrinsic capacity to survive for decades[20,21], these cells provide a long-lasting cellular reservoir for HIV. In immune responders to HAART with normal $CD4^+$ counts, the size of the HIV reservoir decreases very slowly with time, indicating that this cellular reservoir could be partially depleted through the use of intensive antiviral strategies for prolonged periods of time. The second reservoir is harbored by T_{TM} cells and is the main reservoir in individuals with low $CD4^+$ counts, the majority of whom are characterized by persistent immune activation. Our results clearly show that this reservoir persists by homeostatic proliferation of infected T_{TM} cells, ensuring the stability of this viral reservoir in its size and its genetic variability. As the size of this T_{TM} reservoir is reduced in individuals who have been treated early in infection, our findings confirm the importance of early therapeutic intervention, as it limits the size of this proliferating HIV reservoir[8].

Several studies have clearly shown the continuous production of virions during HAART[13,40,41]; a recent analysis of longitudinal plasma samples suggests that this low-level, persistent viremia seems to arise from at least two cellular compartments, one in which viral production decays over time and a second that remains stable for at least 7 years[42]. Our identification of T_{CM} cells and T_{TM} cells as two major reservoirs that are characterized by differing decay rates in HAART-treated individuals supports these findings. Our observations also provide the first evidence, to our knowledge, for the validity of the mathematical model proposed recently, in which bystander proliferation of HIV-infected $CD4^+$ T cells can ensure HIV reservoir persistence without any demonstrable evidence for viral production[43].

The impact of viral production on the replenishment of the HIV reservoir is still controversial[17,18,44–46]. A recent study concluded that persistence of the HIV reservoir is unlikely to result from ongoing viral replication, as sequences from the predominant plasma clones are different from those found in the proviral reservoir[18]. Moreover, genetic analysis of rebounding viruses after treatment interruptions argues against persistence of ongoing low-level replication in individuals on suppressive HAART[47]. Accordingly, our findings suggest a limited role for viral replication in the persistence of the reservoir. First, the nonstochastic distribution of HIV-infected cells among T cell subsets that are permissive to HIV infection suggests that *de novo* infection of $CD4^+$ T cells does not occur. Second, HAART-treated individuals with high levels of immune activation, in whom ongoing replication is most likely to occur, do not show any evidence of genetic evolution in their proviral populations. Altogether, these observations strongly suggest that ongoing viral replication is unlikely to be responsible for the persistence of HIV-infected cells and strengthen the role of cell proliferation as a major mechanism to ensure HIV persistence.

IL-7 has been shown to induce the proliferation and survival of memory $CD4^+$ T cells[25]. We found that $CD4^+$ T cell depletion is accompanied by increased amounts of IL-7, which provides a potential mechanism by which latently infected $CD4^+$ T cells could proliferate in response to this cytokine, as measured by Ki67 expression. Our results are in line with a recent study indicating that $CD4^+$ T cell proliferation in HIV-infected individuals (as assessed by BrdU incorporation) is driven by IL-7 as a homeostatic response to $CD4^+$ T cell depletion[48]. However, our Ki67 measurements may have overestimated the frequency of dividing cells, as $Ki67^+$ cells may not represent true *in vivo* proliferation in HIV-infected individuals, particularly in untreated subjects[49,50]. These results and our observations suggest that a quantitatively stable pool of memory $CD4^+$ T cells that include cells with integrated HIV DNA is maintained through continuous proliferation and apoptosis at low levels in HAART-treated individuals with low $CD4^+$ counts. Our longitudinal analysis shows that the stability of the HIV reservoir size and the conservation of viral sequences over time are associated with IL-7 concentrations in plasma. We observed here that IL-7 is able to induce homeostatic proliferation of $CD4^+$ T cells *in vitro*, thereby ensuring the persistence of HIV proviral sequences. These observations show a role for IL-7 in the maintenance of latently infected $CD4^+$ T cells through cytokine-induced homeostatic proliferation and survival.

Our results indicate that HIV persists in two reservoirs that are maintained by distinct mechanisms. Individuals who have started treatment early in infection carry a viral reservoir of limited size that is harbored mainly by T_{CM} cells, which have the capacity to survive for long periods of time[21,22]. Although these cells that harbor archived proviral DNA are highly stable, the reservoir may decrease at a very slow rate as a consequence of the ability of these cells to mount antigen-induced responses, leading to the elimination of a fraction of infected cells through CTL killing, cytopathic effect and apoptosis during the contraction phase. The HIV reservoir is mainly retrieved in T_{TM} cells in subjects with increased frequencies of proliferating $CD4^+$ T cells because HAART treatment was initiated at a later stage of the disease. This subset of $CD4^+$ T cells continuously proliferates at low levels, leading to the persistence of a genetically stable HIV reservoir through IL-7–induced mitosis of T_{TM} cells. These results suggest that therapeutic strategies relying solely upon antiretroviral molecules will never reach the objective of viral eradication.

Finally, our results indicate that HIV proviral DNA is harbored by cells that express immune activation and proliferation markers such as PD-1 and Ki67. New therapies should target pathways downstream of homeostatic proliferation including inhibitors of the IL-7 pathway or pathways associated with self-renewal and 'stem cell–ness', such as those developed for the treatment of leukemias and cancers[51]. Indeed, by limiting immune activation and affecting long-lived infected $CD4^+$ T cells by targeting IL-7–dependent proliferation and the self-renewal of memory T cells in association with HAART, eradication of virus in aviremic individuals could become a more realistic endeavor.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. Sequences for *Env* genes have been deposited in GenBank with accession numbers EU700514 to EU700943.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

N.C. planned and performed the experiments, analyzed the data and wrote the manuscript. M.E. and F.A.P. assisted with the cell sorting and quantification experiments. P.A., L.T. and B.Y.-D. helped with the flow cytometry experiments and with writing the manuscript. M.-R.B., G.G., J.M.B., T.W.S. and J.-P.R. provided samples from research subjects. J.-P.R. helped with writing the manuscript. M.-R.B. perfomed interleukin-7 measurements. G.B. did the co-receptor prediction usage analysis. B.J.H. and D.C.D. performed lipopolysaccharide measurements. E.K.H. and R.-P.S. planned and supervised all experiments and wrote the manuscript.

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ONLINE METHODS

Patient population. Thirty-four HIV-seropositive subjects on suppressive HAART for an average of 45 months (**Supplementary Table 1**) enrolled in this study and signed informed consent approved by the Royal Victoria Hospital and the CR-CHUM hospital review board. All individuals were receiving various antiviral regimens containing a protease inhibitor, a non-nucleoside reverse transcriptase inhibitor or three nucleoside reverse transcriptase inhibitors. None of these subjects showed any detectable plasma viremia after HAART initiation, as assessed by the Amplicor HIV-1 monitor ultrasensitive Method (Roche). All subjects underwent leukapheresis to collect large numbers of PBMCs.

Isolation of cellular subsets and flow cytometry. We purified total $CD4^+$ T cells by cell sorting after staining with FITC-labeled antibody to CD3 (555339) and allophycocyanin (APC)-labeled antibody to CD4 (340443). To isolate $CD4^+$ subsets, we labeled PBMCs with FITC-labeled antibody to CD3 (555339), APC-labeled antibody to CD4 (340443), APC-Cy7–labeled antibody to CD45RA (624072), phycoerythrin (PE)-Cy7–labeled antibody to CCR7 (557648) and PE-labeled antibody to CD27 (555441). Alternatively, we used PerCP-Cy5.5–labeled antibody to CD4 (341654) and APC-labeled antibody to PD-1 (17-9969-73) to isolate T_{CM} and T_{TM} cells expressing or not expressing the PD-1 marker. We separated T cell subsets by FACSAria (BD Biosciences) to very high purity ($>98.0\%$).

For phenotypic analysis, we stained PBMCs with Pacific Blue–labeled antibody to CD3 (558117), Alexa 700–labeled antibody to CD4 (557922), APC-Cy7–labeled antibody to CD45RA (624072), PE-Cy7–labeled antibody to CCR7 (557648), PE-labeled antibody to CD27 (555441), APC-labeled antibody to CD25 (555434) and FITC-labeled antibody to CD71(347513). Alternatively, we used APC-labeled antibody to PD-1 (17-9969-73) to the surface cocktail and FITC-labeled antibody to Ki67 (556026) for intracellular staining. We purchased all antibodies from BD Biosciences except for APC-labeled antibody to PD-1 (eBioscience). We performed analyses on a LSRII (BD Bioscience) as previously described[52].

HIV DNA quantification. We digested sorted cells as described previously[53] and used the lysates directly for amplification. We used a modified nested *Alu* PCR to quantify both integrated HIV DNA and *CD3* gene copy numbers. As a standard curve for both quantifications, we amplified serial dilutions of ACH2 cells (US National Institute of Allergy and Infectious Diseases Reagent Program) ranging from 3×10^5 to 3 cells at the same time as the experimental samples. We amplified integrated HIV sequences[54] and the *CD3* gene[53] for 12 cycles in triplicate wells. We diluted the PCR products and determined HIV and *CD3* copy numbers in separate second amplification reactions on the Light Cycler instrument (Roche Diagnostics). We determined HIV and *CD3* copy numbers by using appropriate primers and probes[53,54]. We carried out amplification reactions in Jumpstart Mix (Sigma) with 2 U of Taq Polymerase (Invitrogen), 1.25 μM primers and 0.2 μM fluorescent probes. We used similar methods to quantify total HIV DNA and 2-LTR circles, with the appropriate primers and probes[54]. For 2-LTR circle quantifications, we used serial dilutions of a plasmid harboring single copies of the *CD3* gene and 2-LTR junction as a standard curve.

Coculture assays. We cultured sorted $CD4^+$ T cell subsets from aviremic subjects with activated $CD4^+$ T cells and dendritic cells from uninfected donors for their capacity to amplify viral production. We differentiated dendritic cells from monocytes in the presence of 1,500 U ml^{-1} granulocyte-macrophage colony–stimulating factor and 10 ng ml^{-1} IL-4 (R&D Systems) for 4 d. We stimulated purified $CD4^+$ T cells from uninfected donors with 1 μg ml^{-1} phytohemagglutinin (Sigma) and 10 ng ml^{-1} IL-2 (R&D Systems) for 2 d. We cultured 0.5×10^6 cells from sorted $CD4^+$ T cell subsets with 0.1×10^6 dendritic cells and 0.5×10^6 uninfected $CD4^+$ T cells. We collected half of the medium every 3 d and replaced it with fresh medium supplemented with IL-2. After 24 d of culture, we monitored HIV replication by p24 ELISA in supernatants.

***Env* gene amplification.** We performed *Env* gene amplifications, cloning, sequencing and phylogenetic analyses as described previously[55]. We obtained 10–15 independent PCRs from each sample and sequenced 11–16 clones. To exclude the possibility of PCR resampling, we performed a single genome amplification assay in some samples and found similar results. In addition, the calculation of genetic divergence after excluding genetically close sequences obtained from the same cloning reaction gave similar results. All sequences have been submitted to GenBank.

Co-receptor usage prediction. We aligned translated gp120 sequences and did co-receptor usage predictions with the support vector machine analysis tool geno2pheno[co-receptor] version 1.0 (ref. 56) with a false positive rate of 0.05 for CXCR4 detection.

Interleukin-7 determinations. We determined plasma IL-7 concentrations by the ultrasensitive Quantikine HS IL-7 immunoassay Kit (R&D Systems), according to the manufacturer's instructions.

CD3 and CD28 and interleukin-7 stimulations of $CD4^+$ T cells. We isolated $CD4^+$ T cells by negative selection (Miltenyi Biotec) and cultured them at 0.5×10^6 cells per well. We precoated the wells with CD3-specific antibody (1 μg ml^{-1}) (produced by the OKT3 hybridoma clone), and added CD28-specific antibody (2 μg ml^{-1}) (BD Biosciences, 348040) to the culture medium. We used IL-7 (R&D Systems) at final concentrations of 1 ng ml^{-1} or 10 ng ml^{-1}. We cultured cells in the presence of 10% FBS, 2 μM AZT and 50 nM ritonavir. We added fresh medium at day 4. After 8 d, we used the cells for the determination of cell surface phenotypes and *Env* gene amplification. For each condition, we used seven independent PCR products for cloning. We verified the absence of HIV production by p24 ELISA of the supernatants.

Statistical analyses. We performed Spearman's rank correlation and Mann-Whitney *U* tests with Prism 4.0 software. We considered *P* values of less than 0.05 significant.

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