PD-1+ and follicular helper T cells are responsible for persistent HIV-1 transcription in treated aviremic individuals

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The mechanisms responsible for the persistence of HIV-1 after many years of suppressive antiretroviral therapy (ART) have been only partially elucidated. Most of the studies investigating HIV-1 persistence have been performed with blood, although it is well known that germinal centers (GCs) within lymph nodes (LNs) serve as primary sites for HIV-1 replication. We sought to identify the memory CD4 T cell populations in blood and LNs that are responsible for the production of replication-competent and infectious HIV-1, as well as for active and persistent virus transcription in ART-treated (for 1.5–14.0 years), aviremic (<50 HIV RNA copies/ml) HIV-infected individuals. We demonstrate that LN CD4 T cells that express programmed cell death 1 (PDCD1; also known as PD-1), which are composed of about 65% T follicular helper cells as defined by the expression of the cell surface receptors CXCR5 and PD-1, are the major source of replication-competent HIV-1 and of infectious virus, as compared to any other (CXCR5−PD-1− and CXCR5+PD-1−) blood or LN memory CD4 T cell populations. LN PD-1+ cells accounted for 46% and 96% of the total pools of memory CD4 T cells containing inducible replication-competent or infectious virus, respectively. Notably, higher levels of cell-associated HIV-1 RNA were present in LN PD-1+ cells after long-term (up to 12 years) ART than in other memory CD4 T cell subpopulations. These results indicate that LN PD-1+ cells are the major CD4 T cell compartment in the blood and LNs for the production of replication-competent and infectious HIV-1, and for active and persistent virus transcription in long-term-ART-treated aviremic individuals. Thus, these cells may represent a major obstacle to finding a functional cure for HIV-1 infection.

The main mechanisms that hinder HIV-1 eradication despite effective ART are the existence of long-lived latently-HIV-1-infected resting memory CD4 T cells and/or the existence of residual virus replication that replenishes the latent HIV-1 reservoir. Estimates of the half-life for the latent HIV-1 reservoir in the blood indicate that as many as 70 years of ART may be required for the full eradication of the latent reservoir. A pioneering study identified central memory (CM; defined as CD45RA−CCR7+CD27+) and transitional memory (TM; defined as CD45RA−CCR7+CD27+) CD4 T cells as the major cellular compartments of the latent HIV-1 reservoir in blood. More recently, memory CD4 T cells with stem-cell-like properties were identified as a novel but minor latent HIV-1 reservoir. Numerous strategies are currently being evaluated to target and kill HIV-1-infected cells to clear the virus and a functional HIV-1 cure. However, no data are currently available on the role of Tfh cells as a preferential cell compartment for replication-competent virus in long-term-treated aviremic HIV-1-infected individuals.

To address this issue, we investigated the distribution of replication-competent and infectious virus within different memory CD4 T cell populations that were sorted on the basis of the expression of CXCR5 and PD-1—i.e., CXCR5+PD-1−, CXCR5+PD-1− and PD-1+ memory CD4 T cell populations that were isolated from the blood and LNs of long-term-treated aviremic HIV-1-infected individuals. The results shown here demonstrate that LN PD-1+ and Tfh cells (which we refer to as PD-1+/Tfh cells) from long-term-ART-treated aviremic HIV-1-infected individuals are the major source of replication-competent and infectious HIV-1 and constitute the cell compartment responsible for active and persistent virus transcription.

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**RESULTS**

**Characterization of CD4 T cell populations**

We simultaneously collected blood and LNs from 27 ART-treated aviremic HIV-1-infected individuals. These 27 individuals had a documented diagnosis of HIV-1 infection that was between 2 and 27 years old and had been treated with ART for a period ranging between 0.3 and 14 years (Table 1). Mononuclear cells isolated from blood and LNs were then stained with antibodies specific for CD3, CD4, CD45RA, CXCR5 and PD-1 to distinguish between subpopulations of CD4+ T cells. Five populations of memory (CD45RA−) CD4 T cells were identified on the basis of the expression of PD-1 and CXCR5, i.e. CXCR5−PD−1− dual negative (DN) cells, single CXCR5+ (CXCR5−PD−1+) cells, single PD−1+ (CXCR5−PD−1+) cells, and dual CXCR5intPD−1int or CXCR5hiPD−1hi CD4 T cells (Supplementary Fig. 1a). The latter two populations correspond to TFH cells on the basis of their phenotypic, transcription factor expression and functional profiles14. Consistent with a previous study, CXCR5intPD−1int TFH cells were significantly (P < 0.05) enriched in LNs (7.48%) as compared to those in blood (1.28%) (Supplementary Fig. 1a, b), and CXCR5hiPD−1hi TFH cells were only detected in LNs and represented ~2.03% of LN memory CD4 T cells (Supplementary Fig. 1,b). Because of the very low percentages of PD−1+ CD4+ T cells in LNs and blood, and the limited number of LN cells isolated, it was not possible to sort individual PD−1+ T cell populations. Therefore the total PD−1+ CD4 T cell population was sorted and used for the viral outgrowth assay (VOA). Of note, we used the same gating strategy for both blood and LN CD4 T cell populations (Supplementary Fig. 1c), and the mean percentage of total LN TFH cells represented 65% of the total LN PD−1+ CD4+ T cell population (Supplementary Fig. 1d,e).

Consistent with previous studies11,15,16, LN TFH cells from viremic untreated individuals were expanded as compared to those of healthy individuals, and their percentage dropped after prolonged ART to levels observed in healthy individuals (Supplementary Fig. 2a). We also investigated the expression of activation markers such as CD38, HLA-DR, and K+67 (Supplementary Fig. 2b) in the different LN memory CD4 T cell populations and observed significantly higher expression (P < 0.05) of these markers in TFH cells than in the other LN CD4 T cell populations (Supplementary Fig. 2b).

**Isolation of HIV-1 from blood and LN CD4 T cell populations**

We performed a conventional VOA with three single-replicate cell dilutions, (1 × 10^5, 2 × 10^6 and 4 × 10^6 cells) for LN CD4 T cell populations and four single-replicate cell dilutions (5 × 10^3, 1 × 10^4, 2 × 10^4 and 4 × 10^4 cells) for blood CD4 T cell populations. We used the highest common cell concentration (1 × 10^5 cells) to estimate and compare the capacity of the three sorted memory CD4 T cell populations (DN, single CXCR5+ and PD−1+) that were isolated from blood and LNs of long-term-ART-treated aviremic HIV-1-infected individuals in supporting active virus replication and production. We stimulated cell populations with monoclonal antibodies (mAbs) specific for CD3 and CD28, and cultured these cells for 14 d with allogeneic CD8-depleted blood mononuclear cells that were isolated from HIV-uninfected individuals (Fig. 1a). We first compared the levels of HIV-1 RNA and the capsid protein P24 that were produced in the VOA culture supernatants at the highest common cell concentration (1 × 10^5 cells) at days 0, 5 and 14 by using previously validated assays17 (Fig. 1a). The cumulative data showed a significant increase in HIV-1 RNA levels in the culture supernatants that were collected at day 14, as compared to those from day 5, for all memory CD4 T cell populations that were isolated from LNs and for single CXCR5+ and total PD−1+ memory CD4 T cell populations that were isolated from blood (P < 0.05) (Fig. 1b,c). The levels of HIV-1 RNA that we detected in day 5 and day 14 culture supernatants of LN PD−1+/TFH cells were significantly higher than in supernatants from any of the other memory CD4 T cell populations that were isolated from blood or LNs (P < 0.05; 60- to 73,123-fold higher) (Supplementary Fig. 3a-d). Furthermore, only the PD−1+/TFH memory CD4 T cells that were isolated from LNs showed a significant increase (P < 0.05) of HIV-1 P24 in culture supernatants collected at day 14 as compared to that in culture supernatants from day 5 (Fig. 1d,e), suggesting a significant amplification of HIV-1 P24 production in LN PD−1+/TFH cells from long-term-ART-treated aviremic HIV-1-infected individuals. P24 production was not detected in the VOA culture supernatants at day 5 (Fig. 1d,e), whereas it was detected in day 14 culture supernatants from LN memory PD−1+/TFH cells in 6 of the 11 long-term-ART-treated

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**Table 1** Demographic and clinical data

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ETR, etravirine; FTC, efavirenz; TDF, tenofovir disoproxil fumarate; ATV/r, atazanavir boosted with ritonavir; 3TC, lamivudine; ABC, abacavir; EFV, efavirenz; EVG, elvitegravir; RAL, raltegravir; DTG, dolutegravir; LPV/r, lopinavir boosted with ritonavir; AZT, zidovudine; VOA, viral outgrowth assay; CAR, cell-associated RNA; ID, immunohistochemistry; MC, mass cytometry; ND, not determined.
aviremic HIV-1-infected individuals; it was borderline positive in memory PD-1+ CD4 T cells from blood from 1 of the 11 individuals tested. The cumulative data indicated a significant increase (P < 0.05; 13- to 16-fold higher) of HIV-1 P24 levels in day 14 culture supernatants of LN PD-1+/T_FH cells than in those from any of the other memory CD4 T cell populations that were isolated from blood or LNs (Supplementary Fig. 3e,f).

Of note, we have excluded the possibility that the differential viral outgrowth between cells isolated from the LNs and blood has been influenced by different intracellular levels of atazanavir drug (owing to differential penetration of the drug in lymphoid tissue versus blood) (Supplementary Fig. 4).

Moreover, we did not observe a correlation between the frequencies of total LN PD-1+ CD4 T cells and the levels of HIV-1 RNA in the VOA culture supernatants, whereas there was a trend toward correlation (P = 0.05) with the levels of P24 (Fig. 1f,g).

Notably, we observed a strong correlation between both virologic measures and the percentage of LN memory CXCR5hiPD-1hi CD4 T cells (T_FH cells), thus indicating that these cells were largely responsible for the production of HIV-1 RNA and P24 (r = 0.64, P = 0.03 and r = 0.77, P = 0.007, respectively) (Fig. 1h,i).

HIV-1 RNA and P24 levels from LN PD-1+ T_FH cells inversely correlate with duration of treatment

The levels of P24 that were detected in VOA culture supernatants of LN PD-1+/T_FH cells at day 14 correlated with the HIV-1 RNA levels (r = 0.9352, P = 0.0001) (Fig. 2a), thus suggesting a direct association between HIV-1 RNA and P24 production. P24 production was detected only in culture supernatants that had HIV-1 RNA levels >10^4 copies/ml (Fig. 2a), thus indicating that a threshold level of HIV-1 RNA production is necessary for the detection of P24 under the experimental culture conditions used.

We next determined the relationship between the duration of treatment and the levels of HIV-1 RNA and P24 detected at day 14 in the culture supernatants of blood and LN memory CD4 T cell populations. The results indicated a strong inverse correlation between the levels of HIV-1 RNA in the culture supernatants of both LN PD-1+/T_FH
and LN single CXCR5+ CD4 T cells and the duration of treatment \( r = -0.9015, P < 0.0001 \) and \( r = -0.8142, P = 0.0018 \), respectively; Fig. 2b). Similarly, the levels of P24 in the culture supernatants of LN CD4+ T cell populations isolated from blood and LN were inversely correlated with the duration of treatment \( r = -0.8498, P < 0.0007 \); Fig. 2c).

Of note, HIV-1 RNA was consistently detected in culture supernatants of LN CD4+ T cell populations isolated from blood and LN infected individuals \( n = 11 \). NA, not applicable. Dashed line represents the limit of detection.

Undetectable values were arbitrarily defined as 10 HIV-1 RNA copies/ml (a,b,e,g-i). In f,g, histograms correspond to the mean, and error bars (g) denote s.e.m. *P < 0.05 by Spearman rank test (a-c,h,i), Wilcoxon matched-pairs two-tailed signed-rank test (d,e,g) or Chi-squared two-tailed analysis (f).

**Figure 2** The levels of HIV-1 RNA and P24 from LN PD-1+T\(_{FH}\) cells inversely correlate with duration of treatment. (a) Correlation between levels of HIV-1 RNA and P24 in day 14 VOA supernatants of sorted LN memory CD4 T cell populations \( n = 11 \). (b,c) Correlation between duration of ART and levels of HIV-1 RNA (b) or P24 (c) in day 14 VOA supernatants of sorted LN memory CD4 T cell populations \( n = 11 \). (d,e) Levels of HIV-1 RNA in blood (d) or LN (e) memory CD4 T cell populations at days 5 and 14 of an in vitro HIV-1 infection assay \( n = 11 \). (f) Proportion of HIV-1-infected individuals with detectable HIV-1 RNA \( \geq 10 \) HIV-1 RNA copies/ml in blood and LN memory CD4 T cell populations at day 14 of an in vitro HIV-1 infection assay \( n = 11 \). (g) Levels of HIV-1 RNA in blood and LN memory CD4 T cell populations at day 14 of an in vitro HIV-1 infection assay \( n = 11 \). (h,i) Correlation between HIV-1 RNA levels measured from VOAs \((x)\) versus those from in vitro HIV-1 infection assays \((y)\) in culture supernatants of memory CD4 T cell populations isolated from blood (h) or LN (i) of HIV-1-infected individuals \( n = 11 \). NA, not applicable. Dashed line represents the limit of detection.
of the in vitro infection assay showed a significant increase of HIV-1 RNA levels in culture supernatants collected at day 14, as compared to those from day 5, but only from VOA culture supernatants of LN PD-1+/FH cells (P < 0.05) (Fig. 2d,e). Of note, none of the culture supernatants that had been collected at day 0 had detectable levels of HIV-1 RNA (data not shown). We then compared the levels of HIV-1 RNA produced in culture supernatants of the in vitro infection assay from VOA culture supernatants of all memory CD4 T cell populations isolated from blood and LNs at day 14 (Fig. 2f,g). Notably, HIV-1 RNA was significantly (P < 0.05) more frequently detected in culture supernatants of LN PD-1+/FH cells (7 of the 11 long-term-ART-treated aviremic HIV-1-infected individuals tested) as compared to that in any other memory CD4 T cell population isolated from blood or LNs (Fig. 2f). In addition, the levels of HIV-1 RNA detected in culture supernatants of LN PD-1+/FH cells were significantly (P < 0.05) higher than in any other memory CD4 T cell populations that were isolated from the blood or LNs (Fig. 2g). Of note, HIV-1 RNA was detected (at the limit of detection) in culture supernatants of LN memory DN (in 1 of 11 individuals) and single CXCR5+ (in 2 of 11 subjects) CD4 T cell populations and in blood memory PD-1+ (in 1 of 11 individuals) CD4 T cell populations (Fig. 2f). Finally, we determined the relationship between HIV-1 RNA levels that were detected in the VOA and those detected in the in vitro HIV-1 infection assay. No significant association was observed between HIV-1 RNA levels from the VOA and the in vitro HIV-1 infection assay for blood CD4 T cell populations (Fig. 2h). The results, however, indicated a strong correlation between the HIV-1 RNA levels in the culture supernatants of LN PD-1+/FH cells (r = 0.8780, P = 0.0010) (Fig. 2i). Taken together, these data suggest that LN PD-1+/FH cells of long-term-ART-treated aviremic HIV-1-infected individuals represent the major source of infectious HIV-1.

Detection of cell-associated RNA in blood and LN memory CD4 T cell populations

To determine the cell compartment(s) that serve as sites of active and persistent HIV-1 transcription, we then assessed cell-associated HIV-1 RNA in blood and LN memory CXCR5+PD-1−, CXCR5+PD-1− and PD-1+ CD4 T cells isolated from nine HIV-1-infected individuals (Table 1). Of note, blood samples were available for seven of nine HIV-1-infected individuals. In addition to individuals #11 and #24, whose samples were tested in the VOA, we studied an additional seven individuals who had been treated with ART for a period ranging between 1 and 13 years. In eight of the nine individuals tested, we detected cell-associated HIV-1 RNA in LNs. These individuals were treated for up to 12 years (Fig. 3). The levels of cell-associated HIV-1 RNA detected were significantly higher (3.3- to 33-fold) in LN memory PD-1+/FH cells than in blood or LN PD-1− memory CD4 T cells (P < 0.05) (Fig. 3a). No significant associations were observed between the levels of cell-associated RNA detected in blood memory CD4 T cell populations and the duration of treatment (P > 0.05) (Fig. 3b). However, the levels of cell-associated HIV-1 RNA detected in LN PD-1+/FH cells inversely correlated with the duration of treatment (r = −0.7833, P = 0.017) (Fig. 3c). Notably, we observed a decrease in the levels of cell-associated HIV-1 RNA after at least 8 years of ART (Fig. 3c). The levels of cell-associated HIV-1 RNA in the LN CXCR5−PD-1− and CXCR5+PD-1− CD4 T cell populations were stable and detectable for up to 8 years after treatment but did not correlate with the duration of treatment (Fig. 3c). These results indicate that although prolonged ART may reduce the levels of cell-associated HIV-1 RNA over time, LN PD-1+/FH cells serve as a site of active and persistent virus transcription.

Histopathological changes associated with long-term ART in LN tissues

To determine whether the decrease in different virologic measures of HIV-1 replication was associated with changes in the LN structure and in the number and localization of PD-1+ CD4 T cells, we collected LN biopsies from five additional individuals who were on ART treatment that ranged between 0.3 and 12 years. The density of GCs (measured as number of GCs/mm²) in individuals who had been undergoing ART for 0.3 to 10 years ranged between 0.57/mm² and 0.05/mm² (Fig. 4a,b). Only one primary follicle was found in patient #87, who had been treated for 12.2 years (Fig. 4a,b). These results indicate that substantial changes in LN structure are associated with the duration of treatment, reflecting a shift in the LN tissue from an activated to a quiescent state and a strong inverse correlation between the density of GCs and the duration of treatment (R² = 0.99) (Fig. 4a,b).

We then determined the distribution and number of PD-1+ CD4 T cells in the LN tissue. Cells with high expression of PD-1 were almost...
Figure 4  Histopathological changes associated with long-term ART in LN tissues. (a) Representative examples of LNs from three treated aviremic HIV-1-infected individuals (#92, #86 and #87) stained with H&E (top) or a PD-1-specific antibody (using immunohistochemistry) (middle and bottom) at low (top and middle) and high (bottom) magnification (n = 5). Scale bars, 1 mm (top and middle), 100 μm (bottom). (b,c) Correlation between duration of ART and the density of either GCs (b) or PD-1+ cells (c) in LN tissue from aviremic HIV-1-infected individuals (white circles) (n = 5 per group). Correlations (R²) were calculated using a nonlinear one-phase decay model according to GraphPad Prism. Blue dotted curves correspond to the lower and upper confidence intervals at 0.95.

Figures 5a, b and c show the distribution of PD-1+ CD4 T cells in the lymphoid organs. PD-1+ CD4 T cells are exclusively localized within GCs, showing a polarized distribution in the light zone in the best-developed (larger-sized) GCs, and a few scattered and isolated PD1+ cells were seen in mantle zones and in primary follicles (Fig. 4a). Comparison of serial immunostained sections showed that the density and distribution of PD1+ cells in GCs were essentially similar to those of CD4+ lymphocytes, whereas CD8+ cells accounted for only a small fraction of GC cells (Supplementary Fig. 5). Consistent with the reduction in GCs following ART, the number of PD-1+ GC T cells (per mm² of tissue) decreased from 68.16 cells/mm² in patient #92 (who was treated for 0.3 years) to 1.80 cells/mm² in patient #87 (who was treated for 12.2 years) (Fig. 4a,c). As observed for GCs, the number of PD-1+ CD4 T cells negatively correlated with the duration of ART (Fig. 4a,c). Therefore, these results indicate that PD-1+ CD4 T cells are almost exclusively localized within GCs and that their number progressively decreased after prolonged ART.

LN PD-1+/TFH cells are enriched in replication-competent and infectious virus

To estimate the frequencies of HIV-1-infected cells containing inducible replication-competent or infectious virus, the data generated from the single-replicate cell dilutions (1 × 10⁵, 2 × 10⁴ and 4 × 10³ cells) of the 11 individuals, using the conventional VOA, were pooled together, and the frequencies were estimated by using the extreme limiting dilution assay (Fig. 5a). These analyses provide the average RNA units per million cells (RUPM) for each memory CD4 T cell population in the 11 individuals studied. The results indicate that the average RUPM frequency for LN PD-1+/TFH cells following stimulation with CD3-specific and CD28-specific mAbs was significantly increased as compared to those for blood and LN PD-1+ memory CD4 T cell populations, representing about 37 cells containing replication-competent virus per million LN memory PD-1+/TFH cells (P < 0.05) (Fig. 5b). The IUPM frequency in LN memory PD-1+/TFH cells was also significantly increased as compared to that in any other blood and LN memory CD4 T cell populations, representing about six cells containing infectious virus per million LN memory PD-1+/TFH cells (P < 0.05) (Fig. 5c), thus demonstrating that LN PD-1+/TFH cells are enriched in cells containing inducible replication-competent and infectious virus.

The frequencies of inducible replication-competent or infectious virus within each memory CD4 T cell population allowed us to estimate the relative contribution of blood and LN cell populations to the pool of cells containing replication-competent and infectious virus in the blood and LN compartments (Fig. 5d). We estimated the contribution of each CD4 T cell population by using the frequency of each cell population in each compartment and the estimated numbers of CD4 T lymphocytes in the different lymphoid organs. The results indicate that LN PD-1+/TFH cells contributed the most to the pool of cells containing replication-competent virus in the combined blood and LN compartments and that this population represented about 46% of the blood and LN cell reservoir producing replication-competent virus (Fig. 5d).

Finally, we evaluated the contribution of blood and LN memory CD4 T cell populations to the pool of cells containing infectious virus in these compartments. The cumulative data show that LN PD-1+/TFH cells contributed the most to the pool of cells containing infectious virus in the blood and LN compartments, representing about 96% of the blood and LN cell reservoir that produces infectious virus (P < 0.05) (Fig. 5e). Taken together, these results demonstrate that LN PD-1+/TFH cells probably represent the major source of replication-competent and infectious virus in long-term-ART-treated aviremic HIV-1-infected individuals.

DISCUSSION

Our previous study demonstrated that TFH cells serve as the primary CD4 T cell compartment for HIV-1 infection, replication and...
**Figure 5** LN PD-1+ T<sub>FH</sub> cells of long-term-treated aviremic HIV-1-infected individuals are enriched for replication-competent and infectious virus. (a) Schematic representation of estimation of the mean frequencies of inducible RUPM and IUPM. (b,c) Estimated frequencies of inducible RUPM (b) and IUPM (c) in sorted blood and LN memory CD4 T cell populations (n = 11). (d,e) Estimated contribution of blood and LN memory CD4 T cell populations to the pool of cells containing replication-competent virus (d) or infectious virus (e) in both the blood and LN compartments (n = 11). The equation to determine the estimated contribution of a memory CD4 T cell subpopulation from a specific compartment to the pool of cells containing replication-competent or infectious virus in both the blood and LN compartments can be found in the Online Methods. Histograms correspond to the estimated mean (b–e), and red bars correspond to the lower and upper confidence interval at 0.95 (b–e). *P < 0.05; n.s., not significant; by extreme limiting dilution analysis (ELDA) (b,c), or by unilateral or bilateral Z-score<sup>25</sup> (d,e).

The generation of infectious virus in viremic individuals. In the present study, we tested the hypothesis that T<sub>FH</sub> cells serve as the primary site for replication-competent and infectious virus and for active and persistent HIV-1 transcription in long-term-ART-treated aviremic individuals.

We used the conventional VOA to evaluate and compare the capacity of the three memory CD4 T cell populations (i.e., DN, single CXCR5<sup>+</sup> and PD-1<sup>−</sup> cells) that were isolated from blood and LNs of long-term-ART-treated aviremic HIV-1-infected individuals to support active virus replication and produce infectious virus. We showed that LN PD-1<sup>−</sup> CD4 T cells (composed of ~65% T<sub>FH</sub> cells) are the major source of replication-competent HIV-1, as compared to blood or LN PD-1<sup>−</sup> memory CD4 T cell populations from long-term-ART-treated aviremic HIV-1-infected individuals.

We also observed a relationship between the levels of HIV-1 RNA and P24, and between HIV-1 RNA and the transmission of infection to CD4 T cells from HIV-negative individuals<sup>17</sup>. Furthermore, in contrast to the minimal decrease in the pool of latently HIV-1-infected blood CD4 T cells observed over time<sup>10</sup>, there was a substantial decrease in both the levels of HIV-1 RNA and P24, particularly in the LN PD-1<sup>−</sup>/T<sub>FH</sub> cell population, over time. The present results may indicate that because LN PD-1<sup>−</sup> CD4 T cells are mainly effector T cells, the CD4 T cell reservoir containing replication-competent HIV-1 in LNs is much less stable as compared to that in blood and that ART has a major effect on reducing the size of the LN cell reservoir over time.

The demonstration that LN PD-1<sup>−</sup>/T<sub>FH</sub> cells have the highest levels of cell-associated HIV-1 RNA indicates that this cell population is the primary site for HIV-1 transcription. Notably, cell-associated HIV-1 RNA was detected in individuals who were treated for up to 12 years, thus demonstrating that these cells are responsible for persistent HIV-1 transcription. The levels of cell-associated HIV-1 RNA in LN PD-1<sup>−</sup>/T<sub>FH</sub> cells were stable for at least 5 years after ART, and the initial decline was observed only after 8 years of treatment. The levels of cell-associated HIV-1 RNA in the other two LN memory CD4 T cell populations were substantially lower, stable and not influenced by the duration of treatment. These results suggest that the presence of cell-associated HIV-1 RNA within CXCR5<sup>−</sup>/PD-1<sup>−</sup> and CXCR5<sup>+</sup>/PD-1<sup>−</sup> cells may reflect spillover of RNA production as a result of limited and transient release of viral particles, whereas active and persistent virus transcription occurs within the PD-1<sup>−</sup>/T<sub>FH</sub> cells, probably because of their greater state of activation<sup>22</sup> and their location in GCs, which represents a privileged site for virus replication and infection<sup>23</sup>. The persistence of cell-associated HIV-1 RNA in PD-1<sup>−</sup>/T<sub>FH</sub> cells for up to at least 12 years may help explain why virus rebound is consistently observed after ART interruption. Although the present results do not provide formal demonstration for active and persistent HIV-1 replication, they may support the hypothesis of persistent HIV-1 replication during ART and are consistent with the recent study by Lorenzo-Redondo et al.<sup>24</sup>

Long-term ART was also associated with a progressive shift of the LN tissue from a state of activation to a state of quiescence, as indicated by the progressive reduction in the number of GCs that presumably results from the progressive clearance of HIV-1 antigens from the GCs. Along the same lines, the number of PD-1<sup>−</sup>/T<sub>FH</sub> cells that were localized within GCs and were predominantly responsible for active and persistent HIV-1 transcription were greatly reduced after prolonged ART. Taken together, these results indicate that despite the lack of detection of viremia, active and persistent production of HIV-1 RNA occurs in the LN of individuals, even after many years of ART. The persistence of active HIV-1 RNA production is probably driven by the state of activation within the LN tissue, and at least 10 years of therapy are needed to induce a quiescent state in the LN tissue and to result in a substantial reduction in the pool of cells that support active and persistent HIV-1 transcription during ART. Therefore, 10–15 years of ART may be needed to effectively suppress persistent virus replication in LNs.
In conclusion, here we have identified LN PD-1+/TFH cells as the major site for the generation of replication-competent and infectious HIV-1 and for the active and persistent production of HIV-1 RNA in long-term ART-treated HIV-1-infected individuals, demonstrating that LN PD-1+/TFH cells represent a major obstacle to obtaining a functional cure for HIV-1 infection. Therefore, the present results provide a scientific rationale for the development of interventional therapies that target the elimination of PD-1+/TFH cells by using PD-1-specific antibody therapy.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
R.B., F.A.P and A.N. performed the experiments; G. Pollakis performed and analyzed HIV sequencing; M.C. recruited patients; K.O. performed statistical analyses; J.-M.C. performed lymph node biopsies; L.d.L. was in charge of immunohistochemistry experiments; G. Pantaleo and M.P. conceived the study, designed the experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Study group, ethics statement and cell isolation. Twenty seven HIV-1-infected adult volunteers (Table 1) and ten healthy HIV-uninfected individuals were enrolled in the present study. No statistical method was used to predetermine sample size. The sample size was estimated based on a previously published study. The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all individuals gave written informed consent. For inclusion criteria, only individuals undergoing antiretroviral therapy for more than 18 months with undetectable HIV-1 viremia (<50 HIV-1 RNA copies/ml of plasma) were enrolled for VOA analysis, and individuals treated for different durations with ART were enrolled for the additional virological and histopathological analyses. For exclusion criteria, individuals experiencing blips of viremia (>50 HIV-1 RNA copies/ml of plasma) within the last 12 months were not enrolled for VOA analysis. Inguinal lymph node biopsies and blood samples were collected on the same day. Blood mononuclear cells were isolated as previously described, and lymph node mononuclear cells were isolated by mechanical disruption as previously described. Blood mononuclear cells and lymph node mononuclear cells were cryopreserved in liquid nitrogen. Because the inclusion criteria were the same for the individuals studied, there was no need for randomization or blinding.

Cell culture. Cells were cultured in Roswell Park Memorial Institute (RPMI) medium (Gibco; Life Technologies) containing 10% heat-inactivated FBS (Institut de Biotechnologies Jacques Boy), 100 IU/ml penicillin and 100 µg/ml streptomycin (Bio Concept).

Antibodies. The following antibodies were used: allophycocyanin (APC)-H7-conjugated anti-CD3 (clone SK7), Pacific blue (PB)-fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-CF594-conjugated anti-CD4 (clone RPA-T4), V450-conjugated anti-IL-2R-DR (clone G4-6), PE-Cy7-conjugated anti-CD25 (clone M-A251), PerCP-Cy5.5-conjugated anti-CD69 (clone L78), purified coating anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) mAbs were purchased from BD (Becton Dickinson; CA, USA); and energy-coupled dye (phycoerythrin–Texas Red conjugate; ECD)-conjugated anti-CD45RA (clone 2H4) was from Beckman Coulter (CA, USA).

Sorting of CD4 T cell populations. The average number of LN mononuclear cells isolated was about 30 million cells, and the percentage of LN memory CD4 T cells represented about 25% of the total number of isolated LN mononuclear cells. On the basis of the average number of memory CD4 T cells available, it was not possible to sort individual PD-1+ cell populations—i.e., single PD-1+CXCR5+PD-1hiCXCR5hi and PD-1hiCXCR5hi cells—and for these reasons the total PD-1+CD4 T cell population (corresponding to about 9% of memory CD4 T cells) was sorted. It should also be taken into account that to achieve a purity of >98% of the sorted cell populations and due to the loss of cells during sorting, about 150,000 cells per CD T cell population were consistently sorted to use for VOAs. Although the number of blood cells available for sorting was much higher than that of LNs, the limiting factor was the very low percentage (about 0.5%) of PD-1hi cells within the blood memory CD4 T cell population. Cryopreserved blood and lymph node mononuclear cells were thawed, and CD4 T cells were enriched, using EasySep Human CD4 T Cell Enrichment kit (StemCell Technologies, USA). CD4 T cells were then stained with Aqua LIVE/DEAD stain kit (4 °C; 15 min) and then with anti-CD3–APC-H7, anti-CD4–FITC, anti-CD45RA–ECD, anti-PD-1–PE-Cy7 and anti-CXCR5–PE (4 °C; 25 min), and viable memory (CD45RA–CXCR5–PD-1–, CXCR5+PD-1+ and PD-1hiCD4 T cell populations were sorted using FACSaria (Beckton & Dickinson). In all of the sorting experiments, the grade of purity of the sorted cell populations was >98%.

Viral outgrowth assay (VOA). Different cell concentrations (fivefold limiting dilutions, i.e., 1 × 10^2, 2 × 10^2 and 4 × 10^2 cells for LN CD4 T cell populations and 5 × 10^2, 1 × 10^3, 2 × 10^3 and 4 × 10^3 cells for blood CD4 T cell populations) of sorted viable blood and lymph node memory CD4 T cells from HIV-1-infected individuals (Table 1) were cultured with allogeneic fresh CD8-depleted blood mononuclear cells (10^6 cells/ml) from HIV-uninfected individuals. These cells were stimulated for 3 d with anti-CD3 and anti-CD28 mAb-coated plates (10 µg/ml) in presence of IL-2 (50 units/ml). Supernatants were collected at days 0, 5 and 14. Medium was replaced at day 5, and the culture was re-supplemented with cytokines. The presence of P24 antigen was assessed by ECL COBAS HIV Ag (Roche; Switzerland). The presence of HIV-1 RNA was assessed by COBAS AmpliPrep/TaqMan HIV-1 Test (Roche; Switzerland). When required, culture supernatants were diluted (1/10) and then subjected to DNAse treatment (RNAqueous-4PCR Kit, Ambion). RNA samples were extracted from 5 × 10^4 cells/ml of plasma) were referred to as HIV-1 RNA-positive wells. RUPM and IUPM were calculated by using conventional limiting dilution methods using ‘extreme limiting dilution analysis’ (ELDA) (http://bioinf.wehi.edu.au/software/elda/). The estimation of each population’s contribution to the overall pool of HIV-1-infected cells was performed as follows: estimated contribution of memory CD4 T cell population A from compartment X to the pool of cells containing replication-competent virus in both blood and LN compartments = ((percentage of memory CD4 T cell population A from compartment X among total memory CD4 T cells from compartment X) × (estimated RUPM frequency of memory CD4 T cell population A from compartment X)) × (percentage of the estimated number of memory CD4 T cells present in compartment A among the sum of the memory CD4 T cells present in both blood and LN)).

In vitro HIV-1 infection assay. CD8-depleted blood mononuclear cells isolated from HIV-uninfected individuals were activated for 48 h with anti-CD3 and anti-CD28 micro-beads (Miltenyi Biotec) in complete RPMI medium supplemented with IL-2 (30 units/ml). Activated allogeneic CD8-depleted blood mononuclear cells (10^6 cells/ml) from HIV-uninfected individuals were washed and exposed (6 h, 37 °C, 5% CO2) to 100 µl VOA supernatants collected at 14. Following a 6-h exposure, cells were washed twice with complete medium and cultured for 14 additional days in complete RPMI medium. The presence of infectious HIV-1 particles was determined in the culture supernatants at days 0, 5 and 14 after the inoculations, as assessed by the HIV-1 RNA assay (COBAS AmpliPrep/TaqMan HIV-1 Test).

Quantification of cell-associated RNA. Cell-associated HIV-1 RNA from individual samples was extracted from 5 × 10^6 sorted CD4 T cell populations and subjected to DNase treatment (RNAsafe–4PCR Kit, Ambion). RNA standard curves were generated after isolation and quantification of viral RNA from supernatants of ACH2 culture as previously described. One-step cdna synthesis and pre-amplification were performed as previously described by using the following primers ULF1: 5’-ATGCCAGTAAAGGCAACTCGTGTCCTCTGTGTTAGAC-3’; UR1: 5’-CCATCTCTCTCCTTCTAGC-3’. Real-time PCR was performed using a Roche Light Cycler 480II with the following primers: LambdaT: 5’-ATGCCAGTAAAGGCAACTCGTGTCCTCTGTGTTAGAC-3’; UR2: 5’-CTAGGAACTCTTAGTACTCACC-3’; and probes: 56-FAM 5’-CAGTCTAAAGG/ZEN/CAAGCTTTATGGCAGGACGGC-3’ 1ABQF21.

Histopathology. Lymph nodes were measured and cut into slices, which were fixed in 10% buffered formalin for routine processing and embedding in paraffin blocks. 4-µm sections were cut and stained with hematoxylin and eosin (H&E). For each lymph node, serial sections of the most representative block were immunostained according to standard routine protocols by using a Ventana Benchmark platform (Roche) with antibodies against CD20 (L26; Novocastra),...
CD3 (CD3-PS1; Leica), CD4 (SP35; Ventana), CD8 (C8/144B; DAKO), and PD1 (polyclonal goat IgG, RD Systems). PD1-immunostained slides were digitalized using a Hamamatsu Nanozoomer 1.0 scanner (model C9600-01) at 40× with the NDPScan software (v. 2.5.89). Scanning area and focus points were set manually. Image analysis was performed with the Tissue IA-specific module of the Slidepath Software Digital Image Hub (DIH) (version 4.0.7). The surfaces of the entire tissue section, and of individual germinal centers (manually circumscribed), were measured. To quantify PD1-positive cells, we used the ‘measure stained cells algorithm’ at 20×, which was set to take into account strongly stained (PD1+) cells, and quantified nuclei for estimation of cell density.

**Mass cytometry.** Cryopreserved lymph node mononuclear cells were thawed, resuspended (10⁶ cells/ml) in complete RPMI medium and either stimulated or not with 100 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) and 1 µg/ml ionomycin (Sigma-Aldrich) in the presence of golgi plug (BD) for 6 h at 37 °C. Viability of cells in 500 µl of PBS was identified by incubation with 50 µM cisplatin (Sigma-Aldrich) for 5 min at RT and quenched with 500 µl FBS. Next, cells were incubated (30 min; 4 °C) with metal-conjugated antibodies directed against CD3, CD4, CD8, CD45RA, CXCR5, PD-1, CCR7, CD27, CD28, HLA-DR, CD38, CXCR4, CXCR3 and CD57 (Fluidigm/DVS Science). Cells were washed and fixed (10 min; RT) with 2.4% paraformaldehyde (PFA). Cells were then permeabilized (45 min; 4 °C) with Foxp3 Fixation/Permeabilization kit (eBioscience), washed and stained (30 min; 4 °C) with metal-conjugated antibodies against Ki-67, BCL-6, IFN-γ, TNF-α, IL-2, or IL-21 (Fluidigm/DVS Science). Cells were washed and fixed (10 min; RT) with 2.4% PFA. Total number of cells was identified by DNA intercalation (1 µM Cell-ID Intercalator; Fluidigm/DVS Science) in 2% PFA after a 4 °C overnight incubation. Labeled samples were assessed by the CyTOF1 instrument that was upgraded to CyTOF2 (Fluidigm) using a flow rate of 0.045 ml/min. Data were analyzed using Fluidigm Cytobank software package (Cytobank, Mountain View, CA).

**Statistical analyses.** Statistical significance (P values) was either obtained by using two-tailed Chi-square analysis for comparison of positive proportions or by using one-way ANOVA (Kruskal–Wallis test) followed by Wilcoxon matched-pairs two-tailed signed-rank test for multiple comparisons. Spearman rank test, linear regression or nonlinear one-phase decay model was used for correlations. Finally, statistical significance (P value) was obtained by using extreme limiting dilution analysis (ELDA) (http://bioinf.wehi.edu.au/software/elda/), or by unilateral or bilateral Z-score²⁵, for comparison of frequencies of HIV-1-infected cells and contribution of each cell population to the total pool of infected cells, respectively. The analyses of multiple comparisons were taken into account for the calculation of statistical significance.


