PD-1$^+$ and Tfh Cells Are Responsible for Persistent HIV-1 Transcription in Treated Aviremic Individuals

[1]Riddhima Banga, [1]Francesco Andrea Procopio, [1]Alessandra Noto, [2]Georgios Pollakis, [3]Matthias Cavassini, [1]Khalid Ohmiti, [4]Jean-Marc Corpataux, [5]Laurence de Leval, [1,6]Giuseppe Pantaleo and [1]Matthieu Perreau

[1]Service of Immunology and Allergy, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland, [2] Department of Clinical Infection, Microbiology and Immunology (CIMI), Institute of Infection and Global Health (IGH), University of Liverpool, Liverpool, UK, [3]Service of Infectious Diseases, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland, [4]Service of Vascular Surgery, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland, [5]Institute of Pathology, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland, [6]Swiss Vaccine Research Institute, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland

[§] Correspondence should be addressed to:
Dr. Matthieu Perreau matthieu.perreau@chuv.ch
or
Prof. Giuseppe Pantaleo giuseppe.pantaleo@chuv.ch

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Chlorophyll-A Protein ; PerCP-Cy5.5, Peridinin Chlorophyll-A Protein-Cyanin5.5 conjugate ; RPMI, Roswell Park Memorial Institute medium ; RT-PCR, reverse transcriptase-polymerase chain reaction ; Tfh, T follicular helper T cells ; TM, transitional memory ; VOA, viral outgrowth assay.

Abstract

The mechanisms responsible for the persistence of HIV-1 after many years of suppressive antiretroviral therapy (ART) have been partially elucidated. Most of the studies investigating HIV-1 persistence have been performed in 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 population(s) in blood and LNs responsible for the production of replication competent and infectious HIV-1 and for active and persistent virus transcription in ART treated (1.5-14 years) aviremic (<50 HIV RNA copies/ml) individuals. We demonstrate that LN PD-1$^+$ CD4 T cells which are composed of about 65% T follicular helper cells as defined by the expression of CXCR5 and PD-1, are the major source of replication competent HIV-1 and of infectious virus compared to any other (CXCR5$^-$PD-1$^-$, 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 and 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 as compared to the other memory CD4 T cell populations. These results indicate that LN PD-1$^+$ cells serve as the major CD4 T cell compartment in blood and LNs for replication competent and infectious HIV-1 and for active and persistent virus transcription in long-term ART treated aviremic individuals and may represent a major obstacle to HIV-1 functional cure.

Introduction

The main mechanisms that hinder HIV-1 eradication despite effective ART are 1) the existence of long-lived latently HIV-1 infected resting memory CD4 T cells and/or 2) the existence of residual virus replication that replenishes the HIV-1 latent cell reservoir[1-6]. Estimates of the HIV-1 latent reservoir half-life in the blood indicate that as long as 70 years of ART might be required for full eradication of the latent reservoir[5]. A pioneering study identified central memory (CM; defined by $CD45RA^{-}CCR7^{+}CD27^{+}$) and transitional memory (TM; $CD45RA^{-}CCR7^{-}CD27^{+}$) CD4 T cells as major cellular compartments of the latent HIV-1 reservoir in blood[6]. More recently, memory CD4 T cells with stem-cell like properties were identified as a novel but minor latent HIV-1 reservoir[7]. Therefore, numerous strategies are currently being evaluated to target and kill HIV-1 infected cells to ultimately find a cure.

Blood contains only 2% of the total lymphocytes which reside predominantly within lymphoid organs and lymphocyte populations within the tissues are phenotypically and functionally distinct from those in blood[8]. The recently described T follicular helper cells (Tfh) illustrate this difference[9,10]. In this regard, we have recently shown that LN Tfh cells defined by the expression of CXCR5 and PD-1 and to a lesser extent memory $CXCR5^{-}PD\text{-}1^{+}$ CD4 T cells serve as the major CD4 T cell compartments for HIV-1 replication, production and infection in viremic HIV-1-infected individuals[11]. Since, cytotoxic CD8 T cells have limited access to GCs[12] and antiretroviral drug penetration might be reduced in lymphoid tissue[13], HIV-1-infected Tfh cells residing in GCs may represent a major barrier for functional HIV-1 cure and eradication. 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.

In order to address this issue, we have investigated the distribution of replication competent and infectious virus within different memory CD4 T cell populations sorted on the basis of the expression of CXCR5 and PD-1, *i.e.* $CXCR5^{-}PD\text{-}1^{-}$, $CXCR5^{+}PD\text{-}1^{-}$ and $PD\text{-}1^{+}$ memory CD4 T cell populations, isolated from blood and LN of long-term treated aviremic HIV-1-infected individuals. The results shown in the present study demonstrate that LN $PD\text{-}1^{+}$/Tfh cells of long-term ART treated aviremic HIV-1-infected individuals are the major source of replication competent and infectious HIV-1 and the cell compartment responsible for active and persistent virus transcription.

Results

Characterization of CD4 T cell populations

We simultaneously collected blood and LNs of 23 ART treated aviremic HIV-1-infected individuals. The 23 individuals studied had a documented diagnosis of HIV-1 infection between 2 and 27 years and duration of ART between 0.3 and 14 years (**Table 1**). Mononuclear cells isolated from blood and LNs were then stained with CD3, CD4, CD45RA, CXCR5 and PD-1 antibodies. Five populations of memory ($CD45RA^-$) CD4 T cells were identified on the basis of the expression of PD-1 and CXCR5, *i.e.* $CXCR5^-PD\text{-}1^-$ dual negative cells (DN), single $CXCR5^+$ ($CXCR5^+PD\text{-}1^-$) cells, single PD-1^+ ($CXCR5^-PD\text{-}1^+$) cells, dual $CXCR5^{int}PD\text{-}1^{int}$ and $CXCR5^{high}PD\text{-}1^{high}$ CD4 T cells (**Supplementary Fig. 1a**). The latter two populations correspond to Tfh cells on the basis of their phenotypic, transcription factor and functional profiles[14]. Consistent with previous study, Tfh $CXCR5^{int}PD\text{-}1^{int}$ cells were significantly ($P < 0.05$) enriched in LNs (7.48%) as compared to blood (1.28%) (**Supplementary Fig. 1a,b**) and Tfh $CXCR5^{high}PD\text{-}1^{high}$ cells were only detected in LNs representing about 2.03% of LN memory CD4 T cells (**Supplementary Fig. 1a,b**)[11,15]. Because of the very low percentage of PD-1^+ CD4 T cells in LNs and particularly in blood, and limited number of LN cells isolated, it was not possible to sort individual PD-1^+ cell populations. Therefore the total PD-1^+ CD4 T cell population was sorted for the viral outgrowth assay (VOA). Of note, we used the same gating strategy for both blood and LN CD4 T cells (**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 study[11,15,16], LN Tfh cells of viremic untreated individuals were expanded as compared to 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 Ki-67 (**Supplementary Fig. 2b**) in the different LN memory CD4 T cell populations and observed significantly higher expression of these markers in Tfh cells as compared to the other LN CD4 T cell populations ($P < 0.05$) (**Supplementary Fig. 2b**).

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

We performed a conventional virus outgrowth assay (VOA) using three single replicate cell dilutions, (10^5, $2X10^4$ and $4X10^3$ cells) for LN CD4 T cell populations and four single replicate cell dilutions ($5X10^5$, 10^5, $2X10^4$ and $4X10^3$ cells) for blood CD4 T cell populations. We used the highest common cell concentration (10^5 cells) to estimate and compare the capacity of the three sorted memory CD4 T cell populations, *i.e.* DN, single $CXCR5^+$ and PD-1^{high}/Tfh, isolated from blood and LNs of long-term ART treated aviremic HIV-1-infected individuals to support active virus replication and production. We stimulated cell populations with anti-CD3 and anti-CD28 monoclonal antibodies (MAbs) and cultured cells with allogeneic CD8-depleted blood mononuclear cells isolated from HIV-uninfected individuals for 14 days (**Fig. 1a**). We first compared the levels of HIV-1 RNA and of the capsid protein P24 produced in the VOA supernatants at the highest common cell concentration *i.e.* 10^5 cells at day 0, 5 and 14 using previously validated assays[17] (**Fig. 1a**). The cumulative data showed a significant increase in HIV-1 RNA levels in culture supernatants collected at day 14 as compared to day 5 of all memory CD4 T cell populations isolated from LNs and from single $CXCR5^+$ and total PD-1^+ memory CD4 T cell populations isolated from blood ($P < 0.05$) (**Fig. 1b,c**). The levels of HIV-1 RNA detected in day 5 and day 14 culture supernatants were significantly higher as compared to any other memory CD4 T cell populations isolated from blood or LN ($P < 0.05$; 60-73123 fold higher) (**Supplementary Fig. 3a-d**). Furthermore, only PD-1^+/Tfh memory CD4 T cells isolated from LNs showed a significant increase of HIV-1 P24 in culture supernatants collected at day 14 as compared to day 5 ($P < 0.05$) (**Fig. 1d,e**), suggesting a significant amplification of HIV-1 P24 production in LN PD-1^+/Tfh cells of 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 at day 14 in the culture supernatants of LN memory PD-1^+/Tfh cells in six out of the eleven long-term ART treated aviremic HIV-1-infected individuals and was border line positive in one out of the eleven individuals tested in blood memory PD-1^+ CD4 T cells. The cumulative data indicated a significant increase ($P < 0.05$; 13-16 fold higher) of HIV-1 P24 levels in day 14 culture supernatants of LN PD-1^+/Tfh cells as compared to any other memory CD4 T cell populations isolated from blood or LN (**Supplementary Fig. 3e,f**). Of note, we have excluded that the differential viral outgrowth between cells from LN and blood has been influenced by different intracellular levels of atazanavir drug penetration in lymphoid tissue *vs.*

blood[13] (**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 while there was a trend towards correlation (P = 0.05) with the levels of P24 (**Fig. 1f,g**). Interestingly, a strong correlation with both virologic measures was observed when the percentage of Tfh cells was used 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$^+$/Tfh cells inversely correlate with duration of treatment

The levels of P24 detected in culture supernatants of LN PD-1$^+$/Tfh 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 with HIV-1 RNA levels greater than 10^4/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 detected in culture supernatants of LN PD-1$^+$/Tfh and LN single CXCR5$^+$ CD4 T cells and duration of treatment (r = –0.9015, P < 0.0001 and r = –0.8142, P = 0.0018, respectively; **Fig. 2b**). Similarly, the levels of P24 detected in culture supernatants of LN PD-1$^+$/Tfh cells inversely correlated with duration of treatment (r = –0.8498, P = 0.0007; **Fig. 2c**).

Interestingly, HIV-1 RNA was consistently detected in culture supernatants of LN PD-1$^+$/Tfh cells of HIV-1-infected individuals treated for up to 14 years thus indicating that LN PD-1$^+$/Tfh cells may serve as a source of inducible virus after prolonged ART (**Fig. 2b**). However, P24 was detected only within the first 3 years after the initiation of treatment (**Fig. 2c**) and the lack of detection was associated with the decrease of HIV-1 RNA levels in the culture supernatants below 10^4/ml (**Figs. 1c and 2a**). P24 was not detected in the VOA culture supernatants of blood memory CD4 T cell populations or LN PD-1$^-$ memory CD4 T cell populations from the same individuals (**Fig. 1d,e**). Taken together, these data suggest that

177 detection of HIV RNA in VOA culture supernatants is the most sensitive parameter to be tested
178 in blood and LN cell cultures of long-term ART treated individuals.
179
180 **Evaluation of infectious HIV-1 in blood and LN memory CD4 T cell populations**
181 We then assessed the presence of infectious virus in the VOA culture supernatants of
182 blood and LN memory CD4 T cell populations using an *in vitro* HIV-1 infection assay. For these
183 purposes, day 14 VOA culture supernatants of the highest common cell concentration *i.e.* 10^5
184 cells, were used to inoculate activated CD8-depleted blood mononuclear cells isolated from HIV
185 negative individuals. Cells were then cultured for 14 days, and culture supernatants were
186 collected at day 0, 5 and 14 and assessed for the presence of HIV-1 RNA. The cumulative data of
187 the *in vitro* infection assay showed a significant increase of HIV-1 RNA levels in culture
188 supernatants collected at day 14 as compared to day 5 only from VOA culture supernatants of
189 LN PD-1^+/Tfh cells ($P < 0.05$) (**Fig. 2d,e**). Of note, none of the culture supernatants collected at
190 day 0 had detectable levels of HIV-1 RNA (data not shown). We then compared the levels of
191 HIV-1 RNA produced in culture supernatants of the *in vitro* infection assay from VOA culture
192 supernatants of all memory CD4 T cell populations isolated from blood and LNs at day 14 (**Fig.**
193 **2f,g**). Interestingly, HIV-1 RNA was significantly more frequently detected in culture
194 supernatants of LN PD-1^+/Tfh cells (seven out of the eleven long-term ART treated aviremic
195 HIV-1-infected individuals tested) as compared to any other memory CD4 T cell population
196 isolated from blood or LNs ($P < 0.05$) (**Fig. 2f**). In addition, the levels of HIV-1 RNA detected in
197 culture supernatants of LN PD-1^+/Tfh cells were significantly higher as compared to any other
198 memory CD4 T cell populations isolated from blood or LNs ($P < 0.05$) (**Fig. 2g**). Of note, HIV-1
199 RNA was detected (at the limit of detection) in culture supernatants of LN memory DN (one out
200 of eleven individuals) and single CXCR5$^+$ (two out of eleven) CD4 T cell populations and in
201 blood memory PD-1^+ (one out of the eleven) CD4 T cell populations (**Fig. 2f**). Finally, we
202 determined the relationship between HIV-1 RNA levels detected in the VOA and those detected
203 in the *in vitro* HIV-1 infection assay. The results indicated a strong correlation between these
204 HIV-1 RNA levels in the culture supernatants of LN PD-1^+/Tfh cells ($r = 0.8780$, $P = 0.0010$)
205 (**Fig. 2i**). No significant association was observed between HIV-1 RNA levels from the VOA
206 and the *in vitro* HIV-1 infection assay in blood CD4 T cell populations (**Fig. 2h**). Taken together,

these data suggest that LN PD-1$^+$/Tfh 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) serving 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$^+$ cells isolated from nine HIV-1-infected individuals (**Table 1**). Of note, blood samples were available for seven out of nine HIV-1-infected individuals. In addition to individuals #11 and #24 who were tested in the VOA, we studied an additional seven individuals treated with ART for a period ranging between 1 and 13 years. In eight of nine individuals tested, we detected cell-associated HIV-1 RNA in LN. These individuals were treated for up to 12 years (**Fig. 3a,c**). The levels of cell-associated HIV-1 RNA detected were significantly higher (3.3 to 33 fold) in LN memory PD-1$^+$/Tfh cells as compared to blood or LN PD-1$^-$ memory CD4 T cell populations ($P < 0.05$) (**Fig. 3a**). No significant associations were observed between levels of cell-associated RNA detected in blood memory CD4 T cell populations and duration of treatment ($P > 0.05$) (**Fig. 3b**). However, the levels of cell-associated HIV-1 RNA detected in LN PD-1$^+$/Tfh 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 detected in LN CXCR5$^-$PD-1$^-$ and CXCR5$^+$PD-1$^-$ cell populations were stable and detected 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 over time the levels of cell-associated HIV-1 RNA, LN PD-1$^+$/Tfh 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 after initiation of ART ranging between 0.3 and 12 years. The number of GCs/mm^2 ranged between 0.57/mm^2 and 0.05/mm^2 in individuals under ART for a period of time varying from 0.3 to 10 years (**Fig. 4a,b**). Only 1 primary follicle was found in Patient #87 treated for 12.2 years (**Fig. 4a,b**). These results

indicate substantial changes in LN structure associated with the duration of treatment reflecting a shift from an activation to a quiescent state in the LN tissue and a strong inverse correlation between the number of GCs/mm^2 and the duration of treatment ($R^2 = 0.99$) (**Fig. 4a,b**).

We then determined the distribution and the number of PD-1$^+$ CD4 T cells in the LN tissue. Strongly PD-1$^+$ cells were almost exclusively localized within GCs showing a polarized distribution in the light zone in the best developed (larger size) GCs, and few scattered 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 was essentially similar to those of CD4$^+$ lymphocytes, while 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 tissue mm^2 decreased from 68.16 cells/mm^2 in Patient #92 treated for 0.3 years to 1.80 cells/mm^2 in Patient #87 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 their number progressively decreased after prolonged ART.

LN PD-1$^+$/Tfh cells are enriched in replication competent and infectious virus

To estimate the frequencies HIV-1-infected cells containing inducible replication competent or infectious virus, the data generated from the single replicate cell dilutions (10^5, 2.10^4 and 4.10^3 cells) of the eleven individuals using the conventional VOA were pooled together and frequencies were estimated using Extreme Limiting Dilution Assay[18] (**Fig. 5a**). These analyses provide the average RNA-unit per million (RUPM)[19] and Infectious Unit per million (IUPM)[20] for each memory CD4 T cell population in the eleven individuals studied. The results indicate that the average RUPM frequency following anti-CD3/anti-CD28 mAb stimulation of LN PD-1$^+$/Tfh cells was significantly increased as compared to blood and LN PD-1$^-$ memory CD4 T cell populations and represented about 37 cells containing replication competent virus per million of 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 any other blood and LN memory CD4 T cell population and represented about 6 cells containing infectious virus per million of LN memory PD-1$^+$/Tfh cells ($P < 0.05$) (**Fig. 5c**), thus

demonstrating that LN PD-1$^+$/Tfh cells are enriched in cells containing 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 blood and LN compartments (**Fig. 5d,e**). We estimated the contribution of each CD4 T cell population using 1) the frequency of each cell population in each compartment and 2) the estimated numbers of CD4 T lymphocytes in different lymphoid organs[21]. The results indicate that LN PD-1$^+$/Tfh cells contributed the most to the pool of cells containing replication competent virus in the sum of blood and LN compartments and 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 blood and LN compartments and represented about 96% of the blood and LN cell reservoir producing infectious virus ($P < 0.05$) (**Fig. 5e**). Taken together, these results demonstrate that LN PD-1$^+$/Tfh cells likely represent the major source of replication competent and infectious virus in long-term ART treated aviremic HIV-1-infected individuals.

Discussion

Our previous study[11] has demonstrated that Tfh cells serve as the primary CD4 T cell compartment for HIV-1 infection, replication and generation of infectious virus in viremic individuals. In the present study, we tested the hypothesis that Tfh cells serve as the primary site for replication competent and infectious virus and 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 $CXCR5^+$ and $PD\text{-}1^+$, 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 $PD\text{-}1^+$ CD4 T cells (composed of about 65% Tfh cells) are the major source of replication competent HIV-1 as compared to blood or LN $PD\text{-}1^-$ memory CD4 T cell populations of long-term ART treated aviremic HIV-1 infected individuals.

A relationship has also been observed between the levels of HIV-1 RNA, P24 and the generation of infectious HIV-1 as well to transmit infection to CD4 T cells from HIV negative individuals. Furthermore, in contrast to the minimal decrease observed over time in the pool of latently HIV-1-infected blood CD4 T cells[10], there was a significant decrease overtime in both the levels of HIV-1 RNA and P24 particularly in the LN $PD\text{-}1^+$/Tfh cell population. The present results may indicate that since LN $PD\text{-}1^+$ 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 blood and that ART has a major effect on reducing the size of the LN cell reservoir over time.

The demonstration that LN $PD\text{-}1^+$/Tfh cells have the highest levels of cell-associated HIV-1 RNA indicates that this cell population is the primary site for HIV-1 transcription. Interestingly, cell-associated HIV-1 RNA was detected in individuals treated 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 $PD\text{-}1^+$/Tfh 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 significantly lower, stable and not influenced by the duration of treatment. These results suggest that the presence of cell associated HIV-1 RNA within $CXCR5^-PD\text{-}1^-$ and $CXCR5^+PD\text{-}1^-$ cells may reflect spill over of RNA production whereas active and persistent virus transcription within the $PD\text{-}1^+$/Tfh cells.

318 Active and persistent virus transcription occurs preferentially within PD-1$^+$/Tfh cells likely
319 because of their greater state of activation[27] and location in GCs that represent a privilege site for
320 virus replication and infection[28]. The persistence up to at least 12 years of cell-associated HIV-1
321 RNA in PD-1$^+$/Tfh cells may help to explain why virus rebound is consistently observed after
322 ART interruption. Although the present results do not provide formal demonstration for active
323 and persistent HIV-1 replication, they support the existence of persistent HIV-1 replication
324 during ART and are consistent with the recent study by Lorenzo-Redondo *et. al* [22].
325 Long-term ART was also associated with a progressive shift from an activation to a
326 quiescent state of the LN tissue as indicated by the progressive reduction in the number of GCs
327 likely resulting from the progressive clearance of HIV-1 antigens from the GCs. Along the same
328 line, the number of PD-1$^+$/Tfh cells that were localized within GCs and predominantly
329 responsible for active and persistent HIV-1 transcription greatly reduced after prolonged ART.
330 Taken all together these results indicate that despite the lack of detection of viremia, active and
331 persistent production of HIV-1 RNA occurs in the LN many years after ART. The persistence of
332 active production of HIV-1 RNA is likely driven by the state of activation within the LN tissue
333 and at least 10 years of therapy are needed to induce a quiescent state in the LN tissue and the
334 substantial reduction in the pool of cells supporting active and persistent HIV-1 transcription
335 during ART. Therefore, 10-15 years of ART may be needed to effectively suppress persistent
336 virus replication in LNs.
337 We then evaluated the contribution of blood and LN memory CD4 T cell populations to
338 the total pool of cells containing replication competent and infectious virus in these
339 compartments and show that PD-1$^+$/Tfh cells represented a major source of replication
340 competent, *i.e.* about 46%, and of infectious, *i.e.* 96%, virus.
341 In conclusion, the present study has identified LN PD-1$^+$/Tfh cells as the major site for
342 the generation of replication competent and infectious HIV-1 and for active and persistent
343 production of HIV-1 RNA in long-term ART treated HIV-1-infected individuals demonstrating
344 that LN PD-1$^+$/Tfh cells represent a major obstacle to HIV-1 functional cure. Therefore, the
345 present results provide the scientific rationale for the development of interventional therapies
346 targeting the elimination of PD-1$^+$/Tfh cells using PD-1 antibody therapy.

Material and methods

Study group, ethics statement and cell isolation. Twenty seven HIV-1 infected adult volunteers (**Table 1**) and ten 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[23]. The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all individuals gave written informed consent. As inclusion criteria only individuals under antiretroviral therapy for more than 18 months with undetectable HIV-1 viremia (<50 HIV-1 RNA copies/mL) were enrolled for VOA analysis and individuals treated for different durations with ART were enrolled for the additional virological and histopathologial analyses. As exclusion criteria, for VOA analysis individuals experiencing blips of viremia (>50 HIV-1 RNA copies/ml of plasma) within the last 12 months were not enrolled. Inguinal lymph node biopsies and blood samples were collected the same day. Blood mononuclear cells were isolated as previously described[24] and lymph node mononuclear cells were isolated by mechanical disruption as previously described[25]. Blood mononuclear cells and lymph node mononuclear cells were cryopreserved in liquid nitrogen. Since the inclusion criteria were the same for the individuals studied, there was no need for randomization or blinding.

Cell culture. Cells were cultured in RPMI (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: APC-H7-conjugated anti-CD3 (clone SK7), PB- or FITC- or PE-CF594-conjugated anti-CD4 (clone RPA-T4), V450- conjugated anti-HLA-DR (clone (G46-6), 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 ECD-conjugated anti-CD45RA (clone 2H4) 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 total 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-1^{int}CXCR5^{int} and PD-1^{high}CXCR5^{high}, 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 be also 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 CD4 T cell population were consistently sorted for the VOA. Although the number of blood cells available for sorting experiments was much higher than that of LNs, the limiting factor was the very low percentage (about 0.5%) of PD-1^{high} 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 ($CD45RA^-$) $CXCR5^-PD\text{-}1^-$, $CXCR5^+PD\text{-}1^-$ and $PD\text{-}1^+$ CD4 T cell populations were sorted using FACSAria (Beckton & Dickinson). In all sorting experiments the grade of purity of the sorted cell populations was >98%.

Viral outgrowth assay. Different cell concentrations (five-fold limiting dilutions *i.e.* 10^5, $2X10^4$ and $4X10^3$ cells for LN CD4 T cell populations and $5X10^5$, 10^5, $2X10^4$ and $4X10^3$ 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 and were stimulated for 3 days with anti-CD3/anti-CD28 MAb coated plates (10 µg/mL) in presence of IL-2 (50 units/mL). Supernatants were collected at day 0, 5 and 14. Medium was replaced at day 5, and 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) in basement matrix buffer (RUWAG Handels AG). Of note, COBAS® AmpliPrep/TaqMan® HIV-1 Test can detect HIV-1 group O and group M, clades A-H. Wells with detectable P24 (≥ 1 ECL unit/mL) were referred to as P24-positive wells. Wells with detectable HIV-1 RNA (≥ 20 HIV-1 RNA copies/mL) were referred to as HIV-1 RNA-positive wells. RUPM [19] and IUPM[20] were calculated by conventional limiting dilution methods using Extreme Limiting Dilution analysis (http://bioinf.wehi.edu.au/software/elda/) [18]. 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 = [(% of memory CD4 T cell population A from compartment X among total memory CD4 T cells from compartment X) x (estimated RUPM freq. of memory CD4 T cell population A from compartment X)] x [% 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] / [(sum of contribution of memory CD4 T cell populations from compartment X to the pool of CD4 T cells containing replication competent virus) x 100]. Estimated contribution of memory CD4 T cell population A from compartment X to the pool of cells containing infectious virus in both blood and LN compartments = [(% of memory CD4 T-cell population A from compartment X among total memory CD4 T cells from compartment X) x (estimated IUPM freq. of memory CD4 T cell population A from compartment X)] x [% 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] / [(sum of contribution of memory CD4 T cell populations from compartment X to the pool of CD4 T cells containing infectious virus) x 100]. The estimated number of total blood and LN memory CD4 T cells was obtained from Ganusov *et al* [21].

***In vitro* HIV-1 infection assay**. CD8-depleted blood mononuclear cells isolated from HIV-uninfected individuals were activated for 48 hours with anti-CD3/anti-CD28 micro-beads (miltenyi) in complete RPMI medium supplemented with IL-2 (50 units/mL). Activated allogeneic CD8-depleted blood mononuclear cells (10^6 cells/mL) from HIV-uninfected individuals were washed and exposed (6 hours, 37°C, 5% CO_2) to 100 µl VOA supernatants collected at day 14. Following 6 hours 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 day 0, 5 and 14 post inoculations as assessed by 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 $5X10^4$ sorted CD4 T cell populations and subjected to DNase treatment (RNAqueous-4PCR Kit Ambion). RNA standard curves were generated after isolation and quantification of viral RNA from supernatant of ACH2 culture as previously described[26]. One step cDNA synthesis and pre-amplification were performed as previously described[27] using the following primers ULF1: 5'- *ATG CCA CGT AAG CGA AAC TC*T GGG TCT CTC TDG TTA GAC-3' UR1: 5'- CCA TCT CTC TCC TTC TAG C -3'. Real-time PCR was performed using

Roche light Cycler 480II using the following primers : LambdaT: 5'-*ATG CCA CGT AAG CGA AAC T*-3'; UR2: 5'- CTG AGG GAT CTC TAG TTA CC-3' and probes : 56-FAM 5'-CAC TCA AGG/ ZEN/CAA GCT TTA TTG AGG C-3' IABkFQ [28].

Histopathology. Lymph nodes were measured, cut into slices which were fixed in B-plus or formalin prior to routine processing and embedding in paraffin blocks. Four-micron sections were cut and stained with hematoxylin and eosin. For each lymph node serial sections of the most representative block were immunostained according to standard routine protocoles using a Ventana benchmark platform (Roche) using antibodies against CD20 (L26; Novocastra), CD3 (CD3-PS1; Leica), CD4 (SP35; Ventana), CD8 (C8/ 144B; DAKO), and PD1 (polyclonal Goat IgG, RD Systems). PD1-imunostained slides were digitalized using a Hamamatsu Nanozoomer 1.0 Scanner (model C9600-01) at 40x 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. In order to quantify PD1-positive cells, we used the Measure Stained Cells Algorithm at 20X which was set to take into account strongly stained ($PD1^+$) cells, and nuclei as for estimation of cell density.

Mass cytometry. Cryopreserved lymph node mononuclear cells were thawed, resuspended (10^6 cells/ml) in complete RPMI medium and stimulated or not with 100 ng/ml 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 fetal bovine serum. 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, CCR5, CXCR4, CXCR3 and CD57 (Fluidigm/DVS Science). Cells were washed and fixed (10 min; RT) with 2.4% 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 Ki67, Bcl-6, IFN-γ, TNF-α, IL-2, IL-21 (Fluidigm/DVS Science). Cells were washed and fixed (10 min; RT) with 2.4% PFA. Total cells were identified by DNA intercalation (1 µM Cell-ID Intercalator, Fluidigm/DVS Science) in 2% PFA at 4 °C overnight. 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 using two-tailed Chi-square analysis for comparison of positive proportions or 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 non-linear one-phase decay model were used for correlations. Finally, Statistical significance (*P* values) was obtained using Extreme Limiting Dilution analysis (http://bioinf.wehi.edu.au/software/elda/) or by unilateral or bilateral Z score[29] for comparison of HIV-1-infected cell frequencies 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.

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The authors declare no competing financial interests.

Author Contributions.

R.B., F.P., A.N. performed the experiments; G. Pollakis performed and analyzed HIV sequencing, M.C. was responsible for patient recruitment, 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.

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Figure legends

Figure 1. HIV replication and production of blood and LN memory CD4 T cell populations. (a) Schematic representation of the VOA. Levels of HIV-1 RNA in blood (**b**) or LN (**c**) memory CD4 T cell populations at day 5 and 14 (n = 11). Limit of detection of HIV-1 RNA was 20 HIV-1 RNA copies/ml. Undetectable values were arbitrarily defined as 10 HIV-1 RNA copies/ml. Levels of HIV-1 P24 in blood (**d**) or LN (**e**) memory CD4 T cell populations at day 5 and 14 (n = 11). Limit of detection of P24 was 1 ECL unit/ml. Correlation between the *ex vivo* percentage of LN memory CD4 T cell populations with the levels of HIV-1 RNA (**f**) or with the levels of HIV-1 P24 (**g**) produced in culture supernatants at day 14 of VOA of sorted LN memory CD4 T cell populations isolated from long-term treated aviremic HIV-1-infected individuals (n = 11). Correlation between the *ex vivo* percentage of LN $CXCR5^{high}PD\text{-}1^{high}$ memory CD4 T cell populations with the levels of HIV-1 RNA (**h**) or with the levels of HIV-1 P24 (**i**) produced in culture supernatants at day 14 of VOA of sorted LN $PD\text{-}1^{+}$ memory CD4 T cell populations isolated from long-term treated aviremic HIV-1-infected individuals (n = 11). CD4 T cell populations are color coded (**b-i**) and represented by a unique symbol (**b-e**). Red stars indicate statistical significance ($P < 0.05$) and was obtained using Wilcoxon Matched-pairs two-tailed Signed Rank test (**b-e**) or using Spearman rank test (**f-i**).

Figure 2. The levels of HIV-1 RNA and P24 from LN $PD\text{-}1^{+}$/Tfh cells inversely correlate with duration of treatment. (a) Correlation between levels of HIV-1 RNA and P24 produced in day 14 VOA supernatants of sorted LN memory CD4 T cell populations (n = 11). Correlation between duration of treatment and levels of HIV-1 RNA (**b**) or HIV-1 P24 (**c**) produced in day 14 VOA supernatants of sorted LN memory CD4 T cell populations (n = 11). Levels of HIV-1 RNA in blood (**d**) or LN (**e**) memory CD4 T cell populations at day 5 and 14 post HIV-1 *in vitro* infection assay (n = 11). Proportion of HIV-1 infected individuals with detectable HIV-1 RNA ($\geq$ 20 HIV-1 RNA copies/ml) (**f**) or levels of HIV-1 RNA (**g**) in blood and LN memory CD4 T cell populations at day 14 of *in vitro* HIV-1 infection assay (n = 11). (**h**) Correlation between HIV-1 RNA levels measured in VOA and in *in vitro* HIV-1 infection assay in supernatants of blood (**h**) or LN (**i**) memory CD4 T cell populations (n = 11). Undetectable values were arbitrarily defined as 10 HIV-1 RNA copies/ml (**a-b; d-e; g-i**). Histograms correspond to mean (**f-g**); red bars correspond to SEM (**g**). NA corresponds to not applicable (**h**). Red stars indicate statistical significance ($P < 0.05$) (**e-g**) and was obtained using Spearman rank test (**a-c h-i**),

597 Wilcoxon Matched-pairs two-tailed Signed Rank test **(d-e; g),** or using Chi square two tailed
598 analysis **(f)**.

599 **Figure 3. Detection of cell-associated HIV-1 RNA in LN PD-1$^+$/Tfh cells of long-term ART**
600 **treated aviremic HIV-1-infected individuals.** **(a)** Levels of cell-associated unspliced HIV-1
601 RNA (copies/million cells) in matched blood and LN CXCR5$^-$PD-1$^-$, CXCR5$^+$PD-1$^-$ and PD-1$^+$
602 memory CD4 T cell populations isolated from treated aviremic HIV-1-infected individuals (n =
603 9). HIV-1-infected individuals are color coded. **(b)** Correlation between duration of ART and
604 levels of cell-associated unspliced HIV-1 RNA of blood CXCR5$^-$PD-1$^-$, CXCR5$^+$PD-1$^-$ and PD-
605 1$^+$ memory CD4 T cell populations isolated from treated aviremic HIV-1 infected individuals (n
606 = 7). (c) Correlation between duration of ART and levels of cell-associated unspliced HIV-1
607 RNA of LN CXCR5$^-$PD-1$^-$, CXCR5$^+$PD-1$^-$ and PD-1$^+$ memory CD4 T cell populations isolated
608 from treated aviremic HIV-1-infected individuals (n = 9). HIV-1 infected individuals are color
609 coded **(a)**. CD4 T-cell populations are color coded **(b-c)**. Statistical significance (P values) was
610 obtained using One-way ANOVA (Kruskal-Wallis test) followed by Wilcoxon Matched-pairs
611 two-tailed Signed Rank test **(a)** or Spearman rank test for correlations **(b-c)**.

612 **Figure 4. Histopathological changes associated with long-term ART in LN tissues.** **(a)**
613 Representative examples of hematoxylin and eosin (upper panels) and anti-PD-1
614 immunohistochemistry (intermediate and lower panels) staining of LN of three treated aviremic
615 HIV-1-infected individuals (#92, #86 and #87) at low magnification (upper and intermediate
616 panels) and high magnification (lower panels). **(b)** Correlation between duration of ART and the
617 number of GCs per mm^2 of LN tissue of aviremic HIV-1-infected individuals (n = 5). **(c)**
618 Correlation between duration of ART and the number of PD-1-positive cells per mm^2 of LN
619 tissue of aviremic HIV-1-infected individuals (n = 5). Correlations (R^2) were calculated using a
620 non-linear one-phase decay model according to the GraphPad Prism. Blue dotted curves
621 correspond to the lower and upper confidence interval at 0.95.

622 **Figure 5. LN PD-1$^+$/Tfh cells of long-term treated aviremic HIV-1 infected individuals are**
623 **enriched in replication competent and infectious virus.** **(a)** Schematic representation of
624 estimation of the mean inducible RUPM and IUPM frequencies. Estimated frequencies of
625 inducible RUPM **(b)** and IUPM **(c)** frequencies in sorted blood and LN memory CD4 T cell
626 populations (n = 11). Estimated contribution of blood and LN memory CD4 T cell populations to
627 the pool of cells containing replication competent virus **(d)** or infectious virus **(e)** in both blood

628 and LN compartments (n = 11). Estimated contribution of memory CD4 T cell population A
629 from compartment X to the pool of cells containing replication competent or infectious virus in
630 both blood and LN compartments = [(% of population A from compartment X among total
631 memory CD4 T cells from compartment X) x (estimated RUPM or IUPM freq. of population A
632 from compartment X)] x [% of the estimated number of memory CD4 T cells present in
633 compartment X among the sum of the memory CD4 T cells present in both blood and LN
634 obtained from Ganusov *et al*[21]] / [(sum of contribution of memory CD4 T cell populations from
635 compartment X to the pool of CD4 T cells containing replication competent or infectious virus) x
636 100]. Histograms correspond to estimated mean (**b-e**) and red bars correspond to the lower and
637 upper confidence interval at 0.95 **(b-e)**. Red stars indicate statistical significance ($P < 0.05$) and
638 was obtained using ELDA, unilateral or bilateral Z score[29].

639 **Table 1.**

640

Patient ID	Age	Sex	Duration of HIV infection (years)	CD4 Cell Count at enrollment (cells/ul)	Viral Load at Enrollment (copies/ml)	Time on HAART (years)	HAART regimen*	Assays
03	52	F	21	899	<50	8	ATV/r,ABC,3TC	MC
04	60	M	3	424	<50	2	TDF,FTC, EFV	VOA
06	36	M	6	966	<50	3.9	ABC,3TC,ATV	CAR
07	36	M	6	760	<50	6	ATV/r,ABC,3TC	VOA
08	48	M	8	339	<50	2	ABC,3TC,EFV	VOA, MC
10	57	F	27	703	<50	12	TDF,FTC,LPV	MC
11	54	M	17	954	<50	12	ATV/r,FTC,TDF	VOA, CAR
12	41	F	9	1370	<50	5	ATV/r,FTC,TDF	VOA
14	37	M	7	1267	<50	2	TDF,FTC, EFV	VOA
23	55	M	10	430	<50	2	TDF,FTC, EFV	VOA
24	51	M	9	520	<50	3	TDF,FTC, EFV	VOA,CAR
25	55	M	15	500	<50	14	ETR,FTC,TDF	VOA
35	35	M	2	368	<50	1.5	ABC,3TC,EFV	VOA
39	42	F	9	459	<50	7	ATV/r,FTC,TDF	VOA
42	45	M	11	949	<50	7	ATV/r, TDF,FTC	CAR
60	43	F	22	692	<50	8.9	EVG,FTC,EVG	CAR, MC
64	34	M	8	712	<50	5	EVG,FTC,TDF	CAR
67	41	M	16	789	<50	12.9	ABC,3TC,DTG	CAR
68	44	F	4	668	<50	3.3	TDF,FTC,ETR	CAR
71	45	M	2	158	<50	1	TDF,FTC,DTG	CAR
78	38	M	6	509	<50	2.3	ATV/r,TDF,FTC,	MC
80	48	F	22	490	<50	1.2	LPV/r, 3TC,AZT	IHC
82	51	M	22	1064	<50	2	TDF, FTC, EFV,	MC
85	44	M	23	1112	<50	10	ATV,RAL,ABC, 3TC	IHC
86	47	M	15	1025	<50	8.4	ABC,3TC,EFV	IHC
87	56	M	13	593	<50	12.2	TDF,FTC,ATV/r	IHC
92	37	M	4	14	<50	0.3	ABC,DTG,3TC	IHC

641

642 *Abbreviations: ETR, etravirine; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate;
643 ATV/r, atazanavir boosted with ritonavir; 3TC, lamivudine; ABC, abacavir; EFV, Efavirenz;
644 EVG, Elvitegravir; RAL, Raltegravir; DTG, Dolutegrvir; LPV/r, Lopinavir boosted with
645 ritonavir; AZT, Zidovudine; VOA,Viral outgrowth assay; CAR, Cell-associated RNA; IHC,
646 immunohistochemistry; MC, mass cytometry; ND, not determined.

Figure 1

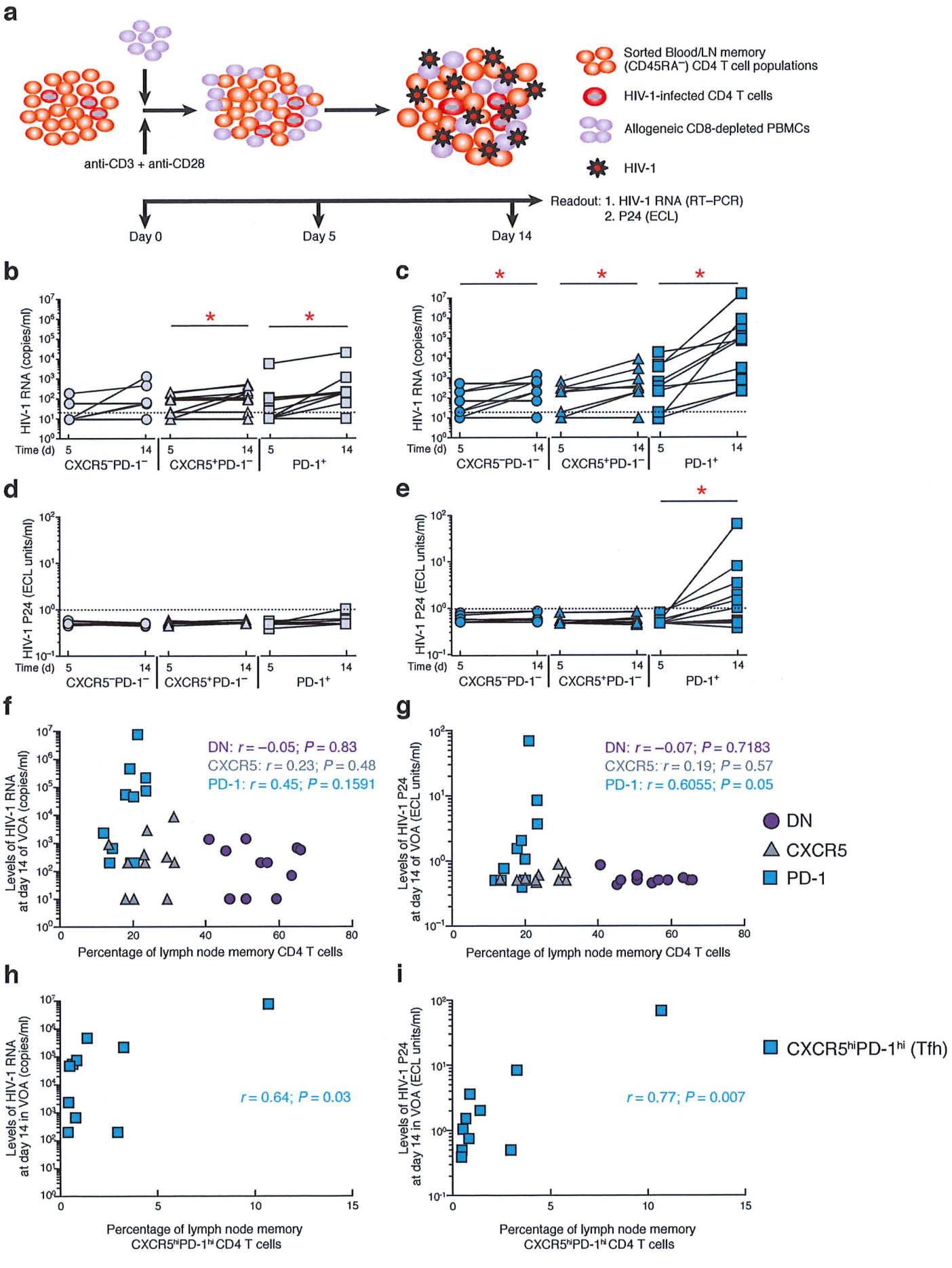

a
anti-CD3 + anti-CD28
Sorted Blood/LN memory (CD45RA⁻) CD4 T cell populations
HIV-1-infected CD4 T cells
Allogeneic CD8-depleted PBMCs
HIV-1
Readout: 1. HIV-1 RNA (RT–PCR)
2. P24 (ECL)
Day 0
Day 5
Day 14
b
c
HIV-1 RNA (copies/ml)
Time (d)
CXCR5⁻PD-1⁻
CXCR5⁺PD-1⁻
PD-1⁺
d
e
HIV-1 P24 (ECL units/ml)
f
g
Levels of HIV-1 RNA at day 14 of VOA (copies/ml)
Levels of HIV-1 P24 at day 14 of VOA (ECL units/ml)
DN: r = –0.05; P = 0.83
CXCR5: r = 0.23; P = 0.48
PD-1: r = 0.45; P = 0.1591
DN: r = –0.07; P = 0.7183
CXCR5: r = 0.19; P = 0.57
PD-1: r = 0.6055; P = 0.05
DN
CXCR5
PD-1
Percentage of lymph node memory CD4 T cells
h
i
Levels of HIV-1 RNA at day 14 in VOA (copies/ml)
Levels of HIV-1 P24 at day 14 in VOA (ECL units/ml)
r = 0.64; P = 0.03
r = 0.77; P = 0.007
CXCR5hiPD-1hi (Tfh)
Percentage of lymph node memory CXCR5hiPD-1hi CD4 T cells

Figure 2

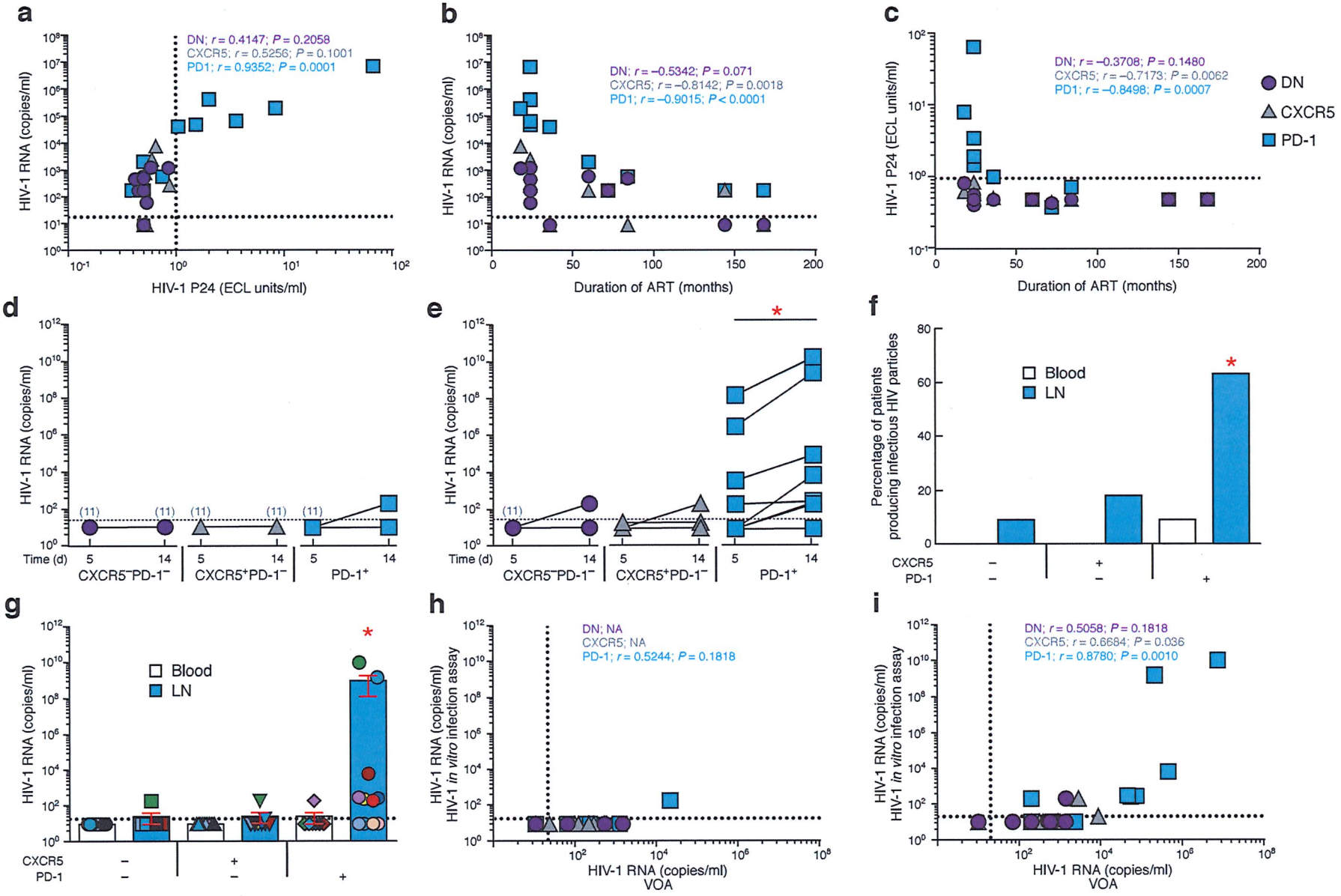

a
DN; r = 0.4147; P = 0.2058
CXCR5; r = 0.5256; P = 0.1001
PD1; r = 0.9352; P = 0.0001
HIV-1 RNA (copies/ml)
HIV-1 P24 (ECL units/ml)
b
DN; r = −0.5342; P = 0.071
CXCR5; r = −0.8142; P = 0.0018
PD1; r = −0.9015; P < 0.0001
HIV-1 RNA (copies/ml)
Duration of ART (months)
c
DN; r = −0.3708; P = 0.1480
CXCR5; r = −0.7173; P = 0.0062
PD1; r = −0.8498; P = 0.0007
DN
CXCR5
PD-1
HIV-1 P24 (ECL units/ml)
Duration of ART (months)
d
HIV-1 RNA (copies/ml)
(11)
Time (d) 5 14 5 14 5 14
CXCR5⁻PD-1⁻
CXCR5⁺PD-1⁻
PD-1⁺
e
*
HIV-1 RNA (copies/ml)
(11)
Time (d) 5 14 5 14 5 14
CXCR5⁻PD-1⁻
CXCR5⁺PD-1⁻
PD-1⁺
f
Blood
LN
*
Percentage of patients producing infectious HIV particles
CXCR5 − + −
PD-1 − − +
g
Blood
LN
*
HIV-1 RNA (copies/ml)
CXCR5 − + −
PD-1 − − +
h
DN; NA
CXCR5; NA
PD-1; r = 0.5244; P = 0.1818
HIV-1 RNA (copies/ml) HIV-1 in vitro infection assay
HIV-1 RNA (copies/ml) VOA
i
DN; r = 0.5058; P = 0.1818
CXCR5; r = 0.6684; P = 0.036
PD-1; r = 0.8780; P = 0.0010
HIV-1 RNA (copies/ml) HIV-1 in vitro infection assay
HIV-1 RNA (copies/ml) VOA

Figure 3

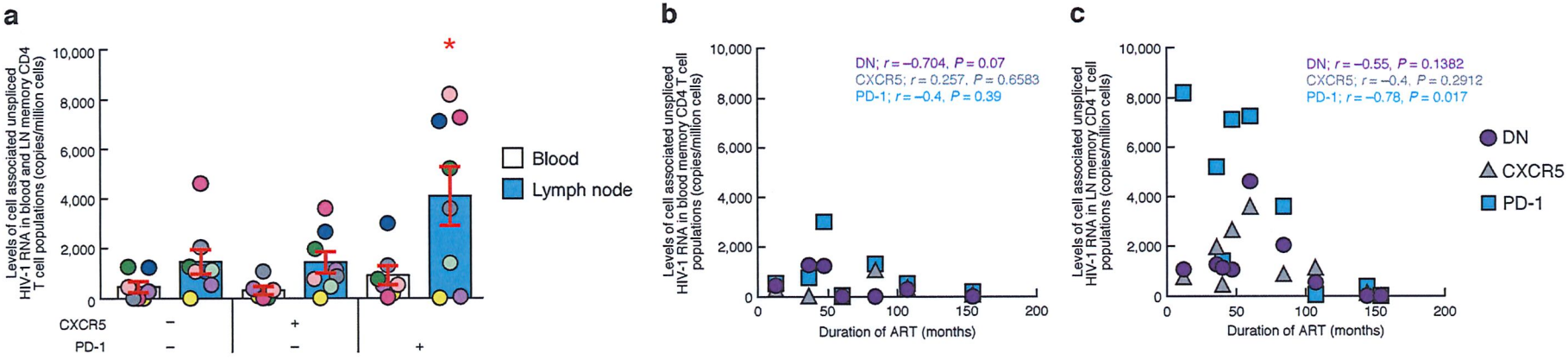

a
Levels of cell associated unspliced HIV-1 RNA in blood and LN memory CD4 T cell populations (copies/million cells)
10,000
8,000
6,000
4,000
2,000
0
CXCR5
PD-1
Blood
Lymph node
b
Levels of cell associated unspliced HIV-1 RNA in blood memory CD4 T cell populations (copies/million cells)
DN; r = –0.704, P = 0.07
CXCR5; r = 0.257, P = 0.6583
PD-1; r = –0.4, P = 0.39
Duration of ART (months)
c
Levels of cell associated unspliced HIV-1 RNA in LN memory CD4 T cell populations (copies/million cells)
DN; r = –0.55, P = 0.1382
CXCR5; r = –0.4, P = 0.2912
PD-1; r = –0.78, P = 0.017
DN
CXCR5
PD-1
Duration of ART (months)

Figure 4

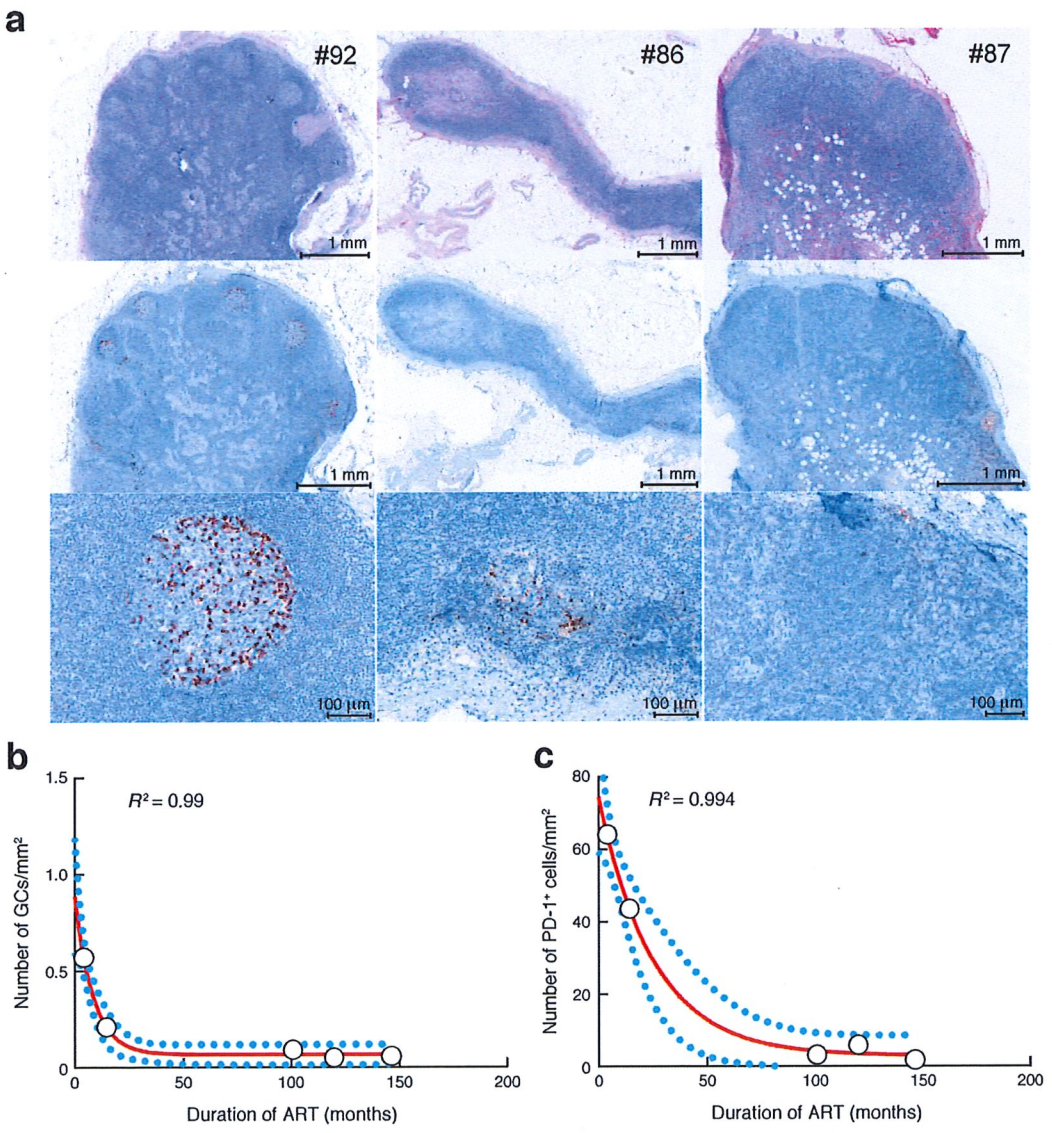
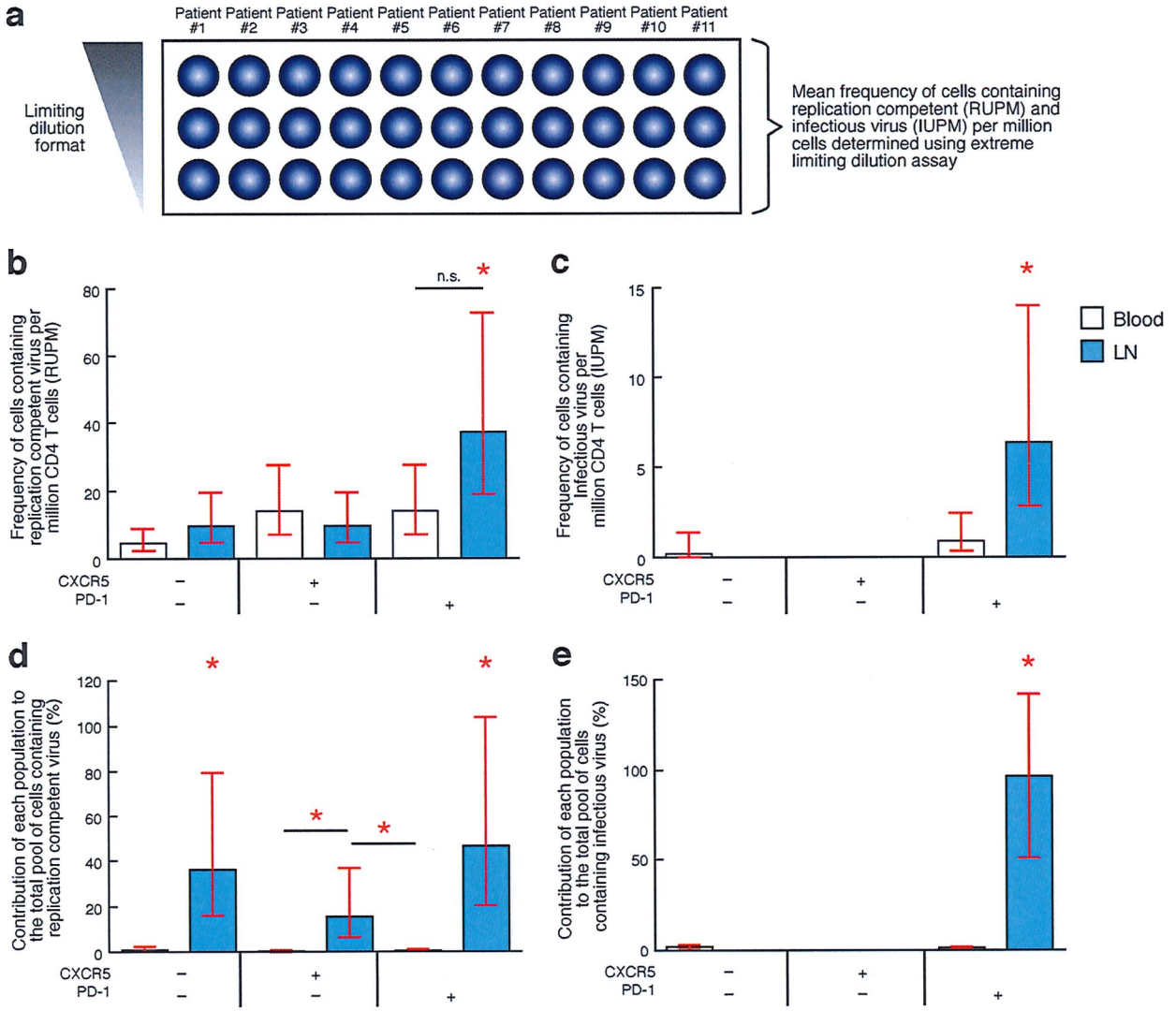

a
#92
#86
#87
1 mm
100 μm
b
$R^2 = 0.99$
Number of GCs/mm²
1.5
1.0
0.5
0
0
50
100
150
200
Duration of ART (months)
c
$R^2 = 0.994$
Number of PD-1⁺ cells/mm²
80
60
40
20
0
0
50
100
150
200
Duration of ART (months)

Figure 5


a
Patient #1
Patient #2
Patient #3
Patient #4
Patient #5
Patient #6
Patient #7
Patient #8
Patient #9
Patient #10
Patient #11
Limiting dilution format
Mean frequency of cells containing replication competent (RUPM) and infectious virus (IUPM) per million cells determined using extreme limiting dilution assay
b
Frequency of cells containing replication competent virus per million CD4 T cells (RUPM)
n.s.
*
0
20
40
60
80
CXCR5
PD-1
c
Frequency of cells containing Infectious virus per million CD4 T cells (IUPM)
*
0
5
10
15
Blood
LN
d
Contribution of each population to the total pool of cells containing replication competent virus (%)
*
*
*
0
20
40
60
80
100
120
e
Contribution of each population to the total pool of cells containing infectious virus (%)
*
0
50
100
150