HIV-1 persistence in CD4+ T cells with stem cell-like properties

Published in final edited form as:
Nat Med. 2014 February ; 20(2): 139–142. doi:10.1038/nm.3445.

HIV-1 persistence in CD4+ T cells with stem cell-like properties

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Abstract

Cellular HIV-1 reservoirs that persist despite antiretroviral treatment are incompletely defined. We show that during suppressive antiretroviral therapy, $CD4^+$ T memory stem cells (T_{SCM}) harbor high per-cell levels of HIV-1 DNA, and make increasing contributions to the total viral $CD4^+$ T cell reservoir over time. Moreover, phylogenetic studies suggested long-term persistence of viral quasispecies in $CD4^+$ T_{SCM} cells. Thus, HIV-1 may exploit stem cell characteristics of cellular immune memory to promote long-term viral persistence.

Antiretroviral combination therapy effectively suppresses HIV-1 replication, but replication-competent virus can persist in memory $CD4^+$ T cells despite treatment [1–2]. The memory $CD4^+$ T cell compartment includes central-memory (T_{CM}), effector-memory (T_{EM}) and terminally-differentiated (T_{TD}) cells, which most likely evolve through a sequential developmental program with progressive commitment to more differentiated cell types[3–4]. The presence of a more immature memory T cell population with stem cell-like properties has previously been hypothesized based on experimental animal studies[5–9], and recently, small proportions of T cells with stem cell characteristics have been discovered in humans[10–11], mice[12] and non-human primates[13]. These cells, termed "T memory stem cells" (T_{SCM}), seem to represent the earliest developmental stage of memory T cells, and can

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Writing of manuscript: MJB, ML
Performance of experiments: MJB, HS, EMG, JL, JZL, TJH,
Data analysis and interpretation: MJB, XGY, ML
Contribution of PBMC samples: FP, BDW, ESR
Technical assistance: CL, AS, KS
Biostatistical assistance RZ, ZO
Critical review of manuscript: JZL, TJH, BDW, ESR, XGY

differentiate into large numbers of effector T cells, while maintaining their own pool size through homeostatic self-renewal. We hypothesized that HIV-1 can use $CD4^+$ T_{SCM} cells as a preferred niche for promoting long-term viral persistence.

To test this concept, we initially investigated the susceptibility of $CD4^+$ T_{SCM} cells to HIV-1 infection. These experiments demonstrated that $CD4^+$ T_{SCM} cells, phenotypically defined as described in previous studies[10,14] and in Supplementary Figure 1, were approximately as susceptible as $CD4^+$ T_{CM} cells to infection with an R5-tropic HIV-1 isolate (Fig. 1a), although their surface expression of CCR5 was slightly lower (Supplementary Fig. 2a/b). In addition, $CD4^+$ T_{SCM} cells were highly susceptible to infection with a VSV-G pseudotyped HIV-1 construct (Fig. 1a, Supplementary Fig. 3), despite comparatively low expression of T cell activation makers (Supplementary Fig. 4). We also observed that HIV-1 RNA was readily detectable in $CD4^+$ T_{SCM} cells from untreated HIV-1 patients (Supplementary Fig. 2c). $CD4^+$ T_{SCM} cells had low sensitivity to cytopathic effects associated with HIV-1 infection (Supplementary Fig. 2d), and expressed reduced levels of the cell-intrinsic HIV-1 restriction factors TRIM5α, APOBEC3G and SAMHD1 (Supplementary Fig. 2e). Together, these data indicate that $CD4^+$ T_{SCM} cells are permissive to HIV-1 infection, and can serve as physiologic target cells for HIV-1.

We next determined the levels of HIV-1 DNA in sorted $CD4^+$ T_{SCM} cells from HIV-1 infected patients, who had been treated with suppressive antiretroviral therapy for a median of 7 years (Supplementary Table). Proportions of $CD4^+$ T_{SCM} cells in these patients did not differ from an HIV-1 negative control cohort (Supplementary Fig. 5). In these HAART-treated patients, per-cell levels of HIV-1 DNA were highest in $CD4^+$ T_{SCM} cells, but their average contribution to the total viral $CD4^+$ T cell reservoir was only approximately 8% (Fig. 1b). Interestingly, the contribution of $CD4^+$ T_{SCM} cells to the total viral reservoir in $CD4^+$ T cells varied considerably among different HAART-treated patients, and was inversely associated with HIV-1 DNA levels in the entire $CD4^+$ T cell compartment (Fig. 1c). Such a negative association was selectively observed for the $CD4^+$ T_{SCM} cell compartment (Supplementary Fig. 6) and resulted in a disproportionately increased contribution of $CD4^+$ T_{SCM} cells to the total viral reservoir in patients with a smaller viral reservoir in $CD4^+$ T_{CM} and T_{EM} cells. This suggests that HIV-1-infected $CD4^+$ T_{SCM} cells represent one of the most stable and durable components of the viral $CD4^+$ T cell reservoir that becomes increasingly visible when viral reservoirs in alternative $CD4^+$ T cell subsets decline. HIV-1 DNA was also detectable in $CD4^+$ T_{SCM} cells from elite controllers[15], although at significantly lower levels than in HAART-treated patients (Supplementary Fig. 7).

Since only a small proportion of $CD4^+$ T cell-associated HIV-1 DNA encodes for replication-competent virus[16], we performed viral outgrowth assays from three study subjects who had been on continuous suppressive antiretroviral combination therapy for a median of 28 months (range 14–42 months). These studies demonstrated that replication-competent virus was retrievable from $CD4^+$ T_{SCM} cells in all three cases, and that the estimated frequency of cells harboring replication-competent HIV-1 in $CD4^+$ T_{SCM} cells exceeded the corresponding frequencies in $CD4^+$ T_{CM} and T_{EM} cells in two of the three patients (Fig. 1d). These findings indicate that HIV-1 DNA in $CD4^+$ T_{SCM} cells is functionally capable of resuming active viral gene expression.

Due to their stem cell-like properties, $CD4^+$ T_{SCM} cells may represent a privileged site for long-term viral persistence. To better investigate this, we longitudinally analyzed HIV-1 DNA in sorted $CD4^+$ T cell subsets from eight individuals who started antiretroviral therapy in primary infection, and then remained on suppressive antiretroviral therapy without treatment interruptions. Using pair-wise comparisons between cell-associated HIV-1 DNA

during earlier stages of antiretroviral therapy (median of 1 year, range: 10–14 months) and during later stages of treatment (median of nine years, range 7–11 years), we observed stable or mildly decreasing viral DNA in $CD4^+$ T_{SCM} cells; viral DNA decline in $CD4^+$ T_{CM} and $CD4^+$ T_{NA} cells was slightly more pronounced (Fig. 1e). In contrast, in the more short-lived $CD4^+$ T_{EM} and T_{TD} cell populations, a significant reduction in per-cell levels of total HIV-1 DNA was noticed over time. Notably, among all $CD4^+$ T cell subsets, the relative longitudinal decline in total HIV-1 DNA at per-cell levels was smallest in $CD4^+$ T_{SCM} cells, although differences between $CD4^+$ T_{SCM} cells and $CD4^+$ T_{NA}/T_{CM} cells did not reach statistical significance in our small study cohort (Fig. 1f). Interestingly, we observed that $CD4^+$ T_{SCM} cells made a relatively small contribution to the total $CD4^+$ T cell reservoir after the first year of suppressive antiretroviral therapy (Fig. 1g). Yet, after long-term antiretroviral treatment, there was a significant increase in the contribution of $CD4^+$ T_{SCM} cells to the total viral reservoir in $CD4^+$ T cells, despite the fact that the numeric contribution of $CD4^+$ T_{SCM} cells to the total $CD4^+$ T cell pool did not change. The contribution of $CD4^+$ T_{CM} cells to the total viral $CD4^+$ T cell reservoir also slightly increased over time, but this did not reach the level of statistical significance. In contrast, the contribution of $CD4^+$ T_{EM} cells to the viral $CD4^+$ T cell reservoir declined, despite a numerically increased proportion of T_{EM} cells in the total $CD4^+$ T cell pool (Fig. 1g). These data, although collected from a limited number of patients, suggest that $CD4^+$ T_{SCM} cells can support long-term viral persistence in patients treated with HAART.

We subsequently performed proviral *Env* sequencing in DNA samples isolated from longitudinally sorted $CD4^+$ T cell subsets of three HIV-1 patients who remained antiretroviral therapy-naïve during the initial years of disease, followed by continuous treatment with suppressive antiretroviral agents (Fig. 2a). We observed substantial variability between viral sequences from $CD4^+$ T_{SCM} cells collected at the beginning of antiretroviral therapy and again several years later, likely reflecting sampling of cells infected with different circulating viral strains during early disease stages (Fig. 2b). Yet, in $CD4^+$ T_{SCM} and $CD4^+$ T_{CM} cells (which were sampled in approximately 10–30-fold higher frequencies than $CD4^+$ T_{SCM} cells), we noticed several identical HIV-1 sequences in samples collected at the beginning of antiretroviral therapy and after 4–8 years of continuous treatment, consistent with long-term viral persistence in these $CD4^+$ T cell subsets (Fig. 2b). Interestingly, we also observed that plasma sequences from early untreated disease stages were phylogenetically most closely related to HIV-1 DNA isolated from $CD4^+$ T_{SCM} and T_{CM} cells collected 6–12 years later, suggesting that viral strains circulating in early infection can persist long-term upon infection of these memory $CD4^+$ T cell subsets (Fig. 2c). In addition, pair-wise sequence comparisons revealed that the genetic distance between early HIV-1 RNA plasma sequences and HIV-1 DNA sequences from $CD4^+$ T cell subsets collected during later stages of infection was lowest for $CD4^+$ T_{SCM} and $CD4^+$ T_{CM} cells (Supplementary Fig. 8). Sequences from $CD4^+$ T_{SCM} cells also showed frequent phylogenetic associations with contemporaneous and ensuing plasma sequences isolated during suppressive antiretroviral therapy, consistent with a possible interchange between viral strains in $CD4^+$ T_{SCM} cells and circulating viral species (Fig. 2c). Finally, we noted viral sequences from $CD4^+$ T_{SCM} cells that were identical to those from $CD4^+$ T_{CM}, T_{EM} and T_{TD} cells isolated several years later, supporting the role of $CD4^+$ T_{SCM} cells as precursor cells for more differentiated $CD4^+$ T cell subsets (Fig. 2c). Although these phylogenetic studies were performed in a limited number of patients, they emphasize the role of $CD4^+$ T_{SCM} and T_{CM} cells as a long-term reservoir for HIV-1.

This study indicates that despite their small frequencies, $CD4^+$ T_{SCM} cells stand out among other memory $CD4^+$ T cell subsets as the cell population in which long-term HIV-1 persistence is particularly evident, likely due to intrinsic cellular programs of these cells that maintain superior abilities to self-renew, resist apoptosis and survive for extremely long

periods of time[10,13]. Interestingly, pharmaceutical inhibition of stem-cell specific molecular pathways is being investigated for targeting cancer stem cells[17], and the specific targeting of cellular pathways responsible for stem cell-like properties of $CD4^+$ T_{SCM} cells may also have adjunct or additive effects on reducing persistence of HIV-1 infected $CD4^+$ T_{SCM} cells. Thus, optimism may be warranted that our increasing understanding of how stem cell-like properties of cellular immune memory maintain HIV-1 persistence despite HAART can be translated into improved clinical strategies for inducing HIV-1 eradication and cure[18].

Patients and Methods

Patients

PBMC samples from HIV-infected individuals were used for this study according to protocols approved by the Institutional Review Board of Massachusetts General Hospital in Boston. All study participants gave written informed consent.

Cell sorting and flow cytometry

$CD4^+$ T_{SCM} cells and other $CD4^+$ T cell subsets were isolated according to a previously described protocol[14] with minor modifications. At least 100 Million PBMC were stained with monoclonal antibodies directed against CD4, CD3, CD45RA, CCR7, CD62L, CD122, CD95. After 20 minutes, $CCR7^+$ $CD45RA^+$ naïve $CD4^+$ T cells, $CCR7^+$ $CD45RA^-$ central-memory $CD4^+$ T cells, $CCR7^-$ $CD45RA^-$ effector-memory $CD4^+$ T cells, $CCR7^-$ $CD45RA^+$ terminally-differentiated $CD4^+$ T cells and $CCR7^+$ $CD45RA^+$ $CD62L^+$ $CD95^+$ $CD122^+$ $CD4^+$ T memory stem cells were live-sorted in a specifically designated biosafety cabinet (Baker Hood), using a FACS Aria cell sorter (BD Biosciences) at 70 pounds per square inch. Cell sorting was performed by the Ragon Institute Imaging Core Facility at Massachusetts General Hospital, and resulted in isolation of live lymphocytes with the defined phenotypic characteristics of >95% purity, as determined by three dedicated experiments in which sorted cells were subjected to repeat flow cytometric analysis (Supplementary Fig. 1b). For phenotypic characterization, cells were additionally stained with CCR5, CXCR4, CD38 or HLA-DR antibodies or Annexin V, and acquired on a LSRII flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Treestar).

Assessment of cell-associated HIV-1 DNA

Isolated $CD4^+$ T cells were digested as previously described[2] to extract cell lysates. We amplified total HIV-1 DNA with primers and probes previously described[19]. As a standard curve, we amplified serial dilutions of chronically infected 293T cells (kindly provided by Dr. Bushman, University of Pennsylvania). Proviral HIV-1 DNA copy numbers were calculated relative to CCR5 gene copy numbers according to standard procedures.

Analysis of cell-associated HIV-1 RNA

Cell-associated HIV-1 RNA in sorted $CD4^+$ T cells was quantified by real-time RT-PCR, using primers and probes previously described[20]. HIV-1 RNA copy numbers were determined according to a standard HIV-1 RNA sample run in serial dilutions, and final results were expressed as the number of HIV-1 RNA copies per microgram of total RNA. The assay used had a detection threshold of 1 HIV-1 RNA copy/μg of total RNA.

Gene expression analysis

Expression of selected gene transcripts in individual $CD4^+$ T cell subsets was analyzed by semiquantitative RT-PCR using Taqman gene expression assays with standardized primers/probes, and normalized to the expression of the housekeeping gene Actb (encoding β-actin) in each $CD4^+$ T cell subset.

In vitro HIV-1 infection assays

Unselected PBMC from HIV-1 negative donors were cultured in RPMI medium supplemented with 10% FCS and 50 U/ml of rhIL-2. A total of 10×10^6 PBMCs were infected with a GFP-encoding VSV-G-pseudotyped virus (MOI=1, unless otherwise indicated) or a GFP-encoding R5-tropic viral strains (Ba-L, MOI=1, both isolates kindly provided by Dr. Littman, New York University). Cells were then washed twice with PBS and cultured at 200,000 cells/well in 96-well round-bottom plates for 5 days. On day 5, cells were stained with surface antibodies to identify individual $CD4^+$ T cell subsets, washed and analyzed on a LSRII flow cytometer.

Analysis of HIV-1 replication products

HIV-1 reverse transcripts were amplified from cell lysates with primers hRU5-F2 and hRU5-R and probe hRU5-P (early RT products), or with primers GagF1 and GagR1 and probe P-HUS-103 (late RT products)[21]. Integrated HIV-1 DNA was detected using nested PCR with Alu-1/Alu-2 primers and HIV-1 LTR primer L-M667 for the first-round PCR and LTR primer AA55M, Lambda T primers, and MH603 probe for the second-round quantitative PCR, as described previously[22]. Serial dilutions of HIV-1 DNA from cell lysates of the HIV-1–infected cell line 293T (provided by F. Bushman, University of Pennsylvania, Philadelphia, PA, USA) were used for reference purposes. Proviral HIV-1 DNA copy numbers were calculated relative to the CCR5 gene previously quantified with the same standard curve. 2-LTR HIV-1 DNA was quantified as previously described[23].

Viral outgrowth assays

Sorted $CD4^+$ T cell populations were seeded at 10,000 cells/well (T_{SCM} cells) or 20,000 cells/well (T_{CM} and T_{EM} cells) in round-bottom 96-well plates. Subsequently, cells were stimulated with PHA (2μg/ml), rh IL-2 (100 units/ml) and irradiated allogeneic PBMCs from HIV-negative healthy donors. CD8-depleted, PHA-stimulated PBMC from HIV-negative donors were added to each well on day 3 and again on day 7 and 14 of culture. Latently HIV-1 infected ACH-2 cells were run as positive control cells, and $CD4^+$ cell-depleted PBMC samples from HAART-treated patients that were otherwise treated identically served as negative controls. The cultures were subjected to removal of 33% of the cell suspension every seven days and replenished with fresh rhIL-2-containing media. After 14–21 days, cell supernatant from each well was harvested and the number of wells containing infectious HIV-1 was assessed by incubation of the supernatant with TZM-bl cells, a permissive HeLa cell clone that contains HIV-1 Tat-responsive reporter genes for firefly luciferase under control of the HIV-1 LTR, permitting sensitive and accurate measurements of infection. Luciferase activity was quantified by luminescence and is directly proportional to the number of infectious virus particles present in the initial inoculum. Estimated frequencies of cells with replication-competent HIV-1 were calculated using limiting dilution analysis as described in[24]; all data were consistent with a single-hit Poisson distribution, as determined using a goodness-of-fit analysis based on a likelihood ratio test[24].

Viral sequencing

Cell lysates from sorted T cell populations and plasma were used for HIV-1 envelope sequencing encompassing the V3 region. For plasma samples, 6 mL of plasma from each time point were ultracentrifuged at 170,000g for 30 min prior to proteinase K digestion and RNA isolation by acid guanidinium isothiocyanate. One-step RT-PCR reaction was then performed in triplicates using outer primers envA/LA17[25]. PCR products were used as a template to generate an amplicon by nested PCR with inner primers LA12 and LA13[25]. For V3 amplification from HIV-1 DNA in cell lysates, two-step nested PCR was performed with

the same primer pairs. For amplification of HIV-1 RNA and DNA sequences, 2–4 separate reactions were conducted for each sample during first-round PCR; these PCR products were then pooled and used as templates for second-round PCR. Amplification products were inserted into TOPO cloning vectors, and used to transform competent bacteria. Individual bacterial colonies were amplified by overnight culture, and extracted DNA was ligated and directly sequenced by T7 or T3 primers on an ABI 3100 PRISM automated sequencer, without prior PCR-based amplification. Sequences were aligned with an HXB2 reference sequence using BioEdit V7.1.9. A neighbour-joining method, as implemented in MEGA4[26], was used to construct phylogenetic trees with phylogenetically informative HIV-1 nucleotide sequences. These sequences omit nucleotide mutations that occur only once and may therefore possibly be introduced by polymerase-induced errors during PCR[27]. Phylogenetically informative sites were identified as described before (http://indra.mullins.microbiol.washington.edu/DIVEIN/insites.html). This conservative approach may slightly underestimate nucleotide diversity relative to single-template amplification methods, but a direct comparison between HIV-1 sequences derived by PCR/cloning and single-genome amplification in a number of our samples demonstrated equivalent population structure (Supplementary Fig. 9), consistent with prior studies[28]. For comparison purposes, viral sequences were analyzed by single genome amplification according to a protocol described before[29].

Statistics

Data are summarized as individual data plots with horizontal lines reflecting the median, or as box and whisker plots indicating the median, interquartile range, and minimum and maximum values. Spearman's correlation coefficient was calculated to analyze correlations. Differences were tested for statistical significance using Wilcoxon rank sum tests, Mann-Whitney U test, Kruskal-Wallis or Fisher's exact test, followed by Bonferroni correction or Dunn's test for multiple comparisons where applicable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the American Foundation for AIDS Research (grant 108302-51-RGRL to ML) and by the National Institutes of Health (grants AI098487 and AI106468 to ML, AI089339 to XGY, AI098480 to TJH, AI100699 to JZL). ML is a recipient of the Clinical Scientist Development Award from the Doris Duke Charitable Foundation (grant number 2009034). MJB is supported by a fellowship award from the European Molecular Biology Laboratory and by the Tosteson postdoctoral fellowship award from Massachusetts General Hospital. Patient blood sample collection was supported by the National Institutes of Health (grant AI074415), by the Mark and Lisa Schwartz Foundation and the Bill and Melinda Gates Foundation.

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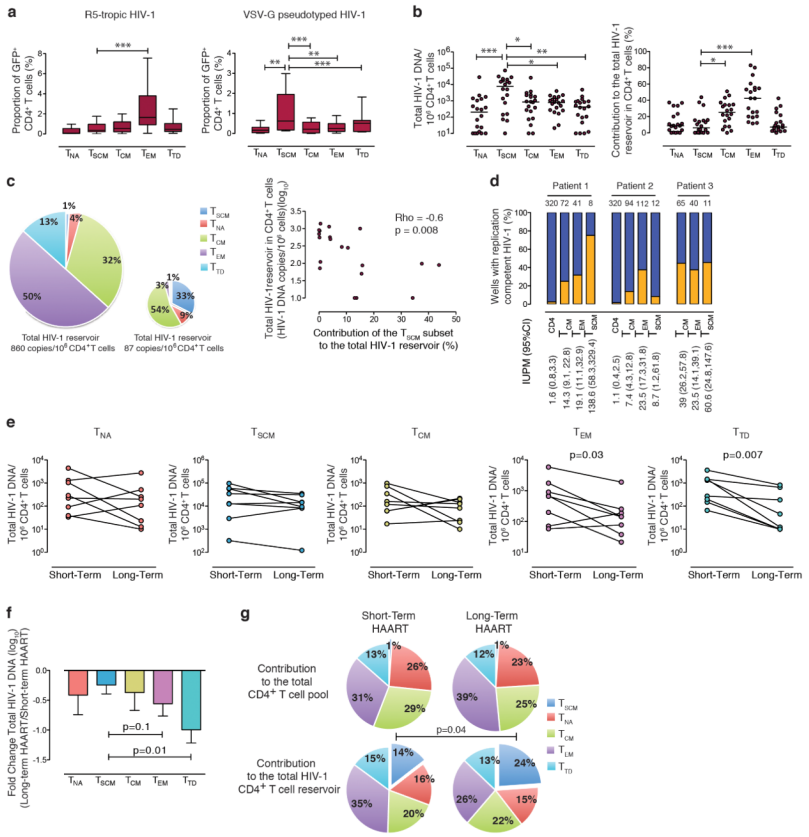

Figure 1. CD4$^+$ T_{SCM} cells represent a long-term reservoir for HIV-1 in HAART-treated patients

(**a**): Proportion of HIV-1 infected cells after ex-vivo infection with GFP-encoding R5-tropic or VSV-G pseudotyped HIV-1 (R5: n=17, VSV-G: n=14). (**b**) Cell-associated HIV-1 DNA in sorted CD4$^+$ T cell populations (left panel) and corresponding contributions to the total HIV-1 reservoir in CD4$^+$ T cells from HAART-treated individuals (right panel). (**c**): Left panel: Representative pie charts reflecting the contribution of CD4$^+$ T_{SCM} cells to the total viral CD4$^+$ T cell reservoir in two persons with large and small HIV-1 reservoirs in total CD4$^+$ T cells, respectively. Right panel: Spearman correlation between contributions of CD4$^+$ T_{SCM} cells to the total HIV-1 CD4$^+$ T cell reservoir, and corresponding size of the HIV-1 reservoir in total CD4$^+$ T cells. (**d**): Reactivation of replication-competent HIV-1 from memory CD4$^+$ T cell subsets. Orange bars reflect proportions of wells with detectable replication-competent HIV-1, blue bars indicate proportions of wells without detectable replication-competent HIV-1. Numbers above columns reflect total numbers of wells analyzed for each CD4$^+$ T cell population; numbers below columns reflect estimated frequencies of cells with replication-competent HIV-1 per million cells (IUPM) based on limiting-dilution analysis. (**e**): Longitudinal evolution of HIV-1 DNA in CD4$^+$ T cell subsets in n=8 study persons who initiated antiretroviral therapy in primary infection. (**f**): Pair-wise fold-differences in HIV-1 DNA measured after short-term and long-term antiretroviral therapy. Mean and standard error are shown. (**g**): Corresponding contribution of individual CD4$^+$ T cell subsets to the total CD4$^+$ T cell pool, and to the total HIV-1 CD4$^+$ T cell HIV-1 reservoir after short-term and long-term antiretroviral therapy. Statistical significance was tested with Wilcoxon rank sum test. *,**, *** reflect p<0.05, p<0.01, p<0.001, respectively, after Bonferroni correction for multiple comparisons in panel a and b.

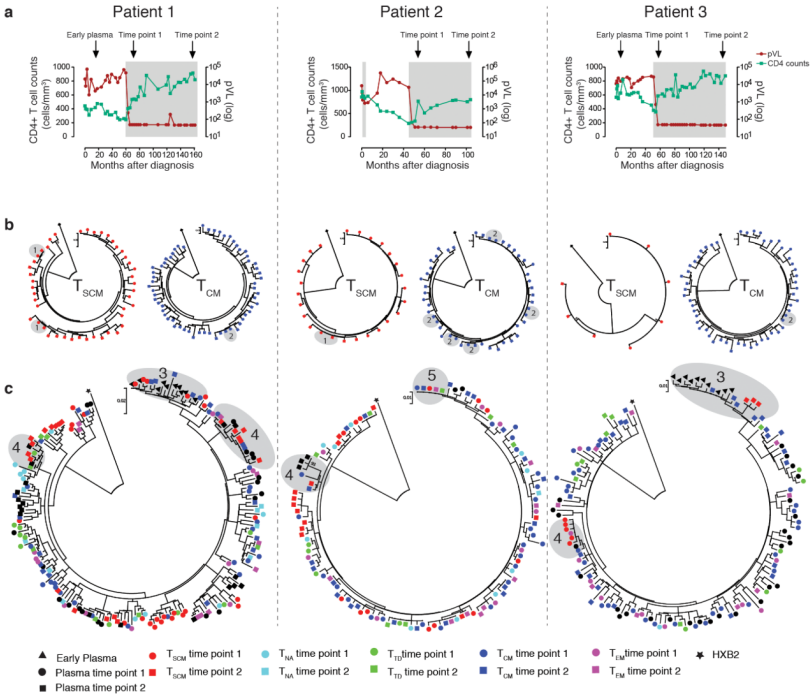

Figure 2. Phylogenetic analysis of HIV-1 sequences isolated from CD4$^+$ T$_{SCM}$ cells

(**a**): Longitudinal evolution of CD4$^+$ T cell counts and viral loads in the three study patients. Shaded areas reflect periods of antiretroviral treatment exposure. Arrows indicate time of CD4$^+$ T cell and plasma sampling. (**b**): Phylogenetic analysis of HIV-1 sequences longitudinally amplified from sorted CD4$^+$ T$_{SCM}$ and TCM cells at the beginning of antiretroviral treatment initiation, and after 4–8 years of continuous suppressive therapy in three study persons. Identical HIV-1 sequences in T$_{SCM}$ cells (1) and TCM cells (2) are highlighted by gray circles. (**c**): Circular phylogenetic trees of HIV-1 sequences amplified from indicated CD4$^+$ T cell subsets and from plasma collected at indicated timepoints. Gray circles reflect: Phylogenetic relationships between HIV-1 DNA sequences from CD4$^+$ T$_{SCM}$ cells and circulating HIV-1 viral RNA sequences isolated during early untreated disease (3) or during contemporaneous and ensuing timepoints (4). Identical HIV-1 sequences isolated from CD4$^+$ T$_{SCM}$, and from CD4$^+$ TCM, TEM, TTD cells isolated at later timepoints, are also highlighted (5).