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Th1/17 Polarization of CD4 T Cells Supports HIV-1 Persistence during Antiretroviral Therapy

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ABSTRACT

The ability to persist long term in latently infected CD4 T cells represents a characteristic feature of HIV-1 infection and the predominant barrier to efforts aiming at viral eradication and cure. Yet, increasing evidence suggests that only small subsets of CD4 T cells with specific developmental and maturational profiles are able to effectively support HIV-1 long-term persistence. Here, we analyzed how the functional polarization of CD4 T cells shapes and structures the reservoirs of HIV-1-infected cells. We found that CD4 T cells enriched for a Th1/17 polarization had elevated susceptibilities to HIV-1 infection in *ex vivo* assays, harbored high levels of HIV-1 DNA in persons treated with antiretroviral therapy, and made a disproportionately increased contribution to the viral reservoir relative to their contribution to the CD4 T memory cell pool. Moreover, HIV-1 DNA levels in Th1/17 cells remained stable over many years of antiretroviral therapy, resulting in a progressively increasing contribution of these cells to the viral reservoir, and phylogenetic studies suggested preferential long-term persistence of identical viral sequences during prolonged antiretroviral treatment in this cell compartment. Together, these data suggest that Th1/17 CD4 T cells represent a preferred site for HIV-1 DNA long-term persistence in patients receiving antiretroviral therapy.

IMPORTANCE

Current antiretroviral therapy is very effective in suppressing active HIV-1 replication but does not fully eliminate virally infected cells. The ability of HIV-1 to persist long term despite suppressive antiretroviral combination therapy represents a perplexing aspect of HIV-1 disease pathogenesis, since most HIV-1 target cells are activated, short-lived CD4 T cells. This study suggests that CD4 T helper cells with Th1/17 polarization have a preferential role as a long-term reservoir for HIV-1 infection during antiretroviral therapy, possibly because these cells may imitate some of the functional properties traditionally attributed to stem cells, such as the ability to persist for extremely long periods of time and to repopulate their own pool size through homeostatic self-renewal. These observations support the hypothesis that HIV-1 persistence is driven by small subsets of long-lasting stem cell-like CD4 T cells that may represent particularly promising targets for clinical strategies aiming at HIV-1 eradication and cure.

Although antiretroviral therapy (ART) is highly effective in suppressing HIV-1 replication and clinical HIV-1 disease manifestations, small reservoirs of HIV-1-infected cells persist despite treatment and can lead to rapid viral rebound once treatment is stopped (1, 2). Identifying the functional and phenotypic characteristics of cell subsets that harbor replication-competent virus during suppressive antiretroviral therapy is a critical step for developing interventional strategies to target residual viral reservoirs. Most available evidence supports the notion that latently infected CD4 T cells containing chromosomally integrated but transcriptionally silent HIV-1 DNA represent the most dominant cell population responsible for HIV persistence despite treatment (3–5). However, it is increasingly recognized that such cells represent a phenotypically diverse cell population that consists of a variety of different cell subsets with distinct developmental profiles and functional properties (6–10), divergent levels of permissiveness to HIV-1 infection and latency, and, most likely, different susceptibilities to clinical strategies or pharmaceutical agents aiming at reversing viral latency and inducing HIV-1 eradication.

From a developmental perspective, memory CD4 T cell evolution can be described as a hierarchical process during which immature, long-lived cells undergo progressive commitment to more differentiated cell types (11). Recent data suggest that the initial, most immature population of memory CD4 T cells consists of a small number of extremely long-lasting cells that phenotypically express a mix of naive and memory cell markers and display superior abilities to self-renew through homeostatic proliferation, while replenishing the pool of more mature memory cells in a stem cell-like fashion (12–15). These cells, termed T memory stem

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TABLE 1 Clinical and demographic characteristics of the study patients

Characteristic	Value for ART-treated HIV-infected patients ($n = 15$)
Median age, yr (range)	52.5 (36.0–63.0)
No. of males/females	10/5
Median cumulative mo of antiretroviral therapy (range)	87 (30–175)
Median CD4 T cell count, cells/μl (range)	942.0 (555.0–1,475.0)
Viral load (log_{10} copies/ml)	<50

cells (T_{SCM}), then give rise to central-memory cells (T_{CM}), which typically represent the largest compartment within the CD4 T cell pool and can serve as precursor cells for effector-memory cells (T_{EM}) specializing in direct antimicrobial immune defense. In addition to this developmental hierarchy, CD4 T cells also differ from a perspective of functional polarization. Polarization of CD4 T cells occurs under the influence of cytokines that induce specific functional profiles characterized by expression of signature cytokines, transcription factors, and phenotypic surface markers (16, 17). Th1 cells, which are associated with surface expression of CXCR3, secrete the antiviral cytokine gamma interferon (IFN-γ) under transcriptional control of T-bet (18), while Th2 cells, which preferentially express CCR4, have roles in antihelmintic and antiparasitic immune defense mediated by interleukin 4 (IL-4) and regulated by the transcription factor Gata-3 (19). Th17 cells represent a more recently discovered population of T helper cells with critical functions for maintaining mucosal antimicrobial immune defense through the production of IL-17; these cells express CCR6 and are governed by RORγt as a master transcriptional regulator (20, 21). T follicular helper cells (Tfh) express the transcription factor BCL6 and represent a CD4 T cell population specializing in supporting B cell development and function, mostly by secreting IL-21 (22); these cells uniformly express CXCR5 and are localized in the germinal center of lymph node B cell follicles, but populations of circulating CD4 T cells with phenotypic and functional characteristics similar to those of Tfh have also been described (23, 24). Importantly, recent studies suggest that the functional commitment to individual patterns of CD4 T cell polarization also affects developmental and maturational aspects, including the ability to survive long term, to proliferate homeostatically, and to serve as precursor cells for effector cell populations. This is particularly obvious in the context of Th17 cells, which represent a long-lasting cell population with increased abilities to self-renew and to repopulate pools of terminally differentiated helper cells of the same, and possibly of other, polarizations (25, 26). In this study, we performed a detailed analysis to investigate the ability of CD4 T cells with different polarization to serve as long-term reservoirs for HIV-1 during antiretroviral treatment.

MATERIALS AND METHODS

Patients. HIV-1-infected study participants treated with suppressive antiretroviral therapy were recruited from the Massachusetts General Hospital (Boston, MA). Clinical and demographic characteristics are summarized in Table 1. Peripheral blood mononuclear cell (PBMC) samples were used according to protocols approved by the Institutional Review Board. Study subjects gave written informed consent to participate in accordance with the Declaration of Helsinki.

Cell sorting and flow cytometry. CD4 T cell populations were stained with monoclonal antibodies to CD4 (clone RPA-T4), CD3 (clone UCHT1), CD45RA (clone 773964D), CCR4 (clone L291H4), CCR6 (clone G034E3), CXCR3 (clone G025H7), and CXCR5 (clone J252D4). After 20 min, cells were washed and the indicated cell populations were live sorted in a specifically designated biosafety cabinet (Baker Hood), using a FACSAria cell sorter (BD Biosciences) at 70 lb/in^2. 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. For phenotypic characterization, cells were additionally stained with CCR5 (clone J418F1) and CXCR4 (clone 12G5) and acquired on an LSRII flow cytometer (BD Biosciences). When indicated, intracellular staining was performed with antibodies against Samhd1 (clone 64326) and Trim5α (clone S2N05101010) after fixation and permeabilization according to standard procedures. Data were analyzed using FlowJo software (TreeStar).

***Ex vivo* HIV-1 infection assays.** Unselected PBMCs from HIV-1-negative donors were cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and 50 U/ml of recombinant human IL-2 (rhIL-2). A total of 10×10^6 PBMCs were infected with a green fluorescent protein (GFP)-encoding vesicular stomatitis virus (VSV) G-pseudotyped virus (multiplicity of infection [MOI] = 1 unless otherwise indicated) or with replication-competent GFP-encoding R5 (Ba-L)- or X4 (NL4-3)-tropic viral strains (both at an MOI of 1). All isolates were kindly provided by D. R. Littman, New York University, New York, NY. After 3 h, cells were washed twice with phosphate-buffered saline (PBS) and cultured for 5 days with IL-2-supplemented medium (50 U/ml). On day 5, cells were stained with surface antibodies to identify individual CD4 T cell populations, washed, and analyzed on an LSRII flow cytometer.

Gene expression analysis. Sorted CD4 T cell populations were activated with CD3/CD28 beads (Dynabeads) for 3 days. RNA was then extracted after the beads were removed from cells, as described by Morita et al. (24). Expression of selected gene transcripts in individual CD4 T cell populations was analyzed by semiquantitative reverse transcription-PCR (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 population.

Assessment of cell-associated HIV-1 DNA. Sorted CD4 T cell populations were digested as previously described to extract cell lysates (7). We amplified total HIV-1 DNA using digital droplet PCR (ddPCR) (Bio-Rad) with primers and probes described previously (7). PCR was performed using the following program: 95°C for 10 min, 45 cycles of 94°C for 30s and 60°C for 1 min, and then 98°C for 10 min. The droplets were subsequently read with the QX100 droplet reader, and data were analyzed using QuantaSoft software (Bio-Rad).

Viral sequencing. Cell lysates from sorted T cell populations were used for single-genome HIV-1 envelope sequencing encompassing the V3 region. After analysis of HIV-1 DNA concentrations in each CD4 T cell population by ddPCR, PCR plates were set up with cellular lysate dilutions yielding <30% positive PCRs for each cell population, according to a protocol described before (27). Nested PCRs were performed in triplicates using outer primers env7/LA11 (28) and inner primers LA12 and LA13 (28). Amplification products were then purified and sequenced using standard procedures. Sequences were aligned with an HXB2 reference sequence using BioEdit V7.1.9. A neighbor-joining method, as implemented in MEGA4 (29), was used to construct phylogenetic trees with phylogenetically informative HIV-1 nucleotide sequences.

Statistics. Data are summarized as individual data plots or as box-and-whisker plots indicating the median, interquartile range, and minimum and maximum values. Differences were tested for statistical significance using Wilcoxon rank sum tests or *t* tests, as indicated, followed by Bonferroni correction for multiple comparisons when indicated.

RESULTS

CD4 T cell polarization influences susceptibility to HIV-1. To investigate how the functional polarization of CD4 T cells affects their ability to serve as a reservoir for HIV-1, we initially analyzed

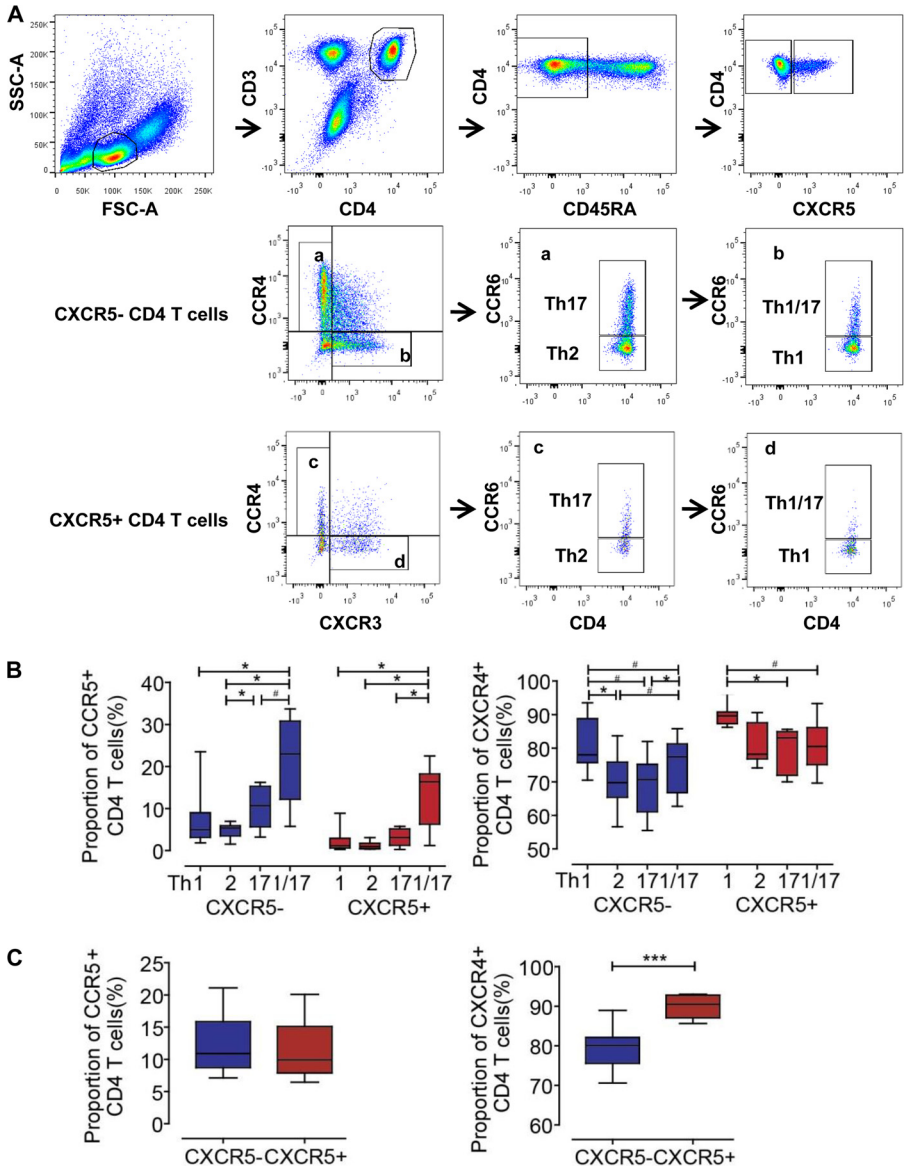

FIG 1 Surface expression of CCR5 and CXCR4 on CD4 T cells enriched for Th1, Th2, Th17, and Th1/17 polarization. (A) Gating strategy for separating CD4 T cell subpopulations enriched for cells with distinct functional polarization within the $CXCR5^-$ and the $CXCR5^+$ compartments. Results from one representative experiment are shown. (B) Box-and-whisker plots reflecting CCR5 and CXCR4 surface expression on the indicated CD4 T cell subpopulations, defined in panel A. Results with cells from 9 HIV-1-negative donors are shown. (C) Surface expression of CCR5 and CXCR4 on peripheral blood $CXCR5^-$ and $CXCR5^+$ CD4 T cells. Differences between cell subpopulations were analyzed using the Wilcoxon signed-rank test (#, $P < 0.05$); the Bonferroni correction for multiple comparisons was used where indicated (*, $P < 0.05$; ***, $P < 0.005$).

the permissiveness of different CD4 T cell populations to HIV. For this purpose, surface expression profiles of the chemokine receptors CXCR3, CCR4, CCR6, and CXCR5 were used to classify cells into CD4 T cell populations with distinct functional polarizations, as described previously (24, 30). Briefly, $CD45RA^-$ memory CD4 T cells from the peripheral blood were initially separated into $CXCR5^+$ and $CXCR5^-$ populations, which allows identification of cells with or without functional T follicular helper cell (Tfh)-like properties, respectively (Fig. 1A) (24, 31, 32). Circulating $CXCR5^+$ CD4 T cells expressed slightly higher levels of BCL6 than their $CXCR5^-$ counterparts (see Fig. S1A in the supplemental material), as described previously (24), and were enriched for cells expressing PD-1, ICOS, and CCR7, markers that were previously associated with a Tfh phenotype (data not shown). In each of the $CXCR5^+$ and $CXCR5^-$ populations, cells were subsequently classified into four distinct subsets: a population of $CXCR3^+$ $CCR4^-$ $CCR6^-$ cells enriched for Th1-polarized cells upregulating the transcription factor T-bet, a $CXCR3^-$ $CCR4^+$ $CCR6^-$ population enriched for Th2 cells overexpressing the transcription factor Gata-3, a $CXCR3^-$ $CCR4^+$ $CCR6^+$ population enriched for Th17 cells with enhanced expression of the transcription factor RORγt, and a population of $CXCR3^+$ $CCR4^-$ $CCR6^+$ cells with Th1/17 polarization expressing higher levels of both T-bet and RORγt (Fig. 1A; see Fig. S1A in the supplemental material).

Interestingly, we observed that classification of memory CD4 T cells along these patterns was associated with a distinct expression

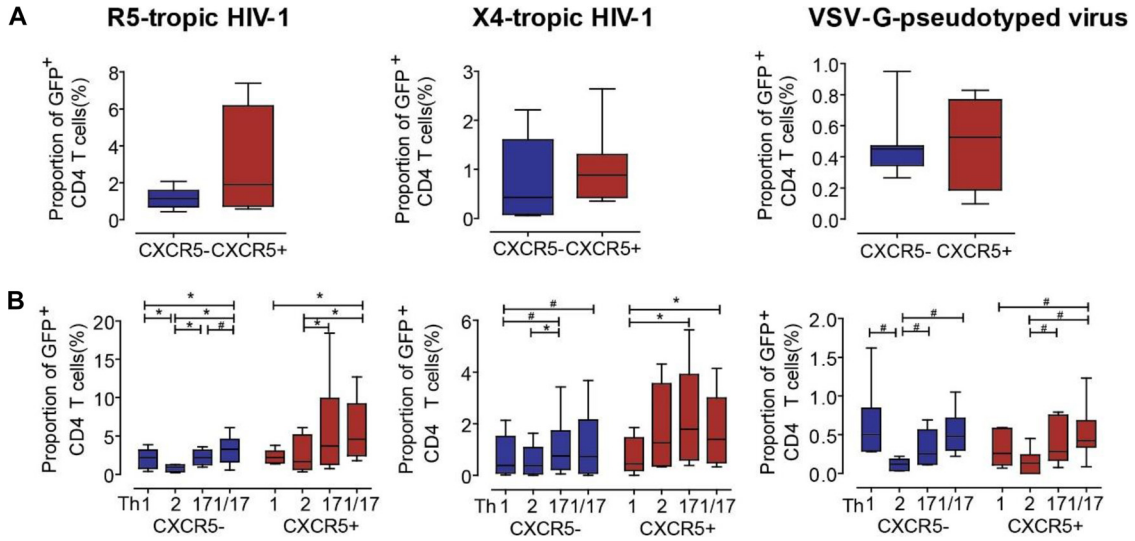

FIG 2 Functional polarization of CD4 T cell subsets influences the susceptibility to *ex vivo* infection with HIV-1. (A) Box-and-whisker plots indicating proportions of GFP^+ cells within the $CXCR5^+$ and $CXCR5^-$ CD4 T cell populations on day 5 after infection with the indicated GFP-encoding HIV-1 in the presence of IL-2-containing medium. (B) Box-and-whisker plots reflecting the proportion of GFP^+ CD4 T cells enriched for the indicated functional polarization on day 5 after *ex vivo* infection in the presence of IL-2-containing medium with the indicated GFP-encoding HIV-1. See Fig. 1 for definitions of gated T cell populations. *Ex vivo* infection data from HIV-1-negative donor cells ($n = 7$ for VSV-G-pseudotyped virus and $n = 8$ for R5- or X4-tropic virus) are shown. Differences between cell subpopulations were analyzed using the Wilcoxon signed-rank test (#, $P < 0.05$); the Bonferroni correction for multiple comparisons was used where indicated (*, $P < 0.05$).

of the HIV-1 coreceptors CCR5 and CXCR4 (Fig. 1B). In line with prior findings (30, 33), expression of CCR5 was highest in cells with preferential Th1/17 polarization, followed by those with preferential Th1 and Th17 polarization; the lowest levels of CCR5 expression occurred in cells enriched for Th2 polarization. CXCR4 expression was highest in cells with preferential Th1 polarization, while levels in cells enriched for Th17, Th1/17, and Th2 polarization were relatively similar. Notably, these patterns were consistent in both the $CXCR5^+$ and the $CXCR5^-$ CD4 T cell populations, but CXCR4 was more strongly expressed in $CXCR5^+$ cells, independently of further subclassification of cells (Fig. 1C).

To further investigate the permissiveness of these CD4 T cell populations to HIV-1, we performed *ex vivo* infection experiments with R5- and X4-tropic virus or with VSV-G-pseudotyped HIV-1 that bypasses coreceptor-mediated entry processes (see Fig. S2 in the supplemental material). Notably, *in vitro* culture conditions did not significantly affect the relative proportions of the analyzed $CXCR5^+$ and $CXCR5^-$ T cell subsets or the expression intensity of CCR5 and CXCR4 on these cell subsets (data not shown). We found that $CXCR5^+$ CD4 T cells generally tended to be slightly more susceptible to R5-tropic HIV-1 than their $CXCR5^-$ counterparts, although this did not reach the levels of statistical significance (Fig. 2A). Among $CXCR5^-$ cells, lowest susceptibilities to R5-, X4-, and VSV-G-pseudotyped infection occurred within cells enriched for Th2 polarization, while cells with preferential Th1/17 polarization seemed to have highest susceptibilities to R5-tropic viral infection (Fig. 2B). Within $CXCR5^+$ cells, the highest levels of *ex vivo* infection with R5- or X4-tropic virus were noted in cells with preferential Th17 and Th1/17 polarization, while infection rates in $CXCR5^+$ cells enriched for Th1 or Th2 polarization were more limited (Fig. 2B). There was a trend for reduced protein expression of the restriction factors Trim5α and Samhd1 in cells with preferential Th17 and Th1/17 polarization, while expression of these molecules appeared to be slightly higher in cells enriched for Th1 and Th2 polarization (see Fig. S1B in the supplemental material). Together, these studies suggest that Th1/17-enriched cells, either with or without surface expression of the Tfh marker CXCR5, have high permissiveness to HIV-1, suggesting that they may serve as preferential reservoirs for HIV-1 in ART-treated HIV-1 patients.

Disproportionately enhanced contribution of Th1/Th17-polarized cells to the viral reservoir. We subsequently sorted CD4 T cells with different polarizations from a cohort of HIV-1-infected patients who had been on suppressive antiretroviral therapy for a median of 7.25 years, which was followed by PCR-mediated detection of HIV-1 DNA levels in these cells. The clinical and demographic characteristics of these patients are summarized in Table 1. Notably, proportions of $CXCR5^+$ and $CXCR5^-$ T cells with preferential Th17 and Th1/17 polarization were preserved in ART-treated patients and were very similar to the corresponding proportions in HIV-1 negative individuals (see Fig. S3 in the supplemental material). In both $CXCR5^+$ and $CXCR5^-$ CD4 T cells, the lowest levels of HIV-1 DNA were detected in cells enriched for Th2 polarization, and the highest HIV-1 DNA levels occurred in cells with preferential Th1/17 polarization (Fig. 3A). To better understand the role of the different CD4 T cell populations in the composition of the reservoir of HIV-1-infected cells, we compared the contribution of each CD4 T cell population to the analyzed memory CD4 T cell pool with the corresponding contribution to the viral reservoir in the analyzed memory CD4 T cells. This analysis indicated a balanced equilibrium between the contributions to the viral reservoir and to the memory CD4 T cell pool for total $CXCR5^+$ and $CXCR5^-$ CD4 T cells (Fig. 3B). However, a discrepancy between those parameters was noted for cells enriched for a Th1/17 polarization, which made a disproportionately

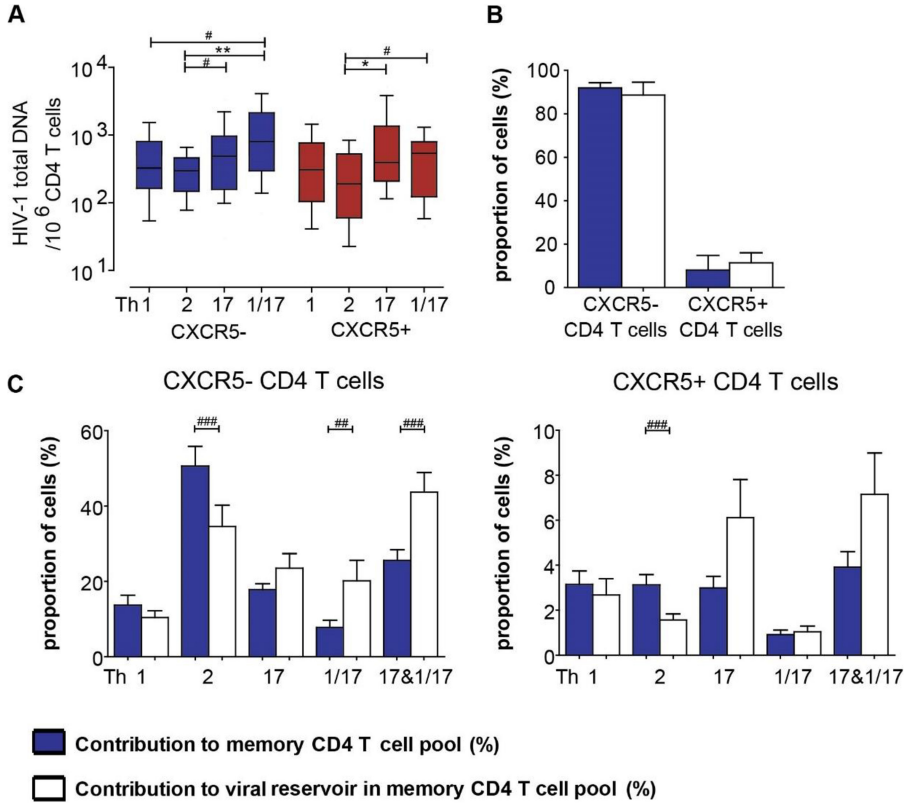

FIG 3 Disproportionately elevated contributions of Th17 and Th1/17 cells to the reservoir of HIV-1-infected cells in patients receiving treatment with suppressive antiretroviral therapy. (A) Levels of total HIV-1 DNA in CD4 T cells enriched for the indicated functional polarization. Cross-sectional data from 15 ART-treated patients are shown. Differences among subpopulations in $CXCR5^-$ or $CXCR5^+$ CD4 T cells were analyzed using the Wilcoxon signed-rank test (#, $P < 0.05$) with Bonferroni correction for multiple comparisons where indicated (*, $P < 0.05$; **, $P < 0.01$). (B and C) Relative contributions of $CXCR5^+$ and $CXCR5^-$ CD4 T cells to the analyzed memory CD4 T cell pool and to the viral reservoir in analyzed memory CD4 T cells. (B) Data from $CXCR5^+$ and $CXCR5^-$ CD4 T cells. (C) Data in cell subsets enriched for the indicated polarization. Data reflect the mean and standard deviation from 15 ART-treated patients (##, $P < 0.01$; ###, $P < 0.005$ [Wilcoxon signed-rank test]).

larger contribution to the viral reservoir than to the memory CD4 T cell pool within the $CXCR5^-$ CD4 T cell compartment (Fig. 3C). A similar but more subtle discrepancy between those parameters was also observed for CD4 T cells enriched for Th17 polarization within the $CXCR5^-$ CD4 T cell pool (Fig. 3C). An opposite finding was made for Th2-enriched cells in the $CXCR5^+$ and the $CXCR5^-$ CD4 T cell pools, which accounted for a larger fraction of the analyzed memory CD4 T cell pools but made only comparatively small contributions to the viral reservoir. Th1-enriched cells in the $CXCR5^+$ and the $CXCR5^-$ compartments made approximately equal contributions to the memory CD4 T cell pool and the viral reservoir (Fig. 3C). These data suggest that the cellular CD4 T cell reservoirs for HIV-1 infected cells in ART-treated persons are biased toward Th1/17 cells and, to a lesser extent, toward Th17 cells.

Increasing contribution of Th1/17 cells to the viral reservoir over time. Increasing evidence suggests that the functional polarization of CD4 T cells involves a maturational aspect and affects the ability of cells to survive and persist long term (25, 34). To better investigate how functional polarization of CD4 T cells influences the ability of cells to serve as a long-term reservoir for HIV-1, we focused on a cohort of six HIV-1 patients who initiated HIV-1 treatment during early HIV-1 infection and then had continuous, uninterrupted antiretroviral therapy over the next 4.5 to 6.7 years. CD4 T cell subsets enriched for different polarizations were sorted after short-term antiretroviral therapy (1.5 to 1.9 years) and after long-term antiretroviral therapy, followed by analysis of HIV-1 DNA. Intraindividual comparisons between early and late treatment time points indicated that HIV-1 DNA was strongly decreasing over time in the Th1-enriched cell populations in most patients, within both the $CXCR5^+$ and the $CXCR5^-$ cell subsets (Fig. 4A). Evolution of HIV-1 DNA in other cell subsets showed a more heterogeneous picture but remained relatively stable on a population level (Fig. 4A). In line with these observations, we observed that the contribution of Th1-enriched CD4 T cells to the viral reservoir declined over time, while the contribution of these cells to the analyzed memory CD4 T cell pool remained fairly constant; this led to a significant discrepancy between the contributions of $CXCR5^+$ and $CXCR5^-$ Th1 cells to the viral reservoir and their respective contributions to the memory CD4 T cell pool after long-term treatment (Fig. 4B; see Fig. S4 in the supplemental material). $CXCR5^-$ T cells enriched for Th1/17-polarized cells, as well the combined pool of cells with preferential Th17 and Th1/17 polarization, showed an opposite pattern; their contribution to the viral reservoir substantially increased during prolonged antiretroviral therapy, in the background of a largely stable contribution to the memory CD4 T cell pool, resulting in a disproportionately increased contribution to the viral res-

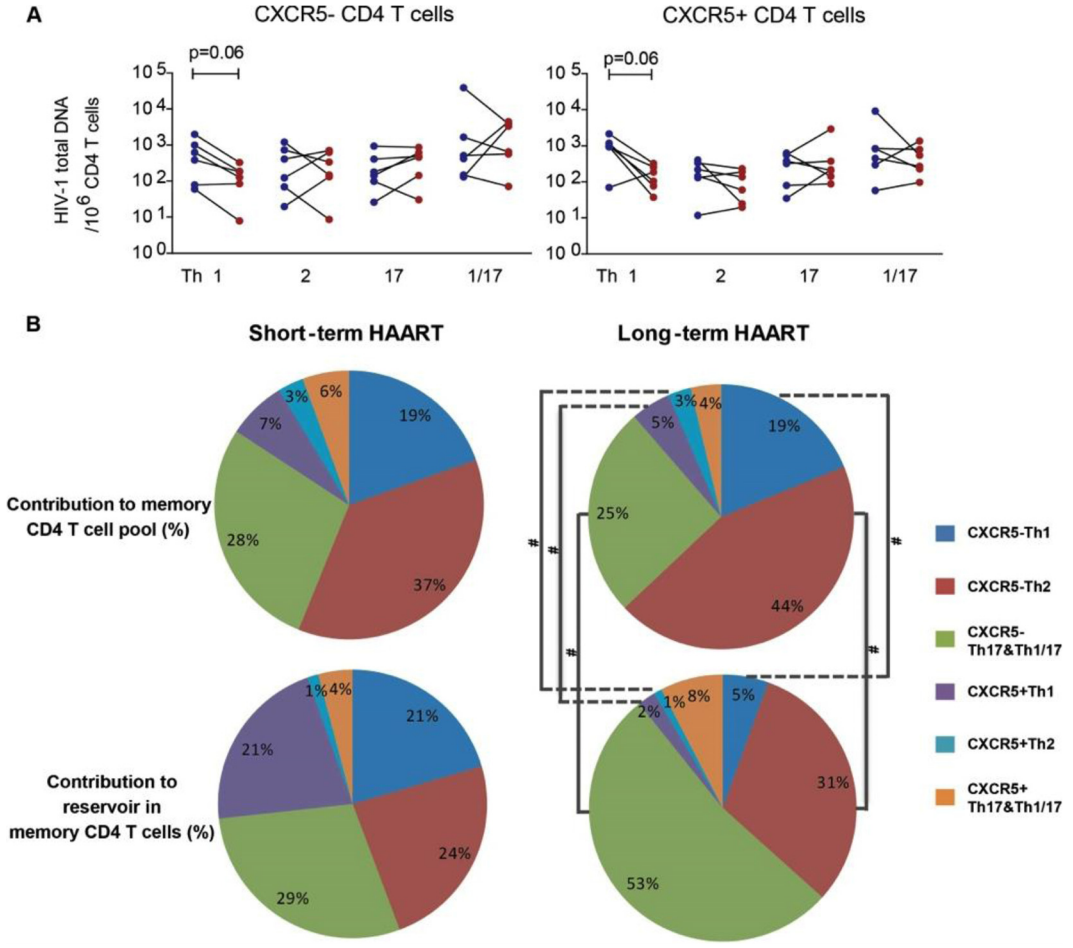

FIG 4 Progressive increase in the combined contributions of Th17- and Th1/17-polarized cells to the viral CD4 T cell reservoir during prolonged antiretroviral therapy. (A) Total HIV-1 DNA levels in CD4 T cell populations enriched for the indicated functional polarization within 1.5 to 1.9 years after initiation of antiretroviral therapy (blue symbols) and after 4.5 to 6.7 years of continuous antiretroviral therapy (black symbols). Differences were analyzed using the Wilcoxon signed-rank test. (B) Pie charts reflecting the contributions of CD4 T cell populations enriched for the indicated polarization to the analyzed memory CD4 T cell pool (upper panels) and to the viral reservoir in analyzed memory CD4 T cells (lower panels). Longitudinal data from 6 ART-treated individuals are shown. Differences were analyzed using the Wilcoxon signed-rank test (#, $P < 0.05$).

ervoir relative to the memory CD4 T cell pool after long-term treatment (Fig. 4B; see Fig. S4 in the supplemental material). The contribution of Th2-enriched cells to the memory CD4 T cell pool and the corresponding contribution to the viral reservoir showed little variation over time. Irrespective of the Th1, Th2, and Th17 subclassification of cells, we observed that the contribution of $CXCR5^+$ CD4 T cells to the memory CD4 T cell pool and to the viral reservoir in memory CD4 T cells declined over time, although such changes did not reach statistical significance (see Fig. S5 in the supplemental material). Together, these data suggest that $CXCR5^-$ Th17 and Th1/17 cells represent a more durable component of the viral reservoir that seems to become increasingly important with prolonged duration of antiretroviral therapy.

Long-term persistence of viral sequences from Th17 and Th1/17 CD4 T cells. We longitudinally analyzed viral sequences in sorted CD4 T cell populations with defined chemokine receptor expression patterns to identify possible phylogenetic associations between sequences obtained from different subsets. For this purpose, we focused on two patients from whom samples collected after short-term (1 to 2 years) and long-term (78 months [patient 1] and 56 months [patient 2]) antiretroviral therapy were available (Fig. 5A). CD4 T cells with different functional polarizations were sorted, and HIV *env* sequences were amplified from genomic DNA. Interestingly, in both study subjects, we noted identical viral sequences that were amplified from Th17- and Th1/17-enriched cells after short- and long-term antiretroviral therapy, suggesting prolonged persistence of HIV-1 in these cell populations (Fig. 5B). In contrast, identical viral sequences after short- and long-term antiretroviral therapy in Th1- and Th2-enriched cells were noted in only one of the two study subjects who had remained on antiretroviral therapy for a shorter cumulative time period (Fig. 5B). Moreover, we observed that in several cases, Th17- and Th1/17-enriched cells from early antiretroviral treatment stages harbored viral sequences identical to those detected in cells with preferential Th1 and Th2 polarization several years later, suggesting that Th17 and Th1/17 may serve as precursor cells for CD4 T cells of alternative polarizations (Fig. 5C). Although these studies were conducted with only a small number of study subjects, these results may support the role of Th17 and Th1/17 as a long-lasting cellular

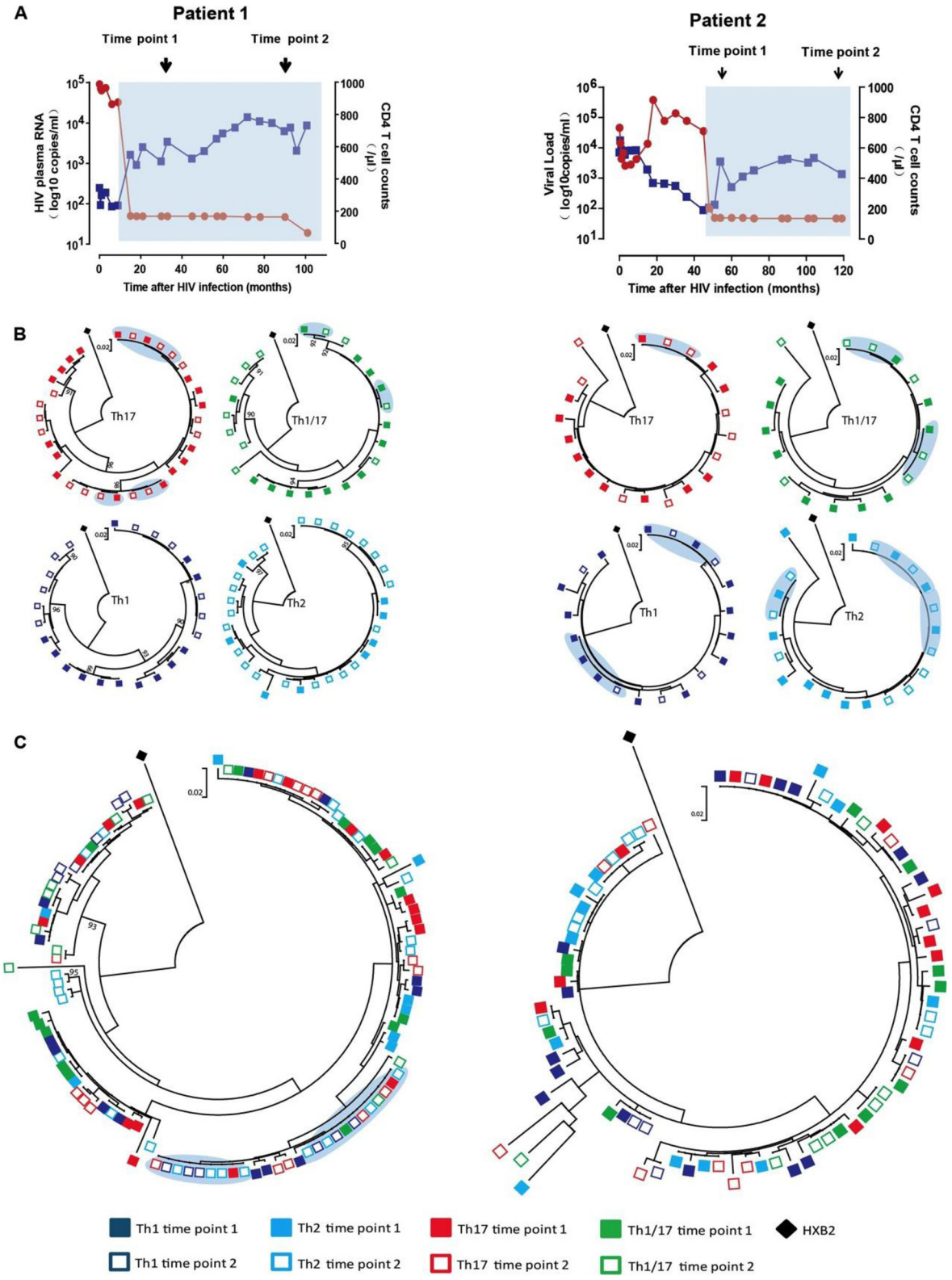

FIG 5 Long-term persistence of identical viral sequences in Th17 and Th1/17 cells during prolonged antiretroviral therapy. (A) Time course reflecting CD4 T cell count, viral load, antiretroviral treatment duration (blue squares), and timing of sample collection in the indicated study patients. (B) Circular phylogenetic trees including viral Env sequences from CD4 T cell populations enriched for the indicated polarizations. Identical viral sequences isolated during short-term (closed symbols) and long-term (open symbols) antiretroviral treatment are highlighted in blue. (C) Circular phylogenetic tree summarizing viral sequences isolated from all analyzed CD4 T cell populations after short- and long-term antiretroviral therapy. Identical viral sequences retrieved from different cell populations and different time points are highlighted in blue.

reservoir that can promote viral persistence during antiretroviral therapy.

DISCUSSION

Increasing evidence indicates that HIV-1 can establish a long-lasting viral reservoir by exploiting biological mechanisms of cellular immune memory (6). However, the ability to effectively support HIV-1 persistence may be restricted to certain subgroups of memory CD4 T cells with specific functional and developmental properties. The identification of such memory CD4 T cell subpopulations that possess cell-intrinsic properties most suitable for maintaining HIV-1 persistence is an important area of investigation to define cellular targets for HIV-1 eradication strategies.

In this study, we have analyzed Th1-, Th2-, and Th17-polarized CD4 T helper cells, with or without concomitant expression of the Tfh surface marker CXCR5, to identify polarization conditions of helper CD4 T cells most closely associated with the persistence of HIV-1 DNA despite ART. Our data suggest that Th1/Th1/17-enriched cells harbor high levels of HIV-1 DNA in ART-treated patients and account for an increasing proportion of the viral reservoir when ART is administered for prolonged periods of time. In part, this may be related to a higher cell-intrinsic susceptibility of Th1/17-polarized CD4 T cells to HIV-1 infection; recent data indeed suggest that Th1/17 cells represent a cellular microenvironment with enhanced abilities to support HIV-1 replication steps (35–37). However, a number of studies also suggest that, independently of cellular permissiveness to HIV-1, Th17 and Th1/17 cells constitutively possess functional and developmental characteristics that make them able to serve as cellular hot spots for viral persistence in the setting of antiretroviral treatment. Notably, although originally characterized as short-lived effector cells (38), Th17 cells have recently been identified as long-lasting cells that overexpress gene transcripts involved in stem cell maintenance and homeostasis, such as TCF-1 and members of the Notch receptor family, have increased abilities to self-renew, and can give rise to more differentiated effector cell populations (25, 26, 34). Moreover, various observations indicate that Th17 cells have a higher degree of developmental plasticity and can acquire functional characteristics of Th1 cells upon encountering context-dependent microenvironmental clues (39, 40). Therefore, Th17 cells may represent a CD4 T cell subset that functionally resembles T memory stem cells, a small subset of CD4 T cells that imitate many of the functional characteristics of classical tissue stem cells and for that reason seem biologically predestined to serve as a preferential niche for HIV-1 long-term persistence (7, 8). Moreover, Th1/17 cells have also been interpreted as a long-lasting subpopulation that may be slightly more mature than pure Th17 cells but still possess a developmental program of long-lasting precursor cells for effector lymphocytes (34). Together, these data support the assumption that stem cell characteristics of cellular immune memory are important for maintaining HIV-1 persistence in the setting of antiretroviral therapy. Whether stem cell-like homeostatic proliferation and developmental plasticity indeed shape the dynamics of the persisting viral reservoir is uncertain at present, but future studies may allow for a deeper analysis of such issues, using longitudinal tracking of chromosomal HIV-1 integration site analysis (41, 42) in sorted T cell subsets.

CXCR5-expressing circulating CD4 T cells have been characterized as a specialized population of memory cells that support B cell function and imitate many of the functional properties of T follicular helper cells encountered in germinal centers of lymph node follicles (24, 43). Prior studies have demonstrated that such T follicular helper cells are highly susceptible to HIV-1 and serve as predominant sites for viral replication and production (44). To what extent the $CXCR5^+$ counterparts of T follicular helper cells circulating in the peripheral blood contribute to HIV-1 replication and persistence has been less certain. Notably, in contrast to Tfh in lymph nodes, blood $CXCR5^+$ CD4 T cells do not express high levels of the activation markers CD69 and PD-1 (24), suggesting that they represent a more resting memory T cell population with possibly improved abilities to support HIV-1 latency. Overall, we observed that due to their low frequencies, $CXCR5^+$ CD4 T cells make only a comparatively small contribution to the viral reservoir and that this contribution tends to decline further during extended periods of antiretroviral therapy. Similarly to the $CXCR5^-$ CD4 T cell compartment, decreases of HIV-1 DNA during antiretroviral therapy appeared to be most prominent in cells with preferential Th1 polarization within the $CXCR5^+$ CD4 T cell pool. Interestingly, the ability of peripheral blood $CXCR5^+$ CD4 T cells to support B cell development through secretion of IL-21 appears to be confined to the subgroups of $CXCR5^+$ cells with Th2 and Th17 polarization, while $CXCR5^+$ Th1-polarized CD4 T cells seem to lack the capacity to help B cells through IL-21 secretion (24). Future studies will be necessary to determine whether such IL-21-secreting Tfh-like peripheral blood T cells may provide an improved cellular microenvironment to support HIV-1 latency and persistence.

Our analysis in this study was limited to an assessment of HIV-1 DNA in different T cell populations. However, large proportions of HIV-1 DNA do not encode replication-competent virus. Therefore, future studies will be necessary to investigate the presence of intact proviruses in T cell subsets with different polarization. Moreover, it will be important to determine whether HIV-1 persistence in CD4 T cell subsets is regulated by transcription factors defining the functional profile of these cells. For instance, the Th2 transcription factor Gata-3 (45) and the Tfh transcription factor BCL6 (46) can bind to the HIV-1 long terminal repeat (LTR) promoter and through this mechanism may directly influence HIV-1 gene transcription. Similar findings were made for FoxP3, the master transcription factor for regulatory T cells (47). As such, it is conceivable that HIV-1 latency and transcriptional activity are directly affected by transcription factors that govern the fate and polarization of T helper cells and that direct linkages exist between regulatory pathways determining the functional profile of T cells and their ability to serve as long-term viral reservoirs.

Efforts to manipulate the reservoir of HIV-1-infected cells in ART-treated patients for clinical purposes currently largely rely on the use of pharmaceutical agents supporting active viral gene transcription and reactivation from viral latency, which may make cells more susceptible to immune-mediated clearance. How susceptible CD4 T cells with different functional polarization may be to such latency-reversing agents is an important aspect of future investigations, and it is possible that combinations of different pharmacological compounds may be necessary to equally target the entire spectrum and the full diversity of latently infected T helper cells. An alternative strategy for targeting the reservoir of HIV-1-infected cells in patients receiving antiretroviral therapy may include the use of nonreactivating pharmaceutical agents that accelerate elimination of latently infected CD4 T cells by interfer-

ing with the growth, homeostatic proliferation, and survival of long-lasting CD4 T cells, such as Th17 or Th1/17 cells. Such efforts would need to consider the individual regulatory pathways that govern the growth behavior of individual CD4 T cell populations and may in part take advantage of existing pharmaceuticals that inhibit long-term persistence of cancer stem cells. As such, our increasing understanding of cell-intrinsic biological characteristics influencing the long-term maintenance of HIV-1 in specific CD4 T cell populations may be informative for designing clinical strategies that reduce persisting HIV-1 reservoirs.

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