Different human resting memory CD4⁺ T cell subsets show similar low inducibility of latent HIV-1 proviruses

Kyungyoon J. Kwon¹, Andrew E. Timmons¹, Srona Sengupta¹, Francesco R. Simonetti¹, Hao Zhang², Rebecca Hoh³, Steven G. Deeks³, Janet D. Siliciano¹, Robert F. Siliciano¹, 4*

The latent reservoir of HIV-1 in resting CD4⁺ T cells is a major barrier to cure. It is unclear whether the latent reservoir resides principally in particular subsets of CD4⁺ T cells, a finding that would have implications for understanding its stability and developing curative therapies. Recent work has shown that proliferation of HIV-1–infected CD4⁺ T cells is a major factor in the generation and persistence of the latent reservoir and that latently infected T cells that have clonally expanded in vivo can proliferate in vitro without producing virions. In certain CD4⁺ memory T cell subsets, the provirus may be in a deeper state of latency, allowing the cell to proliferate without producing viral proteins, thus permitting escape from immune clearance. To evaluate this possibility, we used a multiple stimulation viral outgrowth assay to culture resting naive, central memory (TCM), transitional memory (TTM), and effector memory (TEM) CD4⁺ T cells from 10 HIV-1–infected individuals on antiretroviral therapy. On average, only 1.7% of intact proviruses across all T cell subsets were induced to transcribe viral genes and release replication-competent virus after stimulation of the cells. We found no consistent enrichment of intact or inducible proviruses in any T cell subset. Furthermore, we observed notable plasticity among the canonical memory T cell subsets after activation in vitro and saw substantial person-to-person variability in the inducibility of infectious virus release. This finding complicates the vision for a targeted approach for HIV-1 cure based on T cell memory subsets.

INTRODUCTION

The major barrier to HIV-1 cure is the latent reservoir (1, 2) composed of resting CD4⁺ T cells harboring latent, replication-competent proviruses (3–7). The latent reservoir was first demonstrated using a quantitative viral outgrowth assay in which virus production from resting CD4⁺ T cells was induced upon T cell activation (4, 7–10). The slow decay of the latent reservoir necessitates lifelong antiretroviral therapy (ART) to prevent viral rebound in HIV-infected individuals (1, 11, 12). The HIV-1 latent reservoir is maintained, in part, by memory T cell proliferation (13–16) driven by cytokines, antigens, or effects related to the site of proviral integration (13–27). Latently infected T cells carrying replication-competent proviruses can persist through clonal expansion in vivo (13–15, 28).

Cellular markers of latent infection (29–31) would allow selective targeting of the HIV-1 latent reservoir. Specific subpopulations of CD4⁺ T cells may be enriched in T cells carrying latent, replication-competent proviruses and may differ in propensity for clonal expansion or latency reversal (8–11, 13–16). However, specific markers of latent infection have not been confirmed (32–37), and persistent HIV-1 has been found in all CD4⁺ T cell memory subsets and in lower amounts in naïve CD4⁺ T cells (29, 38–44). Three subsets of memory CD4⁺ T cells are defined by the ce surface proteins CD45, CCR7, and CD27 (45–47); central memory T cells (TCM; CD45RO⁻CCR7⁺CD27⁺), transitional memory T cells (TTM; CD45RO⁺CCR7⁻CD27⁺), and effector memory T cells (TEM; CD45RO⁺CCR7⁻CD27⁻) (48, 49). Naïve T cells (CD45RO⁻CCR7⁺CD27⁺) home to secondary lymphoid organs where they encounter antigen and differentiate into memory T cells (48–54). TCM are long-lived cells that home to secondary lymphoid organs and mediate recall responses (45, 46, 55). TTM are in a transient state between TCM and TEM phenotypes (47, 56). TEM home to inflammatory sites for rapid effector function (48, 56–58).

Early studies demonstrated higher frequencies of HIV-1 in memory CD4⁺ T cells than in naïve T cells (4, 59), consistent with models of virus reservoir formation (4, 60). Proviral DNA has been measured in TCM, TTM, and TEM (38–40) as well as in T helper 1 (Th1) cells (61, 62), T follicular helper (TFH) cells (63), and stem cell–like memory cells (41, 42). Several studies have shown that TCM harbor most of the latent HIV-1 proviruses and have the longest half-life of memory T cell subsets (38, 40, 55, 57, 58, 64–67). However, another study showed that TEM harbor intact HIV-1 proviruses at a higher frequency (39).

One factor contributing to disparate results is the nature of the assays used to measure infected cells (68). The quantitative viral outgrowth assay provides a definitive minimal estimate of latent reservoir size but misses replication-competent proviruses not induced by a single round of activation (4, 8–10, 28). HIV-1 DNA measurements using quantitative polymerase chain reaction (qPCR) markedly overestimate latent reservoir size because they do not discriminate between intact and defective proviruses (69, 70). Near full-length DNA sequencing can distinguish between intact and defective proviruses but is not quantitative and does not provide information on inducibility of viral gene transcription (39, 71, 72). A recently developed intact proviral DNA assay can distinguish between intact proviruses and those with overt fatal defects (73).

It is important to understand the relative inducibility as well as the distribution of proviruses in different T cell subsets. By inducibility, we mean the ability of the viral genes in an intact provirus to be transcribed after T cell stimulation, giving rise to replication-competent virions. Genes involved in T cell activation, migration, and transcriptional regulation are more poised (present in an open chromatin state without active transcription) in resting memory T cells than in naïve T cells (74, 75). DNA methylation decreases within genes that regulate differentiation toward a memory state.

¹Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA. ²Flow Cytometry and Immunology Core, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA. ³Department of Medicine, University of California San Francisco, San Francisco, CA, USA. ⁴Howard Hughes Medical Institute, Baltimore, MD, USA.
*Corresponding author. Email: rsiliciano@jhmi.edu
(76). Because these genes are less transcriptionally restricted in TEM, this memory T cell subset displays a higher proliferative response to interleukin-7 (IL-7) and IL-15 than less differentiated T cell subsets (38, 50, 51, 77).

Initial evidence for differential inducibility of latent HIV-1 proviruses has come from multiple stimulation viral outgrowth assays, demonstrating that many replication-competent proviruses are induced in vitro only after multiple rounds of T cell activation (14, 28). One hypothesis is that inducibility of viral gene transcription depends on the memory T cell subset harboring the latent virus. Repeated activation may drive differentiation to a TEM phenotype with a transcriptional landscape that facilitates proviral transcription. Here, we describe simultaneous analysis of the distribution and inducibility of intact proviruses in CD4+ T cell subsets from 10 patients with chronic HIV-1 infection on ART using the multiple stimulation viral outgrowth assay.

RESULTS
Intact HIV-1 proviruses are similarly distributed across different memory T cell subsets
To understand the distribution and inducibility of latent HIV-1 proviruses in T cell subsets, we isolated resting CD4+ T cells from 10 individuals who had started ART during chronic HIV-1 infection and maintained viral suppression for >6 months (table S1). Resting T cells were stained for the subset-defining surface markers CD27, CCR7, and CD45RO, as well as CD3 and CD4. Resting T cells were then sorted by flow cytometry and analyzed (Fig. 1, A and B).

The intact proviral DNA assay separately quantitates intact and defective proviruses (Fig. 2A) (73). Consistent with previous observations (4, 59, 78), naïve T cells contained nearly 10-fold fewer proviral copies (both intact and defective) than did memory T cells (Fig. 2B). Intact proviral frequency in different T cell subsets varied widely among individuals, but mean values were similar (Fig. 2B).
sorted resting CD4+ T cells from an HIV-1–infected individual on ART. The limit of detection (LOD; four copies per \(10^6\) cells) was used in calculations. All T cell subsets contained at least one type of provirus (intact or defective). Mann-Whitney U tests were performed to compare copies per million between each T cell subset. (C) Frequency of each T cell subset in leukapheresis samples from 10 HIV-1–infected individuals on ART (Fig. 2C). As expected, naïve T cells were the most abundant, followed by TCM, TEM, and TTM. The contribution of each subset to the total pool of intact proviruses was then calculated. We observed large person-to-person variation (Fig. 2D) with no consistent major contribution from any T cell subset.

**T cell subsets differentiate toward an effector phenotype after multiple rounds of stimulation**

To understand the relationship between CD4+ T cell subsets and HIV-1 latency, we examined subset-defining markers and virus induction after multiple rounds of T cell activation (Fig. 1B). T cells that had been separated into subsets by flow cytometry were activated with the mitogen phytohemagglutinin and irradiated allogeneic peripheral blood mononuclear cells (PBMCs) for 24 hours and then were cocultured with MOLT-4/CCR5 cells in which the virions released after induction of latent proviruses could replicate (Fig. 1B). Cultures were split every 9 days, at which time half of each culture was restimulated; the remaining T cells were cultured without further stimulation for 21 days. HIV-1 p24 antigen in culture supernatant was measured 21 days after each stimulation to detect viral outgrowth. Following the initial stimulation, >70% of the T cells retained an activated phenotype throughout the culture period, as determined by CD25 and CD69 expression (Fig. 3A and data file S1). Carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution of each T cell subset after initial activation demonstrated that all T cells underwent proliferation (Fig. 3B). Phenotypic markers of CD4+ T cell subsets assessed every 5 to 7 days showed notable changes after stimulation. naïve T cells expressed CD45RO by 4 days after initial activation (Fig. 3C and data file S2). CCR7 expression on naïve T cells and TCM remained high after initial activation and then decreased as cells approached a TEM phenotype by day 21 (Fig. 3D). TTM and TEM strongly up-regulated CCR7 expression after initial activation from a CCR7− state. CD27 expression after T cell activation showed a more complex pattern that varied among individuals and T cell subsets (Fig. 3D). In general, CCR7 and CD27 expression demonstrated changes consistent with memory T cell subset differentiation from a naïve to an effector phenotype after repeated stimulations (fig. S1). This was expected based on previous studies of T cells from uninfected individuals (47, 56). The marked changes in T cell subset-defining markers upon T cell activation suggested that studies of T cell subsets should be performed on purified resting CD4+ T cells as we did here.
Inducibility of viral gene expression is not dependent on the memory T cell subset

To test the hypothesis that the inducibility of HIV-1 provirus varies with memory T cell subset, we examined viral outgrowth from each subset after each round of T cell activation by measuring the amount of HIV-1 p24 antigen in culture supernatant at 21 days after each stimulation (Fig. 1B). This period was sufficient to allow outgrowth of virus released from individual latently infected cells after induction of viral gene expression (8–10). Thus, we were able to determine how many rounds of T cell activation were required to induce latent proviruses. We first determined the frequency of cells in each T cell subset that gave rise to replication-competent virus after one round of stimulation with mitogen, expressed as infectious units per million cells (IUPM) based on the number of initially plated cells. No viral outgrowth was induced from naïve T cells in 7 of 10 individuals (Fig. 4A), consistent with the lower number of intact provirus copies determined by the intact proviral DNA assay (Fig. 2B). The highest frequencies were observed in TTM and TEM, but there was substantial overlap with frequencies in TCM (Fig. 4A). After normalization based on T cell subset frequency among PBMCs, we observed similar contributions of each subset to the total pool of replication-competent viruses induced after one round of stimulation (Fig. 4B).

To compare provirus inducibility in different memory T cell subsets after one round of stimulation, we calculated an inducibility index, the ratio of IUPM determined by viral outgrowth to intact provirus copies per million cells (ICPM) determined by the intact proviral DNA assay. We found substantial person-to-person variation in virus inducibility and no significant differences among memory T cell subsets (P > 0.13; Fig. 4C). For TTM and TEM, on average, <1% of intact proviruses were induced by one stimulation (Fig. 4C), although this...
ratio varied widely among individuals. This value is similar to that reported for unfractionated resting CD4+ T cells (73).

Because T cell differentiation caused by repetitive stimulation (Fig. 3D and fig. S1B) might alter virus inducibility, we evaluated viral outgrowth at 21 days after each stimulation of T cells. Because most viral outgrowth occurred in MOLT-4/CCR5 cells added to each culture, we could assess changes in virus inducibility as T cells differentiated without the confounding effect of changes in the ability of cells to propagate the infection. Additional viral outgrowth was observed after multiple rounds of T cell stimulation for 9 of 10 HIV-1-infected individuals on ART who were studied (Fig. 5A). For study participant #3147, all viral outgrowth from each T cell subset occurred after the first stimulation. In 7 of 10 individuals, there was no viral outgrowth from naïve T cell cultures even after four stimulations. For other T cell subsets, there were no clear patterns in the number of stimulations required for viral outgrowth (Fig. 5B). For example, TEM required multiple stimulations for viral outgrowth in some individuals but only one stimulation for maximum viral outgrowth in others, and this trend was seen in other T cell subsets (Fig. 5B). IUPM values based on the number of input T cells at the beginning of culture and the cumulative fraction of positive wells after four stimulations are shown in Fig. 5C. Frequencies were higher beginning of culture and the cumulative fraction of positive wells after four stimulations were normalized on the basis of the frequency of T cell subsets present in peripheral blood. There was substantial person-to-person variation and wide overlap between T cell subsets (Fig. 5E). We observed no significant differences among T cell subsets in virus inducibility after four stimulations (P > 0.12; Fig. 5F). Viral outgrowth observed after multiple stimulations of T cells may have resulted from virus production by progeny cells that were generated by in vitro proliferation of the initially plated T cells.

To understand the lack of a relationship between conventional CD4+ memory T cell subsets and the distribution and inducibility of replication-competent proviruses, we examined the phenotypes of T cells at the time of the round of stimulation that led to viral outgrowth. Data for representative individuals are shown in Fig. 5G. For some naïve T cell and TCM cultures, viral outgrowth was detected even when the phenotype at the time of stimulation did not indicate differentiation to TEM (Fig. 5G and fig. S1A). This suggested that CD4+ T cells did not need to have an effector phenotype to produce virus (Fig. 5G and fig. S1B).

**Despite multiple rounds of T cell stimulation, only a few intact proviruses are induced to transcribe viral genes**

Our results suggested that only a small fraction of intact proviruses present in each memory T cell subset could be induced to release replication-competent virus after multiple sequential rounds of stimulation of T cells in vitro. The results of the multiple stimulation viral outgrowth assay and the intact proviral DNA assay are compared in Fig. 6A. The overall fraction of intact proviruses that were induced to release replication-competent virus was low, averaging 1.7% across all T cell subsets (Fig. 5F). There was no strong correlation between IUPM and ICPM as determined by the intact proviral DNA assay (Fig. 6B). Additional rounds of T cell stimulation caused a 30-fold increase in IUPM in TCM from one individual, smaller changes in TCM from six other participants, and no increase in TCM from three other individuals (Fig. 6C). In general, induction of replication-competent virus varied by donor rather than by T cell subset. The variability in number of stimulations required for viral
Fig. 5. Viral outgrowth and inducibility after multiple rounds of T cell stimulation. (A) Cumulative fractions of all wells positive for the HIV-1 p24 antigen 21 days after one, two, three, or four rounds of T cell stimulation with phytohemagglutinin (PHA). Data are graphed separately for T cells from the 10 HIV-1–infected individuals on ART. Transient memory T cells (TTM) were not analyzed for participant #2461 because of an insufficient number of cells. (B) Cumulative fractions of all wells positive for HIV-1 p24 antigen 21 days after one to four rounds of T cell stimulation with PHA. Data are shown for four T cell subsets from 10 HIV-1–infected individuals on ART. (C) Frequency of T cells giving rise to replication-competent virus in the multiple stimulation viral outgrowth assay based on the initial number of T cells seeded. Geometric means ± SD are shown. P values were calculated using a Mann-Whitney U test (**P < 0.01). (D) Frequency of T cells giving rise to replication-competent virus in the multiple stimulation viral outgrowth assay based on the initial number of T cells seeded and the number of wells that were positive for HIV-1 p24 antigen. P values were calculated using the Wilcoxon matched-pairs signed-rank test. (E) Contribution of each T cell subset to the total pool of replication-competent proviruses calculated from IUPM values (C) and the frequency of T cell subsets present in peripheral blood of HIV-infected individuals on ART (C). Means ± SD are shown. (F) The inducibility index was calculated from the ratio of IUPM values to intact copies per million cells for each T cell subset. Geometric means ± SD are shown. Mann-Whitney U tests showed no significant differences in inducibility indices across T cell subsets (P > 0.12). (G) Phenotype of cultured T cells at the time of each stimulation with PHA (left axis) overlaid with viral outgrowth results from that round of stimulation (right axis). Data are shown for two representative HIV-1–infected individuals on ART (top: #2026; bottom: #2669). TN, naïve T cells.

Recent studies suggest that in ART-treated HIV-infected individuals, the population of CD4+ T cells carrying replication-competent proviruses is dominated by large cellular clones with identical proviruses (13–16). To explore the distribution of proviruses with identical sequences among CD4+ T cell subsets, we sequenced the highly variable V3-V4 region of the env gene from viral RNA collected from the supernatants of wells that tested positive for the viral p24 protein (Fig. 7). Of 175 independent isolates of replication-competent virus, 142 (81.1%) had a matching env sequence present in the same blood sample. We also obtained 109 proviral V3-V4 env sequences from unfraccionated resting CD4+ T cells from these individuals. Of these, 56 (51.3%) had a matching proviral env sequence in the same blood sample, which could reflect in vivo clonal expansion given
the clonal prediction score of 96 \((14, 80)\). However, clonality could not be definitively established without integration site analysis. Identical sequences were often obtained from different T cell subsets after different numbers of stimulations. For example, participant #2274 had a large potential clone identified as identical sequences from TCM, TTM, and TEM subsets after different numbers of stimulations that resulted in virus induction. In cases where the identical sequences reflected proliferation of a single infected cell, these findings suggested that members of the clone could display differential replication inducibility and could have resided in different memory T cell subsets.

**DISCUSSION**

It has been unclear whether the HIV-1 latent reservoir is contained mainly in a particular subset of CD4\(^+\) T cells \((29, 38–44)\). If it is, this would allow more specific targeting of T cells hosting the latent reservoir and provide insight into viral persistence. Therefore, we examined the distribution and inducibility of viral gene transcription in intact replication-competent proviruses in resting CD4\(^+\) T cells of the naïve T cell, TCM, TTM, and TEM subsets. In 5 of 10 HIV-infected individuals on ART, we detected no intact proviral DNA in naïve T cells using the intact proviral DNA assay and no viral outgrowth after four rounds of T cell stimulation \((n = 10)\). These results are consistent with previous studies \((4, 59, 78)\) and some models for latent reservoir formation \((60)\). However, for memory T cell subsets, there was no difference in the frequency of T cells with intact proviruses. Because TCM were the most abundant T cell subset, they made the largest contribution to the total pool of intact proviruses, but there was large person-to-person variability in the contribution of T cell subsets, which is notable given the homogeneity of our HIV-infected cohort.
Fig. 7. Neighbor-joining env trees of replication-competent viral isolates. Neighbor-joining env trees of replication-competent viral isolates from the multiple stimulation viral outgrowth assay are shown. Sequences of the V3-V4 region of the env gene in replication-competent viruses from cultures 21 days after T cell stimulation (solid symbols) and proviral sequences from unfractionated resting CD4+ T cells (open circles) are shown for 10 HIV-infected individuals on ART.
We also considered whether there was differential inducibility of replication-competent proviruses in different memory T cell subsets. Previous characterization of the epigenetic landscape and cytokine responsiveness of T cells suggested that proviruses in TEM would be the easiest to induce after T cell stimulation, followed by TTM, TCM, and then naive T cells (38, 50, 51, 57, 74–76). To test this hypothesis, we subjected sorted resting CD4+ T cell subsets to four consecutive rounds of global T cell activation. We found no correlation between T cell subsets and the number of rounds of T cell stimulation needed for viral outgrowth. In addition, we observed virions with identical env sequences in different culture wells after different numbers of stimulations and from different T cell subsets. If sequence identity reflects clonal expansion, then our results suggest that HIV-1–infected T cells can proliferate and differentiate into different memory T cell subsets in vivo without succumbing to viral cytopathic effects or immune clearance. Another study has provided evidence of clonal proviruses with identical integration sites distributed across T cell subsets (80). To better understand the inducibility of proviruses after T cell stimulation, we developed an inducibility index defined as the frequency of T cells that gave rise to viral outgrowth after one or more rounds of T cell stimulation divided by the initial number of T cells with intact proviruses. Average inducibility was 0.74% after one round of T cell activation, similar to previously reported results for unfractiated CD4+ T cells (73). On average, only 1.7% of intact proviruses were induced to release replication-competent virus after four rounds of T cell activation. Low inducibility has also been observed using assays for intracellular HIV-1 RNA (81–83), p24 protein (84), or cell-free HIV-1 RNA (82, 83, 85, 86). For example, Cillo et al. (85) reported that only 1.5% of resting CD4+ T cells with HIV-1 DNA could be induced to produce virions after stimulation with anti-CD3/CD28 antibodies.

The very high fraction (98.3%) of intact proviruses that are not induced even after four rounds of T cell stimulation demands further investigation to determine whether these proviruses contribute to the latent reservoir, residual viremia, and viral rebound. Several biological mechanisms might explain this observation. Stochastic processes may govern whether an intact provirus is induced to release replication-competent virus after T cell activation (87). T cell exhaustion after repeated in vitro stimulations may limit proviral induction (14). Some proviruses may be permanently silenced by epigenetic mechanisms (88, 89), and others may be integrated in genomic locations that affect inducibility (90). Induction of some intact proviruses may be suppressed by transcriptional interference (91–93) or may require signals other than those provided by mitogen stimulation. If a large proportion of these intact noninduced proviruses are replication competent and ultimately inducible in vivo, then they represent a major hurdle to eradicating the HIV-1 latent reservoir.

Our study has several limitations. Participants were mainly Caucasian males over 50 years of age who initiated ART therapy with low CD4+ T cell counts. The induction of latent proviruses and the replication of viruses released after latency reversal may be different in vivo compared to in vitro. For example, viral outgrowth may be limited by the in vitro culture system used. However, viral outgrowth measured using MOLT-4/CCCR5 cells in our study was equivalent to that obtained using the standard assay with donor CD4+ T lymphoblasts (9). The use of a relatively insensitive enzyme-linked immunosorbent assay (ELISA) to score viral outgrowth ensured that our results reflected the induction of proviruses capable of robust in vitro replication. Some noninduced proviruses detected by the intact proviral DNA assay may have had small defects affecting viral fitness. By performing near full-length proviral sequencing, we found that ~70% of proviruses called intact by the intact proviral DNA assay had no obvious defects at the primary sequence level, whereas the remaining 30% had small defects (73). CD4+ T cells that were activated in vivo and T cells that expressed CD25 such as regulatory T cells (Tregs) were not analyzed. We focused on resting CD4+ T cells because their transcriptional environment is nonpermissive for viral gene expression (60) and they have a longer half-life than activated CD4+ T cells (64). Although virus can be found in activated T cells, the stable HIV-1 latent reservoir resides mainly in resting T cells. An additional reason to focus on resting CD4+ T cells was that CD4+ T cell subsets cannot be reliably defined without considering activation status. We show here that expression of markers used to define T cell subsets changes markedly upon T cell activation (Fig. 3D); thus, recent in vivo T cell activation could have caused confounding changes in the expression of canonical markers if activated T cells had been included.

One approach to HIV-1 cure involves identifying markers that would allow selective targeting of latently infected T cells. Our results demonstrate that latent replication-competent proviruses are widely distributed among memory T cell subsets of HIV-infected individuals on ART with no consistent differences in virus inducibility, suggesting that such an approach would be challenging.

**MATERIALS AND METHODS**

**Study design**

This study was designed to determine differences in inducibility of latent HIV-1 across resting CD4+ memory T cell subsets. We obtained deidentified leukapheresis samples from 10 HIV-1–infected adults on ART who were enrolled in the University of California San Francisco SCOPE cohort. The only exclusion criteria were viremia within the previous 6 months and factors related to leukapheresis (vein access). Inclusion criteria were initiation of ART during chronic HIV-1 infection, viral suppression for >6 months, HIV RNA <40 copies/ml, and CD4+ T cell count >350 cells/μl. This study was approved by the Institutional Review Boards at the Johns Hopkins University and the University of California San Francisco. Written informed consent was obtained from all participants.

In this observational study with no intervention, there was no blinding or randomization. We isolated resting CD4+ T cells and sorted them by flow cytometry into naive T cells, TCM, TTM, and TEM subpopulations based on previously described markers (45–47). Frequencies of intact proviruses were assessed using the intact proviral DNA assay. T cell subsets were then cultured in a multiple stimulation viral outgrowth assay to assess differential inducibility to release replication-competent virus. Viral outgrowth was measured by ELISA for the HIV-1 p24 antigen in T cell culture supernatants. Expression of T cell subset–defining markers was analyzed throughout the culture period. Viral RNA from culture supernatants and proviral DNA from sorted resting T cells were sequenced to identify clones.

**Resting CD4+ T cell isolation and sorting strategy**

PBMCs were isolated from leukapheresis samples by Ficoll density centrifugation. CD4+ T cells were isolated from PBMCs using the
Viral RNA was isolated from culture supernatants saved after day 21. Complementary DNA was synthesized using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) and gene-specific primer ES8 (5’-CAGAAGAAGG-3’). The V3-V4 region of env was amplified using primers ES7 (5’-CTGT-1100CAGTGCTTACC-3’) and ES8, and Platinum SuperFi DNA Polymerase (Invitrogen). PCR products were run on a 1% agarose gel, extracted (Qiagen QIAquick Gel Extraction Kit), and then Sanger-sequenced (Genewiz) using sequencing primers Nesty8 (5’-TATAAATTGCTGTATGTATTATC-3’) and DLoop (5’-GTCTAGAGAAGAGG-3’). Sequences were analyzed in BioEdit, aligned by ClustalW, and trimmed to equal lengths. Neighbor-joining trees were generated using the maximum-likelihood method in MEGA6. Provaliral sequences were obtained from the same nested PCRs done on a limiting dilution of template DNA from resting subset cells.

All statistical analyses were conducted using GraphPad Prism. Data were analyzed as log-normal distributions based on D’Agostino-Pearson tests. Comparisons between T cell subsets were analyzed using Mann-Whitney two-tailed U tests. Correlations were assessed using Spearman rank tests. P values <0.05 were considered significant.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Representative flow cytometry plots of T cell subset phenotypes after stimulation.

Fig. S2. Results of the multiple stimulation viral outgrowth assay and intact proviral DNA assay (excluding data points below the limit of detection).

Fig. S3. Illustration of surface marker differences on resting versus activated CD4+ T cell subsets.

Table S1. Characteristics of study participants.

Table S2. Frequencies of proviral copies, infection frequencies, and assay input cell numbers for samples below the limit of detection.

Data file S1. Activation of T cell subsets in four representative participants.

Data file S2. Average expression of CCR7 and CD27 for all T cell subsets from all participants.

*View request a protocol for this paper from Bio-protocol.*

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Competing interests: R.F.S. is an inventor on patent no. 62/126,671 entitled “Novel assay for accurately measuring and characterizing the HIV-1 latent reservoir” filed by Johns Hopkins University and licensed by AccelevirDx. He holds no equity interest in AccelevirDx. R.F.S. consults for Merck and AbbVie on HIV cure–related issues. Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. The env sequences discussed here have been deposited in GenBank under accession nos. MN441770 to MN442053.

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Kyungyoon J. Kwon, Andrew E. Timmons, Srorna Sengupta, Francesco R. Simonetti, Hao Zhang, Rebecca Hoh, Steven G. Deeks, Janet D. Siliciano and Robert F. Siliciano

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Breaching the latent HIV reservoir

The main barrier to curing HIV-1 infection is a latent reservoir of the virus in resting CD4+ T cells, which allows the virus to persist in a form that is not detected by the immune system or affected by antiretroviral drugs. One cure strategy involves inducing viral gene expression so that latently infected T cells can be eliminated. It has been suggested that latent HIV-1 might be enriched in specific subpopulations of CD4+ T cells, which would allow more specific targeting of latency-reversing drugs. In a new study, Kwon et al. now report that surprisingly there was no preferential enrichment or differences in viral gene expression inducibility among naïve CD4+ T cells and three different subsets of memory CD4+ T cells from 10 HIV-infected individuals on antiretroviral therapy. This finding complicates targeted cure strategies based on CD4+ T cell subsets.