Establishment and Reversal of HIV-1 Latency in Naive and Central Memory CD4⁺ T Cells *In Vitro*

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ABSTRACT

The latent HIV-1 reservoir primarily resides in resting CD4⁺ T cells which are a heterogeneous population composed of both naive (T_N) and memory cells. In HIV-1-infected individuals, viral DNA has been detected in both naive and memory CD4⁺ T cell subsets although the frequency of HIV-1 DNA is typically higher in memory cells, particularly in the central memory (T_{CM}) cell subset. T_N and T_{CM} cells are distinct cell populations distinguished by many phenotypic and physiological differences. In this study, we used a primary cell model of HIV-1 latency that utilizes direct infection of highly purified T_N and T_{CM} cells to address differences in the establishment and reversal of HIV-1 latency. Consistent with what is seen *in vivo*, we found that HIV-1 infected T_N cells less efficiently than T_{CM} cells. However, when the infected T_N cells were treated with latency-reversing agents, including anti-CD3/CD28 antibodies, phorbol myristate acetate/phytohemagglutinin, and prostratin, as much (if not more) extra-cellular virion-associated HIV-1 RNA was produced per infected T_N cell as per infected T_{CM} cell. There were no major differences in the genomic distribution of HIV-1 integration sites between T_N and T_{CM} cells that accounted for these observed differences. We observed decay of the latent HIV-1 cells in both T cell subsets after exposure to each of the latency-reversing agents. Collectively, these data highlight significant differences in the establishment and reversal of HIV-1 latency in T_N and T_{CM} CD4⁺ T cells and suggest that each subset should be independently studied in preclinical and clinical studies.

IMPORTANCE

The latent HIV-1 reservoir is frequently described as residing within resting memory CD4⁺ T cells. This is largely due to the consistent finding that memory CD4⁺ T cells, specifically the central (T_{CM}) and transitional memory compartments, harbor the highest levels of HIV-1 DNA in individuals on suppressive therapy. This has yielded little research into the contribution of CD4⁺ naive T (T_N) cells to the latent reservoir. In this study, we show that although T_N cells harbor significantly lower levels of HIV-1 DNA, following latency reversal, they produced as many virions as did the T_{CM} cells (if not more virions). This suggests that latently infected T_N cells may be a major source of virus following treatment interruption or failure. These findings highlight the need for a better understanding of the establishment and reversal of HIV-1 latency in T_N cells in evaluating therapeutic approaches to eliminate the latent reservoir.

A latent HIV-1 reservoir is established in resting CD4⁺ T cells early during acute infection (1–5). This reservoir is unaffected by the immune system or by antiretroviral therapy (ART) but has the potential to produce infectious virus, which may contribute to persistent plasma viremia during ART or viral rebound if ART is interrupted. Therefore, HIV-1 cure strategies will need to include a therapeutic approach that eliminates, or significantly reduces the size of, this reservoir.

The resting CD4⁺ T cell population is heterogeneous and includes naive (T_N), stem cell-like memory, central memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}), and terminally differentiated cells. In HIV-1-infected individuals, viral DNA has been detected in all of these CD4⁺ T cell subsets although the levels of total HIV-1 DNA are typically highest in the T_{CM} and T_{TM} cell compartments, suggesting that they may be the major reservoirs of latent viral infection (6–8). However, recent analyses of the frequency of replication-competent virus in cells from infected individuals on suppressive ART, as measured by the quantitative viral outgrowth assay (QVOA), revealed that while replication-competent HIV-1 was consistently detected within T_{CM} cells, it was not frequently detected in the T_{TM} cell compart-

ment (9) although this finding has not been consistently observed in other studies (6, 10). This finding indicates that quantification of viral DNA alone is not necessarily predictive of the size of the inducible latent reservoir and suggests caution in labeling a cellular reservoir of latent HIV-1 as “major” based solely on the frequency of infection. In addition to the memory CD4⁺ T cell subsets, HIV-1 DNA is almost always detected in T_N cells in both viremic and suppressed individuals, although with a much lower frequency than in the T_{CM} and T_{TM} compartments (6, 7, 10–21). Interestingly, in 2013 Saez-Cirion et al. reported that in some HIV-1-infected individuals who received ART within 10 weeks of

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primary infection, viremia could be controlled for at least 24 months posttreatment interruption (8). In this patient population, HIV-1 DNA was detected in CD4⁺ T_N cells in only 2 of 11 individuals, whereas all the resting memory CD4⁺ T cell subsets (T_{CM}, T_{TM}, and T_{EM}) harbored comparable levels of HIV-1 DNA (8). This finding suggests that the latent HIV-1 reservoir in CD4⁺ T_N cells may be more important than previously considered.

The naive and different resting memory CD4⁺ T cell subsets differ in life span, proliferative capacity, antigen response time, residence throughout the body, and expression levels of their HIV-1 coreceptors, CCR5 and CXCR4 (22–24). In light of this, we hypothesized that the establishment and reversal of HIV-1 latency would differ between naive and memory CD4⁺ T cells and that understanding these phenotypes in different CD4⁺ T cell subsets could facilitate the development of effective cure strategies to purge the latent reservoir. Given the low frequency of latently infected cells in ART-suppressed individuals, approximately 100 copies of HIV-1 DNA or one infectious unit per 10⁶ resting CD4⁺ T cells (25–27), we sought to compare and contrast latent HIV-1 infection using a primary cell model in highly purified T_N and T_{CM} CD4⁺ T cells.

MATERIALS AND METHODS

Purification of T_N and T_{CM} CD4⁺ T cells. A total of 180 ml of blood was obtained from healthy HIV-negative volunteers, which was approved by the University of Pittsburgh Institutional Review Board. Written informed consent was provided for all donors. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque Plus (GE Healthcare) density gradient centrifugation. Resting CD4⁺ T cells were purified by first isolating total CD4⁺ T cells by magnetic bead negative selection using a CD4⁺ T cell purification kit, followed by magnetic bead negative selection using anti-CD25, anti-CD69, and anti-HLA-DR antibodies. T_N cells were isolated from the resting CD4⁺ T cells by magnetic bead depletion of CD45RO⁺ cells. T_{CM} cells were isolated from the resting CD4⁺ T cells by magnetic bead depletion of CD45RA⁺ cells, followed by positive selection of CCR7-expressing cells. Increased labeling times were used to increase cell purity. All magnetic bead purification kits and antibodies were obtained from Miltenyi Biotec. The purity of the T_N and T_{CM} cells was assessed by flow cytometry (LSR II; BD Biosciences) using the following antibodies: CD3-V450, CD4-peridinin chlorophyll protein (PerCP)-Cy5.5, CD45RA-fluorescein isothiocyanate (FITC), CCR7-phycoerythrin (PE), CD27-allophycocyanin (APC)-H7, and CD62L-APC (BD Biosciences). Data were analyzed using FlowJo, version X.0.7. The T_N and T_{CM} cell surface phenotypes were as follows: T_N cells, CD45RA⁺ CCR7⁺ CD27⁺ CD62L⁺; T_{CM} cells, CD45RA[−] CCR7⁺ CD27⁺ CD62L⁺. CD4⁺ T_N and T_{CM} cell purity levels were always found to be ≥98% and ≥96%, respectively (Fig. 1A).

Infection of T_N and T_{CM} CD4⁺ T cells. Purified T_N and T_{CM} CD4⁺ T cells were cultured at a density of 1 × 10⁶ to 2 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.29 mg/ml glutamine (all from Life Technologies). CCL19 (100 nM final concentration) was added to the cells 2 days prior to infection with HIV-1, as described previously (28, 29). Cells were infected with either the CXCR4-tropic strain HIV-1_{LAI} (30) or the CCR5-tropic strain HIV-1_{BaL} at a multiplicity of infection of 1 (titers were determined on GHOST cells) [31] for 2 to 3 h at 37°C. HIV-1_{BaL} was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from S. Gartner, R. C. Gallo, and M. Popovic (32). Cells were then washed twice with fresh medium to remove free virus. Every 2 days following infection, 10 units/ml recombinant interleukin-2 (IL-2; Roche) was added to the medium, in addition to 300 nM efavirenz (EFV; NIH AIDS Reagent Program) to inhibit multiple rounds of HIV-1 infec-

tion. For some experiments, 300 nM raltegravir (RAL; NIH AIDS Reagent Program) was also included to block multiple rounds of HIV-1 infection.

Flow cytometry. T cell activation was assessed by flow cytometry using the following antibodies from BD Biosciences: CD3-V450, CD4-PerCP-Cy5.5, CD25-PE-Cy7, CD69-PE, and HLA-DR-FITC. To measure the expression of the HIV-1 coreceptors CCR5 and CXCR4, T_N and T_{CM} cells were stained with CD3-V450, CD4-PerCP-Cy5.5, and either CCR5-PE or CXCR4-PE (BD Biosciences). Typically, 50,000 to 100,000 cells were collected per sample in the CD3⁺ CD4⁺ gate to adequately measure CCR5 or CXCR4 expression. Dead cells were excluded based on plots of side scatter area (SSC-A) and forward scatter area (FSC-A). For some experiments where noted in the text, cell viability was determined using a Live/Dead fixable cell viability dye for flow cytometry (Invitrogen). The intracellular proliferation marker Ki-67 was stained according to the manufacturer's protocol (BD Biosciences). However, instead of using the cell viability solution (7-amino-actinomycin D [7-AAD]) to discriminate live cells from dead cells, we first stained the cells with Live/Dead-APC (Invitrogen) prior to fixation and permeabilization for Ki-67 staining. All samples were run on an LSR II instrument, and the data were analyzed using FlowJo, version X.0.7.

Extraction and quantification of HIV-1 DNA. Total cellular DNA was extracted from pooled duplicate culture wells and was assayed for total HIV-1 DNA and two-long terminal repeat (2-LTR) circle DNA levels by quantitative PCR (qPCR), as described previously (33, 34). Each sample was run in triplicate using the LightCycler 480 System (Roche). DNA standards were included as described previously (33, 34). HIV-1 DNA and 2-LTR circles were normalized to the total number of cells assayed by quantitative PCR amplification of the CCR5 gene (35).

Integration site sequencing. Genomic DNA (20 μg) was isolated using a DNeasy blood and tissue kit (Qiagen) from resting and phytohemagglutinin (PHA)-activated T_N and T_{CM} CD4⁺ T cells infected with HIV-1_{LAI}. DNA was digested overnight with 100 U each of MseI and BglII and purified using a QIAquick PCR purification kit (Qiagen). Double-stranded asymmetric linkers were prepared by heating 10 μM each oligonucleotide to 90°C in 10 mM Tris-HCl, pH 8.0, and 0.1 mM EDTA and slowly cooling them to room temperature. Linker DNA (1.5 μM) was ligated with digested cellular DNA (1 μg) overnight at 12°C with 800 U of T4 DNA ligase in four parallel reactions, and the DNAs were pooled and repurified using a QIAquick PCR purification kit. Seminested PCR was used to selectively amplify integration sites, with reactions multiplexed into eight separate samples per PCR stage. The first and second rounds of PCR utilized nested HIV-1 U5 primers, whereas the same linker-specific primer was used for both rounds. The linker primer and second-round U5 primer each encoded adapter sequences necessary for Illumina sequencing, as well as for sequencing primer binding sites. To allow the identification of unique library samples from multiplexed sequencing runs, unique bar-coded linker DNAs and linker-specific primers were employed for each sample, and the nested U5 primer additionally encoded a unique 6-bp index sequence. The sequences of the oligonucleotides utilized are provided in Table 1. Each PCR mixture contained 100 ng of template DNA, 1.9 μM U5 primer, and 0.375 μM linker primer. Each reaction was carried out using Advantage 2 polymerase mix (Clontech), according to the manufacturer's instructions, and the reaction mixture was incubated at 94°C for 2 min, followed by 30 cycles at 94°C for 15 s, 55°C for 30 s, and 68°C for 45 s, with a final extension for 10 min at 68°C. Pooled PCR products were purified using a QIAquick PCR purification kit, and second-round reaction products were submitted for sequencing on the Illumina MiSeq platform at the Dana-Farber Cancer Institute Molecular Biology Core Facilities. Resulting sequences were mapped to the hg19 version of the human genome using BLAT, allowing for a minimum unique sequence identity match of 97%. Correlations of integration site distributions relative to various genomic features were conducted using BEDTools (36). Statistical analysis of resulting integration frequencies was determined using R (37, 38), with statistical significance being calculated by Fisher's exact test and a Wilcoxon rank sum test.

FIG 1 Infection of T_N and T_{CM} cells by CXCR4-tropic (HIV-1_{LAI}) and CCR5-tropic (HIV-1_{Bal.}) HIV-1 in the absence and presence of CCL19. (A) T_N and T_{CM} cells were purified from resting CD4⁺ T cells based on the variable cell surface expression of CD45RA, CCR7, CD27, and CD62L. T_N cells were defined as CD45RA⁺ CCR7⁻ CD27⁻ CD62L⁻. T_{CM} cells were defined as CD45RA⁻ CCR7⁺ CD27⁺ CD62L⁺. The purity of each subset was determined by surface

TABLE 1 Primer sequences used for integration site mapping

Oligonucleotide use	Oligonucleotide sequence (5'–3')
First-round HIV-1 LTR primer	TGTGACTCTGGTAACTAGAGATCCCTC
Activated CD4 ⁺ cells	
Linker top strand	PO ₄ -GTCCCTTAAGCGGAG-NH ₂
Linker bottom strand	GTAATACGACTCACTATAGGGCCTCCGCTTAAGGGACT
Linker primer	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTCTGCTGAACCGCTCTCCGATCTGTAATACGA CTCACTATAGGGC
HIV-1 LTR primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTCGATGTGAGATC CCTCAGACCCTTTTAGTCAG
CD4 ⁺ T _N cells	
Linker top strand	PO ₄ -CGAGGCGTCTAATGC-NH ₂
Linker bottom strand	GCTATAGCAGCACATCAGTTAGGCATTAGACGCCTCGT
Linker primer	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTCTGCTGAACCGCTCTTCCGATCTGCTATA GCAGCACATCAGTTAG
HIV-1 LTR primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTTGACCAGAG ATCCCTCAGACCCTTTTAGTCAG
CD4 ⁺ T _{CM} cells	
Linker top strand	PO ₄ -CTATGACGGTGACGC-NH ₂
Linker bottom strand	GAGAATCCATGAGTATGCTCACGCGTCACCGTCATAGT
Linker primer	CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTCTGCTGAACCGCTCTTCCGATCTGAGA ATCCATGAGTATGCTCAC
HIV-1 LTR primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTACAGTGG AGATCCCTCAGACCCTTTTAGTCAG

Quantification of F-actin density. Filamentous actin (F-actin) density was quantified by both flow cytometry and confocal microscopy. For flow cytometry, cells were first surface stained for CD3-V450 and CD4-PerCP-Cy5.5 (BD Biosciences); cells were then fixed in 1% paraformaldehyde (PFA), permeabilized with 0.1% Triton-X (Sigma-Aldrich) supplemented with 5% FBS, and stained with 100 nM phalloidin-FITC (100 nM; Sigma-Aldrich) in phosphate-buffered saline (PBS) supplemented with 5% FBS. Following staining, cells were washed with PBS supplemented with 5% FBS to remove any unbound phalloidin. All samples were run within 24 h on an LSR II system, and the data were analyzed using FlowJo software. A total of 10,000 events were collected per sample in the CD3⁺ CD4⁺ gate. Dead cells were excluded based on side scatter and forward scatter plots. Latrunculin A (Lat-A; Thermo Fisher) was added to cells 6 h prior to the addition of CCL19. At 48 h post-CCL19 treatment, cells were analyzed for F-actin density by flow cytometry as described above. For imaging, cells were fixed in 1% PFA, permeabilized with 0.1% Triton-X supplemented with 5% FBS, washed with PBS supplemented with 5% FBS, and then stained with 1 U/ml phalloidin-Alexa Fluor 488 (AF488) (Life Technologies, Beaverton, OR) in PBS supplemented with 5% FBS. The cells were washed, stained with 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI) (Life Technologies, Beaverton, OR), and washed again. After the second wash, the cell pellets were resuspended in Gelvatol mounting medium and mounted onto microscope slides for imaging. Images were collected using a Nikon A1 R spectral confocal microscope with a 100× (1.4 numerical aperture [NA]) oil immersion objective. Following collection using NIS-Elements software, images were parsed to Imaris (Bitplane) and rendered in three dimensions. The total

actin volume in each cell was calculated following surface extraction and volume rendering in Imaris.

Quantification of cellular dNTP concentrations. Cellular deoxy-nucleoside triphosphates (dNTPs) were extracted from 5 to 8 million cells and quantified as described previously (39).

Reactivation of latent HIV-1 from T_N and T_{CM} CD4⁺ T cells. Seven days postinfection, the T_N and T_{CM} CD4⁺ T cells were washed with medium and plated in a 96-well plate at a density of 100,000 cells/well. Anti-CD3/CD28 antibodies (3 beads per cell; Life Technologies), 10 nM phorbol myristate acetate (PMA; Sigma-Aldrich) with 10 μg/ml PHA (PMA-PHA) (Remel), 5 μM prostratin, or 500 nM suberoylanilide hydroxamic acid (SAHA; Cayman Chemicals) was then added to the well. Unstimulated cells were used as a control. At 3 and 7 days poststimulation, IL-2 and EFV were added to each well. To evaluate the decay of HIV-1-infected cells following latency reversal, HIV-1 DNA was quantified by qPCR, and the result was normalized to cell number as described above at each respective time point. The level of HIV-1 DNA/cell in unstimulated control cells was normalized to 100 for each donor. The level of HIV-1 DNA/cell following treatment with each latency-reversing agent (LRA) was then normalized to the level of HIV-1 DNA/cell from the unstimulated control cells. The log₁₀ of each value was then plotted on a linear scale to generate linear regression curves and decay rates.

Extraction and quantification of extracellular virion-associated HIV-1 RNA. Culture supernatant was centrifuged at 16,100 × g for 70 min to pellet HIV-1 virions. Viral RNA was then extracted using an RNeasy Plus minikit (Qiagen), and quantified using a real-time reverse transcriptase (RT)-initiated PCR assay with single-copy sensitivity, as de-

expression of each marker as measured by flow cytometry. Representative histograms from one donor are shown. (B) Schematic representation of the experimental approach. (C) Quantification of total HIV-1 DNA in T_N cells at 7 days postinfection. (D) Quantification of total HIV-1 DNA in T_{CM} cells at 7 days postinfection. (E) Quantification of HIV-1 2-LTR circles in T_N cells at 7 days postinfection. (F) Quantification of HIV-1 2-LTR circles in T_{CM} cells at 7 days postinfection. For graphs in panels C to F, each dot represents a unique donor, and data are normalized to cell number. ND, not detected; NS, not significant. *P* values for results in HIV-1_{LAI}-infected cells with and without CCL19 were calculated using a Wilcoxon matched-pairs signed-rank test. *P* values between HIV-1_{LAI} and HIV-1_{BaL} results were calculated using a Mann-Whitney test.

TABLE 2 HIV-1 integration site preference in PHA-activated, T_N, and T_{CM} CD4⁺ T cells

Sample	No. (%) of unique integration sites				
	Total	Within RefSeq genes	Within 5 kb (± 2.5 kb) of a transcriptional start site	Within 5 kb (± 2.5 kb) of a CpG island	Avg gene density within 1 Mb (± 0.5 Mb) of integration sites ^a
Activated CD4 ⁺ T cells	133,697	114,110 (85.3) ^c	6,242 (4.7) ^d	7,052 (5.3) ^e	20.3 ^f
CD4 ⁺ T _N cells	729	613 (84.1) ^g	30 (4.1%) ^h	33 (4.5) ⁱ	18.4 ^j
CD4 ⁺ T _{CM} cells	2,620	2,291 (87.4) ^k	109 (4.2%) ^l	112 (4.3) ^m	19.0 ⁿ
MRC ^b	50,000	22,328 (44.7)	2,010 (4.0)	2,100 (4.2)	8.7

^a Based on complete genes.^b Matched random control of 50,000 computer-generated integration sites in proximity to MseI/BglII restriction sites in hg19.^c *P* value (Fisher's exact test) versus values for the following: MRC, $<2.2 \times 10^{-308}$; T_{CM} cells, 0.003; T_N cells, 0.34.^d *P* value (Fisher's exact test) versus values for the following: MRC, 1.7×10^{-9} ; T_{CM} cells, 0.24; T_N cells, 0.53.^e *P* value (Fisher's exact test) versus values for the following: MRC, 1.0×10^{-21} ; T_{CM} cells, 0.02; T_N cells, 0.41.^f *P* value (Wilcoxon rank-sum) versus values for the following: MRC, $<2.2 \times 10^{-308}$; T_{CM} cells, 0.045; T_N cells, 0.0002.^g *P* value (Fisher's exact test) versus values for the following: MRC, 3.5×10^{-106} ; T_{CM} cells, 0.02.^h *P* value (Fisher's exact test) versus values for the following: MRC, 0.85; T_{CM} cells, 1.ⁱ *P* value (Fisher's exact test) versus values for the following: MRC, 0.64; T_{CM} cells, 0.76.^j *P* value (Wilcoxon rank-sum) versus values for the following: MRC, 1.7×10^{-101} ; T_{CM} cells, 0.017.^k *P* value (Fisher's exact test) versus the value for the MRC, $<2.2 \times 10^{-308}$.^l *P* value (Fisher's exact test) versus the value for the MRC, 0.72.^m *P* value (Fisher's exact test) versus the value for the MRC, 0.84.ⁿ *P* value (Wilcoxon rank-sum) versus the value for the MRC, $<2.2 \times 10^{-308}$.

scribed previously (40), using AffinityScript Multiple Temperature RT (Agilent Technologies) in place of Superscript II RT. The primers and probe used to quantify HIV-1 RNA were the same as those used to quantify total HIV-1 DNA. No RT control wells were run for each sample to ensure that amplification was from RNA only and not from DNA.

Statistical analyses. Statistical analysis of integration sites was determined by a Fisher's exact or Wilcoxon rank sum test (Table 2). Statistical comparison between paired samples was analyzed using a Wilcoxon matched-pairs signed-rank test. For all unpaired samples, statistics were determined using a Mann-Whitney test. For all statistical analyses, a *P* value <0.05 was considered significant.

RESULTS

Direct HIV-1 infection of CD4⁺ T_N and T_{CM} cells. Given the low infection frequency of HIV-1 in individuals on ART, it is difficult to use patient-derived cells to perform detailed *in vitro* analyses. Therefore, we first sought to establish appropriate *in vitro* primary cell models of HIV-1 latency in CD4⁺ T_N and T_{CM} cells. To maintain the integrity and authenticity of the T_N and T_{CM} cell populations, we considered only approaches that avoided significant T cell manipulation, including antigen stimulation or T cell differentiation. Following a review of several approaches, we expanded on the assay system developed by Saleh et al., which uses the chemokine CCL19 to enhance the permissiveness of resting CD4⁺ T cells to HIV-1 infection (29). Because prior published studies using this model had characterized the establishment and reversal of HIV-1 latency only in total resting CD4⁺ T cells (28, 29, 41), we first quantified the ability of X4-tropic (HIV-1_{LAI}) and R5-tropic (HIV-1_{BaL}) strains of HIV-1 to infect highly purified CD4⁺ T_N and T_{CM} cells (Fig. 1A) in the absence and presence of CCL19 (Fig. 1B). Both cell types were equally resistant to both HIV-1_{LAI} and HIV-1_{BaL} infection in the absence of CCL19 (Fig. 1C and D). We found that CCL19 significantly enhanced infection of both T_N (Fig. 1C) and T_{CM} (Fig. 1D) cells by HIV-1_{LAI}, as assessed by quantification of total HIV-1 DNA. However, T_{CM} cells were more efficiently infected (mean fold increase, 15.5) than were the T_N cells (mean fold increase, 3.65). CCL19 was also found to increase the ability of HIV-1_{BaL} to infect T_{CM}, but not T_N, cells (Fig. 1C and

D). The result with T_N cells could be due to low surface expression of CCR5 on T_N cells, which was not affected by exposure to CCL19 (Fig. 2). CCL19 expression also did not affect expression of CCR5 on T_{CM} cells or CXCR4 expression on either cell type (Fig. 2). A small percentage of HIV-1 reverse transcripts that fail to integrate are converted to two-long terminal repeat (2-LTR)-containing circles (42), and we accordingly quantified HIV-1 circle levels as a surrogate for unintegrated HIV-1 DNA (Fig. 1E and F). As expected, this analysis revealed that 2-LTR circles constituted only a minor proportion of the total intracellular HIV-1 DNA of both subsets. Moreover, the relative levels of 2-LTR circle DNA mimicked total HIV-1 levels across the different conditions of virus infection (Fig. 1).

Finally, we determined whether CCL19 or direct HIV-1 infection induced T cell activation or cellular proliferation of the purified T_N or T_{CM} cells (Fig. 3). We found that CCL19 treatment did not upregulate surface expression of the T cell activation marker CD25, CD69, or HLA-DR (Fig. 3A). However, a slight increase in CD25 expression was observed in all cells after 7 days of culture, which could not be attributed to HIV-1 infection (Fig. 3A). There was also no evidence of T cell proliferation as assessed by quantification of total cell number (Fig. 3B) or by intracellular staining of Ki-67 (Fig. 3C). Additionally, exposure of the T_N and T_{CM} CD4⁺ T cells to CCL19 or HIV-1 did not induce cell death (Fig. 3D).

Genomic distribution of HIV-1 integration sites in CD4⁺ T_N and T_{CM} cells. We next compared the genomic distribution of HIV-1 integration sites in infected T_N and T_{CM} cells and, as a control, compared these values to those obtained using total CD4⁺ T cells that were broadly stimulated by treatment with phytohemagglutinin (PHA). A total of 729, 2,260, and 133,697 unique integration sites were mapped in the CD4⁺ T_N, T_{CM}, and PHA-activated cells, respectively, with regard to several genomic annotations, including RefSeq genes, transcriptional start sites (TSSs), CpG islands, and gene density (Table 2). The statistical relevance of the observed frequencies versus a matched random control (MRC) data set was determined by Fisher's exact test for RefSeq

FIG 2 CCL19 treatment does not alter the expression of HIV-1 coreceptor expression on primary CD4⁺ T_N or T_{CM} cells. (A) Percent expression of CCR5 or CXCR4 on T_N or T_{CM} cells 2 days following treatment with CCL19 or anti-CD3/CD28 antibodies as determined by antibody staining and flow cytometry. Untreated cells were used as a control. (B) CCR5 and CXCR4 surface density as assessed by mean fluorescence intensity under the same conditions as described for panel A. No significant differences in the number of cells expressing CCR5 or CXCR4 or density of expression were noted between control cells or cells treated with CCL19 (statistics not shown; $n = 5$).

genes, TSSs, and CpG islands and by a Wilcoxon rank sum test for gene density. Distributions relative to CpG islands and TSSs were calculated by counting sites that fell within a 5-kb window (± 2.5 kb) of these features, while gene density was calculated by counting the number of RefSeq genes falling within a 1-Mb window (± 500 kb) of each integration site, and then averaging this value for the entire data set. Our MRC data set revealed that 44.7% of human DNA is comprised of RefSeq genes.

As expected (43), HIV-1 targeted RefSeq genes significantly more frequently than random sequences (T_N cells, 84.1%; T_{CM} cells, 87.4%; PHA-stimulated cells, 85.3%; P values ranging from 3.5×10^{-106} to $<2.2 \times 10^{-308}$). In contrast, the frequency of gene targeting across the different infection conditions was largely similar, with only minor differences noted for the comparisons between T_{CM} and PHA-stimulated cells and between T_N and T_{CM} cells (Table 2). Integration into gene-dense regions of chromosomes was similarly highly significant compared to random, whereas the differences between infected cell data sets were largely similar (P values from 0.0002 to 0.05). As expected (44), the numbers of HIV-1 integration sites nearby CpG islands or TSSs in CD4⁺ T_N and T_{CM} cells were similar to the MRC value although small but significant differences were noted between the MRC and PHA-activated cells (Table 2).

CCL19-mediated HIV-1 infection of CD4⁺ T_N and T_{CM} cells is not due to an increase in F-actin density. The actin cytoskeleton is known to be a key regulator in many early events of HIV-1 infection, including viral entry, reverse transcription, intracellular trafficking, and integration (45–48). In resting CD4⁺ T cells, cortical actin is static and is restrictive to HIV-1 infection due to actin and its regulators being in an inactive state. Additionally, Cameron et al. reported that CCL19 enhanced HIV-1 infection of resting CD4⁺ T cells via rapid dephosphorylation of cofilin and changes in filamentous actin (F-actin) density (28). To validate the role of F-actin density in HIV-1 infection, we first isolated total resting CD4⁺ T cells and exposed them to different concentrations of latrunculin A (Lat-A), a natural product that prevents F-actin assembly, for 6 h prior to the addition of CCL19 (Fig. 4A).

Forty-eight hours later, cell viability (Fig. 4C) and F-actin density (Fig. 4B) were measured by live/dead and phalloidin staining, respectively, and by flow cytometry. After the 48-h treatment, cells were infected with HIV-1_{LAI} and cultured for 7 days (Fig. 4A). HIV-1_{LAI} infection was then assessed by quantification of total HIV-1 DNA (Fig. 4D). Lat-A decreased F-actin density in a dose-dependent manner, as determined by phalloidin staining intensity (Fig. 4B), but did not impact cell viability (Fig. 4C). Importantly, the decrease in F-actin density correlated with a decrease in the ability of HIV-1_{LAI} to infect the resting CD4⁺ T cells (Fig. 4D).

In light of these findings, we next used confocal microscopy (Fig. 5A and B) and flow cytometry (Fig. 5C) to evaluate whether exposure of T_N and T_{CM} cells to CCL19 increased F-actin density. As described by Permanyer et al., we observed a higher baseline of F-actin density in T_{CM} cells than in T_N cells (Fig. 5B and C) (47). However, we found no evidence that CCL19 increased F-actin density in either T cell subset after incubation with the chemokine for 2 days (Fig. 5B and C). It should be noted that Cameron et al. observed a rapid increase in F-actin density within only a few minutes of exposure to CCL19, but after 30 min no significant differences were noted (28). Collectively, these data suggest that the very transient CCL19-mediated increase in F-actin density, reported previously, cannot explain the increased permissiveness of resting CD4⁺ T cells to HIV-1 infection 48 h post-chemokine exposure.

CCL19 does not alter intracellular dNTP levels in CD4⁺ T_N or T_{CM} cells. The sterile alpha motif (SAM) and histidine/aspartic acid (HD) domain-containing protein 1 (SAMHD1) imposes an effective restriction to HIV-1 infection in resting CD4⁺ T cells by enzymatically decreasing cellular dNTP pools and impeding HIV-1 reverse transcription (49–51). It has previously been shown that exogenous addition of dNTPs to resting, naive, or memory CD4⁺ T cells significantly enhances reverse transcription and viral integration without inducing T cell activation (52, 53). Therefore, we examined whether CCL19 could increase intracellular dNTP levels, which in turn would facilitate HIV-1 reverse transcription and viral infection of T_N and T_{CM} cells. Nucleotide

FIG 3 T cell activation, proliferation, and cell viability of purified CD4⁺ T_N and T_{CM} cells. (A) T cell activation markers CD25, CD69, and HLA-DR were assessed by antibody staining and flow cytometry on T_N and T_{CM} cells following purification (day -2), CCL19-treatment (day 0), and HIV-1_{LAI} infection (day 7, left) and on uninfected cells (day 7, right). Data are presented as the means $\pm$ standard errors of the means ($n = 7$). (B) Cellular proliferation of unstimulated cells was measured by qPCR of the *CCR5* gene throughout the time course of the experiment. Data are presented as the means $\pm$ standard errors of the means ($n = 6$). (C) Intracellular staining and flow cytometry for Ki-67. Data are presented as the means $\pm$ standard errors of the means ($n = 2$ performed in duplicate). (D) Cell viability was measured by Live/Dead staining and flow cytometry in freshly isolated, T_N and T_{CM} cells, in cells with and without CCL19 treatment for 2 days, and in cells with and without HIV-1 infection after 7 days, as indicated. Data are presented as the means $\pm$ T_N and T_{CM} cells ($n = 3$ performed in duplicate).

levels in the T_N and T_{CM} cells were much lower than those in anti-CD3/CD28-activated cells (Table 3). However, treatment of T_N or T_{CM} cells with CCL19 did not increase the intracellular dNTP levels compared to those of the untreated controls (Table 3), which suggests that CCL19 does not enhance HIV-1 infection of T_N and T_{CM} cells by increasing the nucleotide pool concentration.

Reactivation of HIV-1_{LAI} from CD4⁺ T_N and T_{CM} cells. We next quantified total virus production (i.e., extracellular virion-associated RNA [vRNA] in the culture supernatant) from latently infected T_N and T_{CM} cells using an ultrasensitive assay capable of single-copy detection of HIV-1 RNA (40, 54) before and after exposure to the following latency-reversing agents (LRAs): (i) anti-CD3/CD28 antibodies (3 beads per cell), (ii) 10 nM phorbol 12-myristate 13-acetate (PMA) plus 10 μ g/ml PHA, (iii) 5 μ M prostratin, or (iv) 500 nM suberoylanilide hydroxamic acid (SAHA) (Fig. 6A). As expected, we saw more total virus production from T_{CM} cells than from the T_N cells (Fig. 6B), which is consistent with the observation that the T_{CM} population contained significantly higher levels of HIV-1 DNA.

To account for differences in HIV-1 infection frequency between the T_N and T_{CM} cell subsets, as well as between different donors, we normalized extracellular vRNA production to the total

HIV-1 DNA copy number/cell at each respective time point (Fig. 6C). Surprisingly our data revealed that T_N cells exposed to anti-CD3/CD28 antibodies, PMA-PHA, or prostratin yielded as much (or more) vRNA as the T_{CM} cells (Fig. 6C). For example, at day 10 the median production of extracellular HIV-1_{LAI} vRNA after exposure to anti-CD3/CD28 antibodies, PMA-PHA, or prostratin was 69.7, 71.5, and 29.6 vRNA copies/infected cell from T_N cells, compared to 18.8, 57.2, and 21.2 vRNA copies/infected cell from T_{CM} cells, respectively. There was, however, significant variation between donors (Fig. 6D). For example, in donors 2, 4, 5, and 6, more extracellular HIV-1_{LAI} vRNA was produced from the T_N cells, whereas more HIV-1_{LAI} was produced from the T_{CM} cells of donor 3 (Fig. 6D). Collectively, these data suggest that donor genetic differences, in addition to the resting CD4⁺ T cell compartment, impact the establishment and reversal of HIV-1 latency. In contrast to the other LRAs, SAHA did not significantly increase vRNA production in either T_N or T_{CM} cells (Fig. 6B and C). This finding is consistent with recent studies that demonstrated the inability of SAHA to increase extracellular HIV-1 production from resting CD4⁺ T cells isolated from infected individuals on suppressive ART (55–57).

Many HIV-1 reverse transcripts fail to integrate, resulting in the accumulation of viral DNA that has the potential to persist in

FIG 4 Inhibition of F-actin polymerization blocks HIV-1 infection of total resting CD4⁺ T cells in a dose-dependent manner. (A) Schematic representation of the experimental approach. (B) Cells were treated with different concentrations of Lat-A for 6 h, followed by treatment with CCL19 for an additional 2 days. F-actin was stained with phalloidin and measured by flow cytometry. Cells stimulated with PMA plus IL-2 were used as a positive control. (C) Following the same experimental conditions as in described for panel B, cell viability was assessed by flow cytometry using Live/Dead staining. Untreated cells were used as a negative control, and cells heated at 56°C for 1 h prior to staining were used as a dead cell control. (D) F-actin density and HIV-1 infection of resting CD4⁺ T cells are plotted. Following the experimental approach shown in panel A, HIV-1 infection was measured at 7 days postinfection by quantification of total intracellular HIV-1 DNA, normalized to cell number. HIV-1 DNA and F-actin density were normalized to treatment with CCL19 only. Data shown for panels B to D are representative of two independent experiments and, error bars represent standard deviations. MFI, mean fluorescence intensity.

FIG 5 CCL19 does not have an effect on F-actin density in T_N or T_{CM} cells. (A) Representative confocal microscopy images of T_N and T_{CM} cells in the absence or presence of CCL19 or PMA plus IL-2 for 2 days. Phalloidin and DAPI were used to stain F-actin and nuclei, respectively. (B) Total F-actin volume was quantified in T_N and T_{CM} cells from confocal microscopy images using Imaris software. (C) Flow cytometric analysis of F-actin density was measured by phalloidin staining in T_N or T_{CM} cells under the same conditions as described for panel A. Data are representative of three independent experiments. MFI, mean fluorescence intensity.

resting CD4⁺ T cells (58–61). To exclude the possibility that some forms of this unintegrated HIV-1 DNA become integrated upon T cell activation, resulting in productive infection and virus particle production, we next assessed the contribution of unintegrated HIV-1_{LAI} DNA to extracellular vRNA production from both T_N and T_{CM} cells by including the integrase inhibitor raltegravir (RAL) at the same time as anti-CD3/CD28 (Fig. 6E and F). This analysis suggested that unintegrated viral DNA did not significantly contribute to the extracellular vRNA quantified in the supernatant following reversal of latency in our model system.

Reactivation of HIV-1_{BaL} from CD4⁺ T_N and T_{CM} cells. We

also evaluated extracellular HIV-1_{BaL} RNA production from infected T_N and T_{CM} cells exposed to the same LRAs; however, we excluded SAHA, given that we did not see an effect in our previous experiments (Fig. 6B and C). HIV-1_{BaL} vRNA was produced from T_{CM} cells, with no differences noted compared to production by HIV-1_{LAI} (Fig. 7). In contrast, almost no detectable HIV-1_{BaL} vRNA was produced from the T_N cells (Fig. 7). However, as shown by the data in Fig. 1, these cells were largely refractory to infection by HIV-1_{BaL}.

Decay of HIV-1_{LAI}-infected CD4⁺ T_N and T_{CM} cells after exposure to latency-reversing agents. We next measured the decay

TABLE 3 Intracellular dNTP levels in T_N and T_{CM} CD4⁺ T cells in the absence and presence of 100 nM CCL19

Cell type and treatment	Nucleotide concn (fmol/10 ⁶ cells) ^a			
	dATP	dGTP	dCTP	dTTP
T _N cells	8.70 (<4–10.7)	32.0 (14.6–44.5)	27.7 (<4–43.1)	5.90 (<4–7.60)
T _N cells + CCL19	4.70 (<4–6.10)	28.2 (17.5–29.4)	14.7 (<4–30.6)	4.70 (<4–7.30)
T _N + anti-CD3/CD28	379 (112–513)	484 (404–622)	88.6 (62.7–111)	649 (244–735)
T _{CM} cells	3.70 (<4–5.8)	30.0 (19.5–77.1)	11.5 (5.75–21.0)	4.40 (<4–6.90)
T _{CM} cells + CCL19	4.10 (<4–4.90)	24.6 (11.6–61.6)	10.0 (4.80–17.3)	4.90 (<4–12.3)
T _{CM} + anti-CD3/CD28	285 (57.2–294)	232 (217–345)	41.0 (31.9–61.8)	164 (106–188)

^a Data are presented as the median (range) of three independent experiments. A value of <4 indicates a level below the limit of detection based on the number of cells used for quantification.

FIG 6 Reversal of HIV-1 latency in CD4⁺ T_N and T_{CM} cells infected with HIV-1_{LAI} following treatment with LRAs. (A) Schematic representation of the experimental approach. (B) Total copies of extracellular virion-associated HIV-1_{LAI} RNA produced from T_N or T_{CM} cells after exposure to anti-CD3/CD28 antibodies, PMA-PHA, prostratin, or SAHA. Background HIV-1 RNA from unstimulated controls at each time point is also shown. Data are the means $\pm$ the standard errors of the means from 6 donors. (C) Copies of extracellular virion-associated HIV-1_{LAI} RNA produced per infected T_N or T_{CM} cell after exposure to anti-CD3/CD28 antibodies, PMA-PHA, prostratin, or SAHA, normalized to the level of infection at each respective time point. Background HIV-1 RNA from unstimulated controls is shown. Data are shown as the means $\pm$ standard errors of the means from 6 donors. (D) Copies of extracellular vRNA produced per infected T_N or T_{CM} cell after exposure to anti-CD3/CD28 antibodies from 6 donors. The contribution of unintegrated HIV-1_{LAI} DNA to the total vRNA copy number after exposure of infected T_N cells (E) or T_{CM} cells (F) with anti-CD3/CD28 antibodies was determined with or without EFV only or EFV-RAL treatment throughout the experiment. Unstimulated cells treated with EFV only were used as a control. Cells stimulated in the absence of any antiretroviral drugs were used as a positive control. Data are representative of two independent experiments.

FIG 7 Reversal of HIV-1 latency in CD4⁺ T_{CM} cells infected with HIV-1_{BaL} following treatment with LRAs. The experimental approach was the same here as shown in Fig. 5A. The number of copies of extracellular virion-associated HIV-1_{BaL} RNA produced per infected T_{CM} cell after exposure to anti-CD3/CD28 antibodies, PMA-PHA, or prostratin, normalized to the level of infection at each respective time point, is shown. Background HIV-1 RNA from unstimulated controls at each time point is shown. Data are shown as the means $\pm$ standard errors of the means from 3 donors. For comparative purposes, data for HIV-1_{LAI} RNA from infected T_{CM} cells (Fig. 6C) are included.

kinetics of HIV-1_{LAI}-infected cells in both T cell populations after exposure to the different LRAs (Fig. 8A and B). The rates of decay (half-life [$t_{1/2}$]) of the HIV-1_{LAI}-infected T_N cells treated with anti-CD3/CD28, PMA-PHA, or prostratin were 4.2, 3.5, and 4.8 days, respectively (Fig. 8A), similar to the values calculated for the T_{CM} cells (2.6, 2.5, and 5.2 days for cells treated with anti-CD3/CD28, PMA-PHA, and prostratin, respectively) (Fig. 8B). No decay was observed in either T_N or T_{CM} cells treated with SAHA. Of note, the anti-CD3/CD28 antibodies, PMA-PHA, and prostratin all induced cell activation in both T cell subsets, as evidenced by increased expression of CD69, CD25, and HLA-DR (Fig. 8C). The levels of cellular activation were also similar between T_N and T_{CM} cells and likely do not contribute to the differences observed in virus production between these cell types.

DISCUSSION

Latently infected resting CD4⁺ T cells constitute the major reservoir of persistent HIV-1 infection, and significant reduction or elimination of this reservoir could lead to either a functional or sterilizing cure, respectively. It has been hypothesized that therapeutic approaches that reactivate latent HIV-1 infection will promote death of the infected cell by viral cytopathic effects and/or by host cell effector mechanisms (62). This strategy is typically referred to as the “kick-and-kill” approach (63). The resting CD4⁺ T cell population, however, is heterogeneous and consists of different T cell subsets, including naive and memory cells. It is not known whether the kick-and-kill approach will be equally effective in the different T cell subsets, which differ in life span, proliferative capacity, antigen response time, residence throughout the body, and CCR5 and CXCR4 expression levels (22–24). Therefore, mechanistic insights into the establishment and reversal of latent HIV-1 infection in different resting CD4⁺ T cell subsets could provide important clues to eradicating this persistent reservoir.

In this study, we compared the establishment and reversal of HIV-1 latency in resting CD4⁺ T_N and T_{CM} cells using a primary cell model of latency that utilizes direct infection of highly purified cells. Prior studies demonstrated that HIV-1 latency could be established *in vitro* in resting CD4⁺ T cells pretreated with chemo-

kines that bind to the CCR6, CCR7, or CXCR3 receptor (28). From an important biological perspective, the concentration of CCL19 (the chemokine ligand for CCR7 used in this study) significantly increases during the acute phase of HIV-1 infection in which the latent reservoir is established and correlates with disease progression (64–67). Furthermore, pretreatment of T_N or T_{CM} cells with CCL19 does not induce T cell activation or proliferation (Fig. 3). Thus, the integrity of the purified T cell subsets is largely preserved in the experiment, which was an important prerequisite for the objectives of this study.

We show that CCL19 pretreatment of T_N and T_{CM} cells significantly increased the capacity for X4-tropic HIV-1 to infect these cells (Fig. 1). However, as reported in other *in vitro* systems (10, 53, 68), the levels of HIV-1 infection in the T_{CM} cells, as assessed by quantitation of total viral DNA, were higher than in the T_N cells. In contrast, CCL19 increased R5-tropic virus infection of only T_{CM} cells and not T_N cells, a finding which may be attributable to the extremely low levels of CCR5 expression on T_N cells isolated from healthy donors (Fig. 2). Interestingly, several studies have demonstrated that R5-tropic virus can be isolated from CD4⁺ T_N cells from HIV-infected individuals (15, 17, 69, 70). There are two possible explanations that could account for the discrepancies between *in vitro* studies and the *in vivo* observations: (i) HIV-1 infection systematically upregulates CCR5 expression on T_N cells, thus making them more susceptible to infection (71–74), and/or (ii) R5-tropic virus may be more efficiently transferred to T_N cells by plasmacytoid dendritic cells (75–77). Further studies, however, are needed to elucidate the mechanism(s) by which T_N cells become infected *in vivo*.

We also attempted to address the mechanism by which CCL19 increases the ability of HIV-1 to infect T_N and T_{CM} cells. In this regard, Cameron et al. (28) reported that ligation of the CCR6, CCR7, and CXCR3 receptors led to changes in cortical actin, allowing rapid migration of the preintegration complex to the nucleus and efficient nuclear localization and integration. While our data show a critical role for F-actin density in the ability of HIV-1 to infect resting CD4⁺ T cells (Fig. 4) and suggest that T_N cells may be less susceptible to HIV-1 infection than T_{CM} cells due to a lower F-actin density, as reported previously (46, 47), our comprehensive imaging and flow cytometry data do not support a role for CCL19 in increasing F-actin density in either cell type (Fig. 5). SAMHD1 has also been identified as an effective restriction factor to HIV-1 infection in resting CD4⁺ T cells by enzymatically decreasing cellular dNTP pools and impeding HIV-1 reverse transcription (22, 49, 51, 78). Interestingly, blockade or degradation of SAMHD1 has been shown to greatly enhance the susceptibility of resting CD4⁺ T cells to HIV-1 infection, especially in T_N cells (50, 68). These findings are consistent with earlier studies showing that addition of dNTPs to T_N or memory cells significantly enhanced reverse transcription and integration (52, 53). In this study, we found that CCL19 does not alter intracellular dNTP concentrations and that HIV-1 can infect both T_N and T_{CM} cells despite low nucleotide concentrations. Collectively, these data suggest that CCL19 pretreatment could potentially impact an as yet undocumented restriction factor in resting CD4⁺ T cells.

An unexpected finding from this study was that T_N cells exposed to LRAs produced as much (if not more) extracellular virion-associated RNA per infected cell as the T_{CM} population (Fig. 6). This observation was found to be independent of the LRA used. These data would suggest that more latently infected T_N cells

FIG 8 Decay of HIV-1_{LAI}-infected cells and T cell activation posttreatment of latently infected CD4⁺ T_N and T_{CM} cells. Decay of HIV-1_{LAI}-infected T_N (A) or T_{CM} (B) cells was measured over 10 days of treatment with anti-CD3/CD28 antibodies, PMA-PHA, prostratin, or SAHA. Total intracellular HIV-1 DNA was quantified as described in the legend of Fig. 1. Data are presented as the means $\pm$ standard errors of the means from 6 donors (except for the SAHA data, where $n = 4$). (C) Cellular proliferation of T_N and T_{CM} cells was measured by qPCR of the *CCR5* gene following stimulation with anti-CD3/CD28 antibodies, PMA-PHA, prostratin, or SAHA. Background samples represent unstimulated CD4⁺ T_N and T_{CM} cells. Data are presented as the means $\pm$ standard errors of the means ($n = 6$, except for SAHA data, where $n = 4$). (D) T cell activation was measured by antibody staining and flow cytometry of the surface expression of CD25, CD69, and HLA-DR on T_N or T_{CM} cells at 3, 7, and 10 days after treatment with anti-CD3/CD28 antibodies, PMA-PHA, prostratin, or SAHA. Untreated cells were used as a negative control. Data are representative of two independent experiments.

could produce virus than T_{CM} cells. However, we observed no major differences in the genomic distribution of HIV-1 integration sites between the two T cell subsets. Given these differences in virus production between T_N and T_{CM} cells, we also evaluated

whether HIV-1 reactivation resulted in death of the infected cell. We found that the rates of decay of the HIV-1-infected cells in the T_N and T_{CM} populations treated with anti-CD3/CD28 antibodies, PMA-PHA, or prostratin were largely equivalent (Fig. 7). Using a

different *in vitro* primary cell model of latency, Shan et al. reported that if the LRA induced T cell activation, reactivation of latent HIV-1 resulted in death of the infected cell (79). Our data support this finding. However, Shan et al. (79) reported that following administration of SAHA, which does not induce T cell activation, the HIV-infected resting CD4⁺ T cell population survived, even in the presence of autologous cytolytic T lymphocytes. In other words, SAHA facilitated the kick but not the kill. In contrast, we observed that SAHA promoted neither reactivation of latent HIV-1 nor death of the infected cell. Alternatively, in both primary cell models of latency, the decrease in frequency of HIV-1-infected cells could be due to preferential expansion of only the uninfected cell population after T cell activation.

In conclusion, this study highlights the differences in regard to the establishment and reversal of HIV-1 latency in T_N and T_{CM} cells. Importantly, the data reveal that despite low infection frequency, T_N cells produce as much (if not more) extracellular virion-associated vRNA per infected cell as T_{CM} cells. This suggests that T_N cells may be an important reservoir of latent HIV-1 infection and should not be ignored simply because the frequency of infection of these cells is lower than that in the memory T cell subsets in infected individuals on ART. Importantly, we have presented a novel approach to study HIV-1 latency in a primary cell model, focusing specifically on CD4⁺ T_N and T_{CM} cell subsets that can further be used to understand the establishment, maintenance, and reversal of latency in both subsets.

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