HIV-1 Vpr drives a tissue residency-like phenotype during selective infection of resting memory T cells

Reuschl et al. show that HIV-1 cell-to-cell spread promotes productive infection of resting T cells, bypassing the need for in vitro activation. They discover that HIV-1 Vpr reprograms resting T cells phenotypically and transcriptionally to gain characteristics of tissue-resident memory T cells, suggesting that HIV-1 drives changes that may affect viral reservoir establishment and persistence.

Highlights

- Cell-to-cell spread makes resting CD4+ T cells permissive to HIV-1 infection
- Resting memory T cells are productively infected by HIV-1
- HIV-1 Vpr drives resting memory T cells to gain characteristics of tissue residency
- Vpr induces widespread transcriptional reprogramming of infected T cells

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SUMMARY

HIV-1 replicates in CD4+ T cells, leading to AIDS. Determining how HIV-1 shapes its niche to create a permissive environment is central to informing efforts to limit pathogenesis, disturb reservoirs, and achieve a cure. A key roadblock in understanding HIV-T cell interactions is the requirement to activate T cells in vitro to make them permissive to infection. This dramatically alters T cell biology and virus-host interactions. Here we show that HIV-1 cell-to-cell spread permits efficient, productive infection of resting memory T cells without prior activation. Strikingly, we find that HIV-1 infection primes resting T cells to gain characteristics of tissue-resident memory T cells (TrM), including upregulating key surface markers and the transcription factor Blimp-1 and inducing a transcriptional program overlapping the core TrM transcriptional signature. This reprogramming is driven by Vpr and requires Vpr packaging into virions and manipulation of STAT5. Thus, HIV-1 reprograms resting T cells, with implications for viral replication and persistence.

INTRODUCTION

Resting primary CD4+ T cells cannot be efficiently infected by cell-free HIV-1 virions in vitro and require robust mitogenic stimulation to support viral replication (Stevenson et al., 1990; Swiggard et al., 2005; Zack et al., 1990). This has led to the notion that T cell activation is necessary for HIV-1 replication. However, mitogenic T cell activation in vitro results in widespread phenotypic and functional reprogramming, which dominates changes in gene and protein expression (Howden et al., 2019; Szabo et al., 2019a; Wolf et al., 2020), concealing and potentially altering authentic virus-host interactions. This presents a significant challenge for understanding the cellular response to HIV-1 infection and the consequences of the virus-host interaction for HIV-1 replication and persistence. While it is clear that HIV-1 efficiently infects and replicates in activated T cells, the outcomes of the virus-host interaction with resting T cells have been reported to be cell death (Doitsh et al., 2010) or latency (Agosto et al., 2018). However, previous data demonstrating that HIV-1 cell-to-cell spread is highly efficient and drives widespread changes in protein phosphorylation status in both infected and target cells (Hübner et al., 2009; Jolly et al., 2004; Len et al., 2017; Sattentau, 2008; Soursseau et al., 2007) suggested that cell-to-cell spread may overcome the barrier to productive infection of resting T cells. Here, we comprehensively show for the first time that HIV-1 exploits cell-to-cell spread to efficiently infect resting memory CD4+ T cells, and we have used this to uncover a hitherto unknown consequence of HIV-1 infection for T cell reprogramming driven by the accessory protein Vpr, inducing cells to gain characteristics of tissue-resident memory T cells.
Figure 1. HIV-1 exploits cell-to-cell spread to preferentially infect resting memory CD4+ T cells
(A) HIV-1 NL4.3 infected mitogenically activated primary CD4+ donor T cells co-cultured with resting autologous primary CD4+ target T cells separated by a 0.4 μm transwell (cell-free) or in direct co-culture (cell-cell). Target cell infection was measured by intracellular staining for HIV-1 Gag protein. Representative flow cytometry plots are shown. Bar graphs show mean of independent experiments (n = 4).
(B) Cell-to-cell spread into resting or αCD3/αCD28-activated CD4+ target T cells measured by intracellular Gag expression (n = 5).
(C and D) Cell-to-cell spread from activated primary donor CD4+ T cells to resting primary target CD4+ T cells preferentially infects CD45RA- memory CD4+ T cells. A representative flow cytometry plot and quantification are shown (n = 4).
(E) Quantification of infection performed as in (C) (n = 11).
(F) HIV-1 infection of target CD4+ T cells as part of the total resting CD4+ T cell population (total) compared with pre-isolated naive and memory CD4+ target T cells (isolated) (n = 9).
(G and H) Quantification of infection of CXCR4 (X4)- and CCR5 (R5)-tropic viruses (n = 4) (G) and transmitter-founder viruses HIV-1 CH040 and CH077 (n = 7) (H).
(I) Representative flow cytometry plots of cell-to-cell infection of resting CD4+ T cells with CCR5-tropic HIV-1 NL4.3 BaL and transmitter founder viruses HIV-1 CH040 and CH077 as performed in (C).
(J and K) Cell-to-cell infection of resting CD4+ T cells is reduced by the HIV-1 fusion inhibitor T20 (n = 6) (left) and the reverse transcriptase inhibitor efavirenz (n = 6) (right) measured by intracellular Gag staining (median fluorescence intensity, MFI) (J) or HIV-1 LTR-driven GFP-reporter gene expression (n = 4) (K).
(L) HIV-1 infection downregulates CD4 expression. Shown is the percentage of CD4+ cells in the total CD3+ target cell population (n = 6).
(M–O) Resting CD4+ memory T cells were isolated after 72 h of cell-to-cell spread by fluorescence-activated cell sorting (FACS) and cultured for 4 days. HIV-1 infection was measured by intracellular Gag staining (M) and virus release was measured by culture supernatant reverse transcriptase (RT) activity (N) (n = 4–7).
RESULTS

HIV-1 cell-to-cell spread drives productive infection of resting memory CD4+ T cells

To test whether cell-to-cell spread allows for productive infection of resting T cells, HIV-1-infected primary CD4+ T cells were cocultured with uninfected autologous resting CD4+ T cells (Figures 1A and S1A–S1E). We confirmed that CD4+ T cells isolated from peripheral blood display a resting phenotype by staining for Ki67, CD69, CD25, CD38, HLA-DR, and MCM2 (Figures S1C–S1E). Infection of resting target cells in the absence of mitogenic or cytokine activation was measured. Direct co-culture of infected and uninfected cells resulted in significant levels of HIV-1 infection of resting CD4+ target T cells (Gag+) measured by intracellular flow cytometry staining (Figures 1A and S1F). By contrast, resting CD4+ T cells were not infected (<1%) when cell-cell contact was prevented by separating the two cell populations by a transwell (Figures 1A and S1F), a condition that allows for only cell-free infection. As expected, mitogenic activation of primary target T cells made them more permissive to HIV-1 infection (Figures 1B and S1F–S1H), but as previously shown, infection was still substantially boosted by direct co-culture allowing for cell-to-cell spread (Figures S1F–S1H) (Hubner et al., 2009; Jolly et al., 2004; Sourisseau et al., 2007). Resting T cells remained refractory to cell-free infection even when incubated with high doses of virus (Figure S1I) (excluding the concentration of virus detected in a cell-to-cell co-culture Figure S1J), while, as expected, activated CD4+ T cells could be infected by cell-free virus (Figure S1I). Thus, we show that resting T cells are highly refractory to cell-free HIV-1, but this can be overcome during infection mediated by cell-to-cell spread.

Infection of resting CD4+ target T cells by cell-to-cell spread was preferentially detected in CD45RA− resting memory T cell populations rather than CD45RA+ naive T cells, which are both abundant in peripheral blood (Figures 1C–1E and S2A). Co-staining for CD62L confirmed that the infected CD45RA− cells were mainly naive rather than TEmRA (Figures S2B and S2C) (Sallusto et al., 1999). The preferential infection of CD45RA− memory T cell populations rather than the CD45RA+ naive population is in agreement with HIV-1 being predominantly detected in memory CD4+ T cells in vivo (Brenchley et al., 2004; Chomont et al., 2009; Shan et al., 2017). This was not due to competition between naive and memory cells, because the same effect was observed when CD45RA+ and CD45RA− resting CD4+ target T cells were separated prior to cell-to-cell infection (Figures 1F and S2D). Of note, when measuring infection only in the permissive resting memory T cell population, cell-to-cell spread resulted in up to 60% infection (Figures 1D–1F). As expected this is much higher than observed in the total resting T cell population (Figure 1A), in which the analysis did not distinguish between naive (non-permissive) and memory (permissive) CD4+ target T cells. Cell-to-cell infection of resting memory T cells was observed with the CXCR4-tropic strain NL4.3 and CCR5-tropic viruses NL4.3 BaL and two transmitter-founder (T/F) primary isolates (CH040 and CH077) (Figures 1G–1I and S2E), demonstrating that increased permissivity was not unique to a particular virus or receptor tropism. Preventing viral entry with a fusion inhibitor (T20), or blocking reverse transcription (efavirenz), inhibited the appearance of Gag+ and also GFP+ cells (the latter using a replication-competent GFP-reporter virus) (Figures 1J, 1K, and S2F–S2I), demonstrating that this signal reflects productive infection and not simply virus capture (Hubner et al., 2009; Jolly et al., 2004; Len et al., 2017; Sourisseau et al., 2007). Consistent with productive infection, we also observed downregulation of CD4 expression on target cells that was most pronounced in the resting memory T cell population (Figures 1L, S2J, and S2K). To confirm that productively infected resting memory T cells can make new virus and propagate viral spread, we recovered HIV-1-infected resting memory target cells from co-cultures by flow sorting (Figures S1M and S2L) and returned them into culture without activation to measure infected cells and virus output over time. This confirmed that these cells supported viral replication by (1) spreading infection within the resting T cell population as evidenced by an increase in the number of Gag-positive resting T cells over time (Figure 1M), (2) producing new virus that was detected in culture supernatants (Figure 1N), and (3) transmitting infection to fresh target cells added to the culture (Figure 1O). Activating the recovered resting memory T cells by T cell receptor (TCR) cross-linking further boosted virus production, confirming that the cells remained responsive to stimuli (Figures S2M and S2N). Interestingly, resting T cells infected by cell-to-cell spread were also longer lived than their matched activated counterparts and showed improved persistence after restimulation with αCD3/αCD28 (Figures S1K and S1L), suggesting these cells may contribute to the establishment of a longer-lasting infected T cell reservoir. Collectively, these data demonstrate that cell-to-cell spread drives productive infection of resting CD4+ T cells that have the capacity to disseminate infection.

HIV-1 infection induces resting memory CD4+ T cells to gain characteristics of tissue-resident T cells by synergizing with interleukin-7

We confirmed that HIV-1+ target T cells infected by cell-to-cell spread maintained their resting phenotype and did not upregulate Ki67 or MCM2, two markers of cell-cycle progression (Figures S3A and S3B). Therefore these cells are not simply being activated and driven into the cell cycle by either infection or bystander effects during co-culture. Intriguingly, expression of CD69 on HIV-1-infected resting memory target T cells was significantly increased compared with mock-treated (uninfected) target T cells (Figures 2A and 2B). Importantly, this was not due to preferential infection of a minor pre-existing population of CD69+ CD4+ T cells in blood (Figure S1C). We confirmed this by removing the small proportion of CD69+ blood T cells recovered at day 1 or 4 post-isolation were then cultured with uninfected eFluor450+ target Jurkat T cells, and infection of Jurkat T cells was measured after 72 h (O) (n = 3). All measurements were made after 72 h or at the indicated time post co-culture. Data are the mean ± SEM. Paired two-tailed t test or one-way ANOVA with Bonferroni post test was used. For (I), the median + IQR is shown and Friedman test with Dunn’s post test was used. For (O), unpaired one-tailed t test was used. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not significant.
Figure 2. HIV-1 infection induces a TRM-like phenotype in resting memory CD4+ T cells
(A) CD69 expression on resting memory CD4+ target T cells following co-culture with HIV-1-infected primary donor T cells or uninfected donor T cells (mock) (n = 17).
(B) Representative flow cytometry plots from (A).
(C) CD69 expression on infected resting memory CD4+ T cells ± IL-7 and T20 (n = 7).
(D) CD69 expression on infected resting memory CD4+ T cells ± IL-7 and ruxolitinib (n = 8).
(E) CD69 expression on infected resting memory CD4+ T cells in response to IL-7 and IL-15 (n = 11).
(F) CD69 expression on infected Gag+ resting memory CD4+ T cells and uninfected Gag+ bystander cells in response to IL-7 and IL-15 (n = 11).
(G) CXCR6 surface expression from (F) (n = 11).
(H) Representative flow cytometry plots of CD69 and CXCR6 co-expression in the presence of IL-7.
(I) Co-expression of CD69 with CXCR6, CD49A, or PD-1 on infected resting memory CD4+ T cells (n = 5–7).
(J) As for (I) in the presence of IL-7 (n = 4–7).
(K) As for (I) comparing infected Gag+ memory CD4+ T cells and uninfected Gag+ bystander cells.
(L and M) Blimp-1 expression in CD69+ HIV-infected resting memory CD4+ T cells and infected CD69- cells in the presence of IL-7 (n = 8).
(N) Total lymphocytes from cellularized tonsils co-cultured with HIV-1-infected Jurkat T cells. Infection of resting CD4+ T cells shown as CD45RO versus Gag.

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by flow cytometry sorting to recover pure CD69^+ CD4^+ T cells, co-culturing these cells with HIV-1-infected donor T cells, and observing de novo upregulation of CD69 on the newly infected resting memory target cells (Figures S3C and S3D). While CD69 is classically thought of as a marker of early T cell activation, expression can occur independent of cell-cycle progression and T cell activation (Corneau et al., 2017; Lea et al., 2003; Szabo et al., 2019b). Consistent with this, we did not detect activation concomitant with CD69 upregulation in HIV-1-infected resting CD4^+ memory T cells, and CD69^+ cells remained HLA-DR negative (Figure S3E).

Functionally, CD69 is crucial for T cell retention in tissues by interfering with S1P receptor-mediated egress and has been identified as a hallmark of tissue-resident memory (TRM) T cells (Kumar et al., 2017). Recently it has been demonstrated that although TRM cells are largely absent from peripheral blood (Figure S3F), precursor cells poised to adopt a TRM phenotype are present in the circulation (Almeida et al., 2022; Fonseca et al., 2020; Kok et al., 2020). Interestingly, while HIV-1 infection alone induced upregulation of CD69, additional exposure of these HIV-1-infected resting memory T cells to the homoeostatic T cell cytokine interleukin-7 (IL-7) further boosted CD69 upregulation 4-fold, compared with HIV-1 or IL-7 alone (Figures 2C and S3J). IL-7 secreted by stromal cells is required for long-term maintenance of CD4^+ TRM cells (Adachi et al., 2015; Amezcue Vesely et al., 2019; Yeon et al., 2017). Although IL-7 can enhance HIV-1 infection of T cells (Coiras et al., 2016) (Figure S3G), infection of resting memory T cells mediated by cell-to-cell spread does not require IL-7 (Figure 1); furthermore, IL-7 increased CD69 expression on infected cells even when added 48 h post-infection (Figure S3J). HIV-1-induced CD69 upregulation was abrogated by suppressing infection with the fusion inhibitor T20 (Figure 2C) or by treating cells with ruxolitinib, which blocks IL-7-mediated JAK-STAT signaling (Figure 2D), demonstrating that the enhanced CD69 induction requires both infection and cytokine signaling. Similar enhancement of CD69 expression on HIV-1-infected resting cells was also observed in response to the γc-chain cytokine IL-15 (Figures 2E and 2F), but not IL-12 or TGF-β (Figures S3H and S3I). Alongside CD69, resting memory T cells also increased co-expression of the TRM marker CXCR6 (Kumar et al., 2017) during HIV-1 infection (Figures 2G, 2H, S3K, and S3N). Similarly, we also observed an increase in the population of CD69^+ cells co-expressing CD49a, PD-1, and CD101, thus generating a population of resting memory T cells co-expressing multiple TRM marker proteins that are associated with the defined core TRM phenotypic signature (Kumar et al., 2017) (Figures 2I, 2J, and S3L). This suggests that HIV-1 infection primes CD4^+ T cells to gain characteristics that are associated with TRM cells and adopt a TRM-like phenotypic signature. Similar to ex vivo TRM cells, we saw no upregulation of CX3CR1 expression (Figure S3O) and no transcriptional upregulation of S1PR1 or KLF2 by RT-PCR (Figure S5I); however, comprehensive RNA-sequencing (RNA-seq) analysis revealed a 2-fold downregulation of KLF2 and S1PR1 in HIV-1-infected compared with uninfected (mock) cells (Data S1), consistent with their suppression under conditions of TRM induction (Kumar et al., 2017). Critically, induction of the TRM-like phenotypic markers did not occur in uninfected Gag^+ bystander cells (Figures 2K, S3M, and S3N). By contrast to CD8^+ TRM cells, CD103 was barely detectable and not upregulated (Figures S3P and S3Q), consistent with the observation of limited CD103 expression on CD4^+ T cells (Kumar et al., 2017). Induction of CD69 expression was also concomitant with upregulation of the TRM-associated transcription factor Blimp-1 (Hombrink et al., 2016; Mackay et al., 2016; Pallett et al., 2017) (Figures 2L and 2M). Similar upregulation of TRM-associated markers was also observed when unstimulated CD4^+ T cells from tonsil (Figures 2N, 2O, and S4A) or mediastinal lymph nodes (Figures S4B and S4C) were infected with HIV-1 via cell-to-cell spread and exposed to IL-7, demonstrating that induction of this TRM-like phenotype occurs in tissue-derived T cells following HIV-1 cell-mediated infection.

Functionally, TRM cells are poised for rapid production of cytokines (IFN-γ, IL-2, and TNF) following antigenic-stimulation (Christo et al., 2021; FitzPatrick et al., 2021; Wiggins et al., 2021). Notably, we found that HIV-1-induced TRM-like cells produced these cytokines faster and to higher levels following recall stimulation compared with HIV-1-infected CD69^+ non-TRM cells (Figure 2P) and also showed a greater propensity to produce multiple cytokines per cell (Figure 2Q). Taken together, these data suggest that HIV-1 infection of resting memory CD4^+ T cells reprograms cells by upregulating expression of a combination of cell-surface proteins and inducing T cells to gain phenotypic and functional characteristics that are associated with tissue residency, which we term “TRM-like” cells.

Vpr is required for induction of the TRM-like phenotype in HIV-1-infected T cells

HIV-1 expresses four accessory proteins, Vif, Vpu, Vpr, and Nef, which directly and indirectly manipulate host cell factors to facilitate efficient viral replication in vivo and drive pathogenesis (Malim and Emerman, 2008). Co-culture of resting target T cells with donor T cells infected with HIV-1 accessory protein mutants showed that deletion of Vpr (HIV-1ΔVpr) prevented induction of the TRM-like phenotype following HIV-1 infection, evidenced by no CD69 upregulation and no increase in the CD69^+CXCR6^+ CD49a^+ triple-positive TRM-like memory population (Figures 3A, 3B, 3D, S5A–S5E, and S6). By contrast, deletion of Vpu or Nef did not affect HIV-1 induction of these marker proteins on infected resting T cells (Figures 3A, 3B, S5A–S5E, and S6). HIV-1 ΔVif could not be tested because Vif is required to antagonize...
APOBEC3-mediated viral restriction and allow infection (Sheehy et al., 2003). HIV-1 Vpr is not required for infection of T cells in vitro (Balliet et al., 1994; Rogel et al., 1995), and concordantly, lack of changes to TRM-marker protein expression was not due to lack of infection of resting target cells by HIV-1 ΔVpr virus nor reduced Gag expression (Figures 3C, S5F, S5G, and S5H). Like WT virus,
ΔVpr virus also maintained a preferential tropism for resting memory T cells over naive T cells (Figure 3C). Critically, Vpr was required for induction of CD69 expression observed at the mRNA level, as well as induction of CXCR6 and Blimp1 (PRDM1) mRNA (Figure 3E). As expected, there was no upregulation of S1PR1 or KLF2 mRNA by either HIV-1 WT or ΔVpr virus (Figure S3I). Vpr also mediated spontaneous production of IFN-γ by infected CD4+ memory T cells (Figure 3F), characteristic of TRM cells (FitzPatrick et al., 2021; Wiggins et al., 2021). Importantly, Vpr-dependent changes were also observed using tonsil-derived tissue memory T cells as targets for cell-to-cell spread (Figures 3G, SSK, and SSL). While a proportion of tonsil-derived target cells already express residency markers at baseline as expected, expression was further increased on WT HIV-1-infected cells but not ΔVpr-infected cells (Figures 3G, SSK, and SSL), confirming the requirement for Vpr in driving TRM-like phenotypic changes in tissue cells ex vivo.

Vpr is a multifunctional protein that is packaged into viral particles and is present during the early stages of infection, in which it plays an important, but as yet poorly defined, role in HIV-1 pathogenesis. Among the best-defined functions of Vpr are its ability to (1) bind the Cul4A-DDB1 (DCAF1) complex, leading to an interaction with the ubiquitinylation and proteasomal machinery; (2) induce G2/M cell-cycle arrest; and (3) drive apoptosis in infected cells (Jowett et al., 1995; Laguette et al., 2014; Schröfelbauer et al., 2007; Wen et al., 2007; Wu et al., 2016). We abrogated these functions individually by introducing the Vpr mutations Q65R, S79A, or R80A, respectively, into HIV-1 and confirmed that each Vpr mutant is packaged into virions and is present during the early stages of infection, in which the Gag-p6 Vpr packaging sequence was mutated to resemble its ability to (1) bind the Cul4A-DDB1 (DCAF1) complex, leading to an interaction with the ubiquitinylation and proteasomal machinery; (2) induce G2/M cell-cycle arrest; and (3) drive apoptosis in infected cells (Jowett et al., 1995; Laguette et al., 2014; Schröfelbauer et al., 2007; Wen et al., 2007; Wu et al., 2016). We abrogated these functions individually by introducing the Vpr mutations Q65R, S79A, or R80A, respectively, into HIV-1 and confirmed that each Vpr mutant is packaged into virions (Figure 3H). Co-culture of resting memory target T cells with HIV-1 WT cells infected with different Vpr mutants revealed the Vpr mutations Q65R, S79A, or R80A, respectively, into virions (Figure 3H). Vpr also mediated spontaneous production of IFN-γ by infected CD4+ memory T cells (Figure 3F), characteristic of TRM cells (FitzPatrick et al., 2021; Wiggins et al., 2021). Importantly, Vpr-dependent changes were also observed using tonsil-derived tissue memory T cells as targets for cell-to-cell spread (Figures 3G, SSK, and SSL). While a proportion of tonsil-derived target cells already express residency markers at baseline as expected, expression was further increased on WT HIV-1-infected cells but not ΔVpr-infected cells (Figures 3G, SSK, and SSL), confirming the requirement for Vpr in driving TRM-like phenotypic changes in tissue cells ex vivo.

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Vpr-mediated induction of a TRM-like phenotype was not inhibited by potently suppressing HIV-1 integration into resting memory CD4+ T cells using the integrase inhibitor raltegravir (Figures 4A and 4B). The presence of integrated provirus in resting CD4+ T cells at approximately one provirus per infected cell (Figure 4B) supports the observation that highly efficient cell-to-cell spread results in resting CD4+ T cell infection (Figure 1), but notably without leading to multiple proviral integrations. Vpr is packaged into HIV-1 virions and as such is delivered into the target cell during the earliest steps of infection. To determine whether incoming virion-associated Vpr was sufficient to drive the phenotypic changes in the presence of IL-7, we took three approaches (Figure 4C). First, we used an HIV-1 mutant in which the Gag-p6 Vpr packaging sequence was mutated to prevent Vpr incorporation into virions (Radestock et al., 2013; Wanaguru and Bishop, 2021) (Figures 4D and 4E). This mutant (which we termed VprPM) cannot package Vpr into virions, but retains an intact vpr gene, allowing for de novo Vpr synthesis from the viral genome following infection, and maintains the capacity for cell-to-cell spread (Figure S7A). Preventing Vpr packaging into virions abrogated upregulation of CD69 and CXCR6 on resting memory T cells compared with the parental WT control (termed WTPM) (Figure 4E). Second, complementing the ΔVpr virus with Vpr in trans allowed for Vpr packaging into virions and delivery into cells without de novo Vpr synthesis during infection, and fully rescued upregulation of CD69 and CXCR6 spinooculation of resting CD4+ T cells (Figures 4D, 4F, 5B, and 5C). Finally, we made infectious but non-replicative HIV-1 Env pseudotyped virus-like particles (Env-VLPs) that do not contain an HIV-1 genome but can package Vpr expressed in trans during VLP production (Env-VLP-Vpr), and confirmed sufficient Vpr delivery for degradation of a known Vpr target, UNG2, upon spinooculation (Figures S7D, 5E, and S7F). Env-VLPs allow efficient particle entry into resting CD4+ T cells, which are not permissive to VSVg-mediated transduction. Using Env-VLP-Vpr particles to deliver Vpr in the absence of viral replication was sufficient to induce co-expression of CD69 and CXCR6 in the presence (Figures 4G and 4H), but not in the absence (Figure S7G), of IL-7. Taken together, we conclude that incoming Vpr in virions is sufficient to drive T cells to upregulate TRM markers in synergy with IL-7.

Vpr induces widespread transcriptional reprogramming in HIV-1-infected resting T cells and a core TRM transcriptional signature

Next we performed transcriptional profiling by RNA-seq analysis of flow-cytometry-sorted resting memory CD4+ T cells infected with HIV-1 WT or ΔVpr virus by cell-to-cell spread in the presence and absence of IL-7. HIV-1 infection alone induced widespread changes in gene expression in resting memory T cells compared with uninfected cells (226 differentially expressed genes [DEGs], fold change >1.2, adjusted p < 0.01) (Figure 5). Hierarchical clustering and principal-component analysis revealed that the gene expression patterns in response to HIV-1 WT infection were clearly distinct from those induced following infection with ΔVpr virus (Figures 5A–5C, 5E, and 5F) demonstrating that Vpr deletion suppresses the global transcriptional response to HIV-1 infection. Specifically, infection with ΔVpr virus resulted in only 13 genes showing statistically significant changes compared with uninfected cells, in contrast to 226 genes for HIV-1 WT virus (Data S1; Figure 5E). In fact, much of the transcriptional response to HIV-1 was regulated by Vpr, as evidenced by striking differences in the number of DEGs in the presence and absence of Vpr (Figures 5E and 5F). The requirement for Vpr in driving many of the changes in DEGs was also observed when infected cells were exposed to IL-7, implicating the virus as the dominant driver of T cell reprogramming in our experiments (Figures 5A, 5B, 5D, and 5E).

To gain greater insight into the native effects of HIV-1 and Vpr on T cells, gene set enrichment analysis (GSEA) (Figure 5G) and ingenuity pathway analysis (IPA) (Figures 5H and 5I) were performed on data from infected cells in the absence of IL-7 treatment, since IL-7 was not required for productive infection of resting memory T cells (Figure 1). Consistent with Vpr manipulating the T cell response to HIV-1 infection (Data S1), these
data revealed enrichment of numerous cellular signaling pathways following HIV-1 infection that appeared Vpr dependent, most notably, pathways associated with cytokine and inflammatory responses as well as immune signaling (Figures 5G and S8; Data S1). This was further evidenced by upstream regulator analysis that showed significant enrichment for genes associated with cytokine signaling and transcriptional regulators that were again largely Vpr dependent (Figures 5J, 5K, and S8). For comparison the same analysis of HIV-1 WT and ΔVpr virus-infected cells in the presence of IL-7 is shown (Figure S8). Taken together,
Figure 5. Transcriptional profiling of HIV-1-infected resting memory CD4+ T cells

(A) Heatmap showing hierarchical clustering of 226 differentially expressed genes (DEGs) of infected (HIV-1 WT) over uninfected (mock) resting memory CD4+ T cells at 72 h post co-culture (adjusted p < 0.01, fold change ≥1.2). Mean log2 TPM of four biological repeats are shown. Cytokine indicates presence or absence of IL-7. Virus indicates infection with HIV-1 WT, HIV-1 ΔVpr, or uninfected (mock) condition.

(B) Principal-component analysis (PCA) of (A), with ellipses indicating 95% CI.

(C and D) Scatterplots of mean log2 TPMs of DEGs from HIV-1 WT/mock (gray circles) or HIV-1 ΔVpr/mock (orange circles) in the absence (C) or presence (D) of IL-7 (adjusted p < 0.01, fold change ≥1.2). Lines indicate line of identity (LOD). Genes above or below the LOD are up- or downregulated, respectively.

(E and F) Venn diagrams showing overlap of DEGs comparing expression profiles of HIV-1 WT/mock with HIV-1 ΔVpr/mock (E) or HIV-1 ΔVpr/HIV-1 WT (F).

(G) GSEA was performed on expression profiles comparing HIV-1 WT/mock (black) or HIV-1 ΔVpr/HIV-1 WT (gray). Normalized enrichment scores (NES) are shown for significantly enriched hallmark gene sets (false discovery rate [FDR] q < 0.05 and NES > 1.75).

(H and I) Top 10 significantly enriched canonical pathways predicted by ingenuity pathway analysis (IPA) of DEGs in HIV-1 WT/mock (H) or HIV-1 ΔVpr/HIV-1 WT (I) (adjusted p < 0.05).

(J and K) Cytokines (J) and transcription regulators (K) predicted to be upstream regulators by IPA of gene expression signatures for HIV-1 WT/mock (black) or HIV-1 ΔVpr/mock (gray); line indicates p = 0.05. TPM, transcripts per million.
these data reveal that HIV-1 induces dramatic reprogramming during infection of resting memory CD4+ T cells driven largely by Vpr.

Tissue residency of T cells has been associated with a 31-gene core transcriptional signature (Kumar et al., 2017). We took advantage of this dataset and our RNA-seq analysis to determine whether this core TRM gene signature was enriched in our transcriptome of HIV-1-infected resting memory T cells.

Hierarchical clustering of our data comparing with the core gene signature of TRM cells (Kumar et al., 2017) showed that HIV-1-infected memory T cells exposed to IL-7 were grouped distinctly and clustered with bona fide CD69+ TRM cells (Figure 6A; Data S1) and were distinct from non-TRM T cells (CD69- T cells isolated...
Figure 7. Vpr enhances STAT5 activation to drive a TRM-like resting memory CD4+ T cell phenotype

(A) GSEA enrichment plot of the hallmark IL-2 STAT5 signaling pathway for HIV-1 WT-infected resting memory T cells versus mock.
(B) CD69 expression on infected resting memory CD4+ T cells ± ruxolitinib at 72 h (n = 4).
(C) CD127 MFI on infected resting memory CD4+ T cells ± IL-7 (n = 7) at 72 h.
(D) Representative histogram of intracellular STAT5 phosphorylation in resting memory T cells infected by cell-to-cell spread at 72 h.
(E) Quantification of (D) shown as P-STAT5 MFI (n = 10).
(F) P-STAT5 MFI (left) and %P-STAT5+ (right) in Gag+ resting memory T cells 24 h post spinoculation with the indicated viruses (n = 12).
(G) Representative western blot analysis of P-STAT5 and total STAT5 levels in resting T cells at 0 and 24 h post spinoculation with HIV-1 WT and ΔVpr virus (n = 2).

Values indicate P-STAT5 or total STAT5 levels normalized to β-actin and mock at 0 h.
(H) Quantification of P-STAT5/STAT5 levels normalized to β-actin from western blots of total CD4+ T cells at 24 h post spinoculation with HIV-1 WT and ΔVpr virus (n = 4).

(legend continued on next page)
from tissue and blood from Kumar et al., 2017). We further corroborated this finding by calculating a T\text{RM} gene enrichment score, using the gene expression data in the published core T\text{RM} transcriptional signature. Extracting this 31-gene set from our RNA-seq data and performing a statistical comparison of gene expression between our data and that of Kumar et al. (2017) showed that HIV-1-infected resting memory T cells (±IL-7) harbor a T\text{RM} signature score (and thus gene expression profile) that approximated closely with that of bona fide T\text{RM} cells (Figure 6B) and was statistically significantly different from non-T\text{RM} T cells. Critically, this was Vpr dependent, with mock- and ΔVpr-infected cells showing an enrichment score that was more closely aligned with and not statistically different from non-T\text{RM}. Thus, we conclude that HIV-1 Vpr induces resting T cells to gain a constellation of features that define T\text{RM} cells, including co-expression of key phenotypic T\text{RM} surface markers, functional characteristics associated with T\text{RM} T cell recall responses, and, as our comprehensive RNA-seq reveals, a transcriptional T\text{RM}−like signature via the accessory protein Vpr.

Vpr activates STAT5 to synergize with IL-7 signaling for T cell reprogramming

Having shown that HIV-1 Vpr drives a T\text{RM}−like phenotype in resting memory T cells and primes cells to become hyperresponsive to IL-7, we hypothesized that Vpr may do so by manipulating JAK-STAT signaling, the pathway downstream of IL-7 signals. In support of this, GSEA of our RNA-seq data revealed increased expression of genes regulated by the transcription factor STAT5 (Figures S5G, S7A, and S8C), including CD69 (Kanai et al., 2014), as well as enrichment of common-gamma-chain cytokine signaling (Figures S5 and S8), consistent with the hypothesis that HIV-1 manipulates STAT5 in resting memory T cells. Furthermore, HIV-1-dependent upregulation of the T\text{RM} marker CD69 was inhibited by ruxolitinib treatment, which blocks JAK-STAT signaling, in a Vpr-dependent manner (Figure 7B). Testing this further, we found that infection also downregulated the IL-7 receptor \( \gamma \) subunit (CD127) from the cell surface (Figure 7C) and transcriptionally (fold change = 0.693, adjusted \( \rho = 5.89 \times 10^{-5} \); Data S1) (Park et al., 2004), collectively indicative of HIV-1 inducing enhanced JAK-STAT signaling even in the absence of IL-7. Importantly, Vpr also mediated STAT5-activation during HIV-1 cell-to-cell infection of resting memory CD4\(^+\) T cells as evidenced by an increase in the intracellular levels of phosphorylated STAT5 (P-STAT5) (Figures 7D and 7E) under conditions where cells were not exposed to IL-7. A similar, Vpr-dependent increase in P-STAT5 was also seen when Vpr was delivered into resting T cells by spinoculation of infectious HIV-1 (Figures 7F–7J) or using Env-Vpr-VLPs (Figure 7K), confirming again that incoming Vpr is sufficient to drive reprogramming of resting memory T cells. Given that STAT5 drives CD69 gene expression, we next treated resting memory T cells with the selective STAT5 inhibitor AC-4-130 (Wingelhofer et al., 2018). Strikingly, blocking STAT5 activity in this way completely inhibited induction of the T\text{RM} phenotype by HIV-1 infection, preventing upregulation of CD69 and CXCR6 (Figures 7L and S5O). AC-4-130 inhibited CD69 upregulation by HIV-1 Vpr in both the absence and the presence of IL-7, consistent with HIV-1 alone activating STAT5 via Vpr, resulting in increased CD69 expression. Taken together, these data suggest a mechanism by which Vpr manipulates cellular signaling pathways and STAT5 phosphorylation, making T cells hypersensitive to IL-7, which works in synergy with HIV-1 to drive induction of a T\text{RM}−like phenotype (Figure 7I).

**DISCUSSION**

Our discovery that resting CD4\(^+\) T cells can be productively infected by cell-to-cell spread, allowing for viral integration, replication, and dissemination, transforms our ability to determine how T cells respond to and support HIV-1 replication without confounding activation-induced changes. Here, employing our coculture model, we have revealed that HIV-1 infection of resting memory T cells induces T cell reprogramming, resulting in these cells gaining characteristics that are associated with T\text{RM} T cells, a phenotype that we have termed T\text{RM}−like. This is evidenced by the upregulation and co-expression of T\text{RM}−associated marker proteins on infected cells (e.g., CD69/CXCR6/CD49a triple-positive cells), induction of a core T\text{RM} transcriptional signature, and the gain of functional characteristics associated with T\text{RM} cells. HIV-1 establishes cellular and tissue reservoirs, both active and latent, that ultimately prevent cure with anti-retroviral therapy. Importantly, T\text{RM} cells are long-lived and are thought to be largely confined to tissue (Kumar et al., 2018), providing an alternate model for a tissue-associated reservoir driven by the virus itself. Our results suggest that HIV-1 persistence and the establishment of tissue reservoirs may be driven, in part, through direct viral induction of a T\text{RM}−like phenotype via transcriptional reprogramming. Recently, T\text{RM} cells in cervical tissue were found to be preferentially infected by HIV-1 and can harbor an HIV-1 reservoir in vivo (Cantero-Pérez et al., 2019). The relative contribution of pre-existing versus HIV-1-induced T\text{RM} cells to viral reservoirs and their relative abundance in different anatomical compartments in vivo remain to be quantified, but we expect T\text{RM} cells harboring virus to be important contributors to viral persistence. In light of these findings it is possible that HIV-1-infected cells circulating in peripheral blood may in fact represent cells that have failed to become part of the tissue reservoir, leading to an...
underestimation of the true viral burden. Having shown that HIV-1 infection of resting T cells by cell-to-cell spread results in productive infection, while being less sensitive to HIV-1-mediated cytotoxicity, we hypothesize that induction of a T RM-like phenotype in infected cells may also play additional roles in establishing and maintaining viral reservoirs by sequestering infected cells in tissue sites where susceptible target T cells are in abundance, thus supporting localized viral replication and spread. Indeed we have shown that infected resting memory T cells support spreading infection to disseminate virus. Given the importance of T RM cells as a population that is increasingly recognized to be critical in providing localized immunity and immunosurveillance (Fonseca et al., 2020; Kok et al., 2020), future work should focus on understanding the contribution of HIV-1-induced T RM-like cells in pathogenesis and persistence. While HIV-1 infection directly reprograms T cells to gain core features shared across human T RM populations in vivo, these virus-induced cells might still reveal functional differences from the host’s various subsets of “native” CD4+ T RM cells. Dissecting and comparing these further to better understand the causes and consequences for T cell fate and function will be important. More broadly, it is now emerging that committed T RM precursors, imprinted with the capacity to become mature T RM, pre-exist in blood and that, when exposed to the appropriate cues in tissues or ex vivo, can become tissue-resident (Fonseca et al., 2020; Kok et al., 2020). In fact, studies of long-term chimerism after hematopoietic transplantation have recently further confirmed this in humans (Almeida et al., 2022). Thus the ontogeny, derivation, and maintenance of T RM cells, and their heterogeneity in vivo, appear more complex that initially appreciated. Our discovery that HIV-1 induces a T RM-like phenotype in CD4+ T cells provides an opportunity to gain new understanding of mechanisms behind CD4+ T RM induction and maintenance.

Recently, it has been reported that cell-to-cell spread can also facilitate latent infection of resting T cells without productive infection (Agosto et al., 2018). Together, our work and that of Agosto et al. highlight the distinct advantages of co-culture models that do not require mitogenic, experimental stimulation of T cells to drive infection to study native HIV-1-host cell interactions, permisiveness, and the cellular response to infection. Having shown that resting memory T cells are preferentially infected by cell-to-cell spread compared with naive cells, future studies should address how cell-to-cell spread drives permisiveness and what regulates the selective permisiveness of resting memory cells (for example, whether this is influenced by the expression of surface receptors involved in cell-cell spread and/or other factors downstream of viral entry) to shed new light on the interaction between HIV and host T cells.

We found that HIV-1 infection of resting memory T cells was associated with striking transcriptional reprogramming that was driven by Vpr, thus identifying a novel function for this enigmatic HIV accessory protein. Crucially, Vpr is packaged into HIV-1 virions and is thus present upon virus entry into the target cell and during the earliest events of infection. Using a series of complementary but distinct approaches, we showed that incoming Vpr is in fact sufficient to drive T RM remodeling of T cells, independent of viral integration, and that Vpr induction of this phenotype does not require de novo Vpr transcription and protein synthesis, allowing HIV-1 to directly and immediately reshape the niche in which it resides. Vpr-mediated induction of the T RM-like phenotype was dependent on residue Q65, and Vpr is reported to drive widespread remodeling of the cellular proteome via its recruitment of DCAF1 through Q65 (Greenwood et al., 2019). Whether this requirement for Q65 in induction of a T RM-like phenotype is DCAF1 dependent remains unclear, because DCAF1 knockout in primary T cells made cells hyperresponsive to HIV-1-induced cell death (Figures S5M and S5N). Moreover, given that Q65R did show a partial reduction in Vpr packaging, it is possible that the reduced Vpr effect is due to reduced Vpr delivery. We also cannot discount the effects of Q65R on mislocalizing Vpr (Khan et al., 2020), resulting in loss of function and thus T cell reprogramming. Thus we cannot at present formally exclude other functions of Vpr Q65 in the process of T RM induction. However, our data showing that Vpr manipulates the JAK-STAT pathway through which IL-7 signals and activates STAT5 suggests a mechanism by which Vpr can work in synergy with IL-7, driving T cells to gain characteristics of T RM cells.

Notably, HIV-1 induction of a T RM-like phenotype via Vpr was accompanied by induction of a T RM transcriptional signature that aligned closely with a published core T RM signature (Kumar et al., 2017). Vpr deletion abolished not only the induction of this T RM signature, but also many HIV-1-induced changes to gene expression following infection of resting T cells. This is in keeping with widespread proteome remodeling by Vpr in activated T cells (Greenwood et al., 2019), but suggests that these changes may be driven in part by a hitherto unappreciated role for Vpr in modulating the host cell gene expression profile. Whether the reprogramming of resting memory T cells into T RM-like cells reflects widespread epigenetic changes mediated by Vpr or manipulation of key upstream regulators remains to be determined. In this regard, a recent elegant study has revealed that Vpr function is associated with epigenetic remodeling of the host cell (Dupont et al., 2021). Moreover, having found that Vpr manipulates STAT5, a crucial transcriptional factor for T cell function and survival, to mediate T RM-like phenotypic changes in resting memory T cells, it is intriguing to note that persistent STAT5 activity has been implicated in altering the epigenetic landscape of CD4+ T cells (Ding et al., 2020). While to date a formal role for STAT5 has not been described in T RM formation, we propose that a contribution of this transcriptional factor to this process should be investigated further. Our data show that HIV-1 Vpr poises T cells for increased responsiveness to external stimuli by manipulation of immune signaling pathways, including innate and inflammatory responses. This is particularly intriguing and suggests that HIV-1 manipulates crucial immune signaling pathways to benefit the virus, in this case by priming resting memory T cells for T RM-like induction.

Notably, a rare case of laboratory-derived infection with Vpr-defective HIV-1 was characterized by markedly delayed seroconversion, suppressed viremia, and normal CD4+ T cell counts (Ali et al., 2018), consistent with reduced pathogenesis and failure to establish and maintain a significantly large tissue reservoir. We envisage therapeutic targeting of Vpr to manipulate persistence and pathogenesis. To achieve an HIV-1 cure, it is essential to understand the nature and establishment of HIV-1 reservoirs and how to manipulate them. By demonstrating that HIV-1 infection drives a T RM-like phenotype during infection of resting
memory T cells, we have taken a significant step to help accelerate the quest for an HIV-1 cure.

Limitations of the study
Here we have shown that HIV-1 infection of resting memory CD4+ T cells in vitro induces T cells to differentiate and gain characteristics that align closely with a phenotypic and transcriptional program of TRM T cells. We are mindful to term these cells “TRM-like” T cells since no single phenotypic marker might faithfully identify a cell as tissue-resident. While differentiation of TRM cells in vitro has been reported previously (Pallett et al., 2017; Wiggins et al., 2021), bona fide TRM cells in vivo are defined by their anatomical location in tissues. Future work will be required to determine to what extent HIV-1 infection and/or expression of Vpr alone is able to induce this TRM-like state in vivo and whether HIV-1 infection reprograms resting T cells to adopt a TRM profile and localization in situ. Currently, no appropriate in vivo models are available to mimic human TRM biology. Thus, while these experiments will be challenging, and will necessitate the development of new humanized mouse models that allow us to capture human TRM cell generation and localization while also supporting HIV-1 infection, they will be important to what extent this HIV-1-induced T cell reprogramming overlaps TRM cells in vivo or reflects other T cell differentiation states.

STAR+METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at [https://doi.org/10.1016/j.celrep.2022.110650](https://doi.org/10.1016/j.celrep.2022.110650).

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AUTHOR CONTRIBUTIONS
A.K.R. and C.J. conceived the project. A.K.R. and C.J. designed the experiments. A.K.R., M.S., D.M., and M.V.X.W. performed the experiments. A.K.R., C.J., L.J.P., M.K.M., M.S., D.M., M.V.X.W., A.G.-A., and M.N. analyzed the data. L.J.P. and M.K.M. provided reagents. J.P.T., C.H., S.F., R.M., K.J.D., and A.S. provided lymphoid tissue samples. A.G.-A. and M.N. performed the core TRM transcriptional mapping analysis. A.K.R. and C.J. prepared the manuscript. All authors provided critical review of the manuscript.

DECLARATION OF INTERESTS
L.J.P. participates in advisory boards and provides consultancy to SQZ Biotech.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Ultra-LEAF™ Purified anti-human CD3 Antibody (clone: OKT3) | Biolegend | Cat# 317326; RRID: AB_11150592 |
| Ultra-LEAF™ Purified anti-human CD28 Antibody (clone: CD28.2) | Biolegend | Cat# 302934; RRID: AB_11148949 |
| Brilliant Violet 510™ anti-human CD3 Antibody (clone: UCHT1) | Biolegend | Cat# 300448; RRID: AB_2563468 |
| Brilliant Violet 711™ anti-human CD3 Antibody (clone: UCHT1) | Biolegend | Cat# 300464; RRID: AB_2566036 |
| FITC anti-human CD3 Antibody (clone: UCHT1) | Biolegend | Cat# 300406; RRID: AB_314060 |
| PE anti-human CD8 Antibody (clone: SK1) | Biolegend | Cat# 344706; RRID: AB_1953244 |
| Brilliant Violet 605™ anti-human CD8 Antibody (clone: SK1) | Biolegend | Cat# 344742; RRID: AB_2566513 |
| APC/Fire™ 750 anti-human CD4 Antibody (clone: SK3) | Biolegend | Cat# 344638; RRID: AB_2572097 |
| PE/Dazzle™ 594 anti-human CD45RA Antibody (clone: HI100) | Biolegend | Cat# 304146; RRID: AB_2564079 |
| Brilliant Violet 421™ anti-human CD45RA Antibody (clone: HI100) | Biolegend | Cat# 304130; RRID: AB_10965547 |
| PerCP/Cyanine5.5 anti-human CD45RO Antibody (clone: UCHL1) | Biolegend | Cat# 304222; RRID: AB_2174124 |
| Brilliant Violet 785™ anti-human CD62L Antibody (clone: DREG-56) | Biolegend | Cat# 304830; RRID: AB_2629555 |
| APC/Fire™ 750 anti-human CD69 Antibody (clone: FN50) | Biolegend | Cat# 310946; RRID: AB_2616709 |
| PE/Dazzle™ 594 anti-human CD69 Antibody (clone: FN50) | Biolegend | Cat# 310942; RRID: AB_2564277 |
| PE/Dazzle™ 594 anti-human CD186 (CXCRI6) Antibody (clone: K041E5) | Biolegend | Cat# 356016; RRID: AB_2563974 |
| Anti-MCM2 antibody | Abcam | Cat# ab4461; AB_304470 |
| PerCP/Cyanine5.5 anti-human HLA-DR Antibody (clone: L243) | Biolegend | Cat# 307630; RRID: AB_893567 |
| PE/Dazzle™ 594 anti-human CD25 Antibody (clone: M-A251) | Biolegend | Cat# 356126; RRID: AB_2563562 |
| PE/Cyanine7 anti-human CD38 Antibody (clone: HIT2) | Biolegend | Cat# 303516; RRID: AB_2072782 |
| PE/Cyanine7 anti-human CD49a Antibody (clone: TS2/7) | Biolegend | Cat# 328312; RRID: AB_2566272 |
| PE/Cyanine7 anti-human CD279 (PD-1) Antibody (clone: EH12.2H7) | Biolegend | Cat# 329918; RRID: AB_2159324 |
| Brilliant Violet 711™ anti-human Ki-67 Antibody (clone: Ki-67) | Biolegend | Cat# 350516; RRID: AB_2563861 |
| PE anti-human Ki-67 Antibody (clone: Ki-67) | Biolegend | Cat# 350504; RRID: AB_10660752 |
| PE Rat Anti-Blimp-1 (clone: 6D3) | BD Biosciences | Cat# 564702; RRID: AB_2738901 |
| PE/Cyanine7 anti-human CD101 (BB27) Antibody (clone: BB27) | Biolegend | Cat# 331013; RRID: AB_2716108 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| PE/Dazzle™ 594 anti-human CX3CR1 Antibody (clone: 2A9-1) | Biolegend | Cat# 341623; RRID: AB_2687151 |
| Brilliant Violet 711™ anti-human CD103 (Integrin αE) Antibody (clone: Ber-ACT8) | Biolegend | Cat# 350221; RRID: AB_2629650 |
| PE/Cyanine7 anti-human CD127 (IL-7Rα) Antibody (clone: A019D5) | Biolegend | Cat# 351320; RRID: AB_10897098 |
| PE anti-human IFN-γ Antibody (clone: B27) | Biolegend | Cat# 506507; RRID: AB_315440 |
| PE Mouse Anti-Stat5 (pY694) (clone: 47) | BD Biosciences | Cat# 612567; RRID: AB_399858 |
| PE anti-DYKDDDDDTag Antibody (clone: L5) | Biolegend | Cat# 637310; RRID: AB_2563148 |
| HIV-1 core antigen-FITC (clone: KC57) | Beckman Coulter | Cat# 6604665; RRID: AB_1575987 |
| HIV-1 core antigen-RD1 (clone: KC57) | Beckman Coulter | Cat# 6604667; RRID: AB_1575989 |
| Antiserum to HIV-1 p24 (ARP432) donated by Dr G. Reid and obtained from the CFAR | | Cat# 0432 |
| HIV-1 NL4-3 Vpr Antiserum donated by Dr. Jeffrey Kopp, and obtained from the NIH ARP | | Cat# 11836 |
| Phospho-Stat5 (Tyr694) (D47E7) XP® Rabbit mAb | Cell Signaling Technologies | Cat# 4322; RRID: AB_10544692 |
| Stat5 (D3N2B) Rabbit mAb | Cell Signaling Technologies | Cat# 25658; RRID: AB_2798908 |
| UNG Mouse Monoclonal Antibody (clone: OTI2C12) | OriGene Technologies | Cat# TA503563; RRID: AB_11266624 |
| VPRBP Polyclonal antibody (DCAF1) | Proteintech | Cat# 11612-1-AP; RRID: AB_2216933 |
| Anti-Actin antibody | Sigma-Aldrich | Cat# A2066; RRID: AB_476693 |
| Anti-α-Tubulin antibody (clone: DM1A) | Sigma-Aldrich | Cat# T6199; RRID: AB_477583 |
| Alexa Fluor® 488-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Human IgG (H+L) | Jackson ImmunoResearch | Cat# 709-546-149; RRID: AB_2340569 |
| Alexa Fluor® 488-conjugated AffiniPure F(ab')2 Fragment Donkey Anti-Rabbit IgG (H+L) | Jackson ImmunoResearch | Cat# 711-546-152; RRID: AB_2340619 |
| Alexa Fluor® 488-conjugated AffiniPure F(ab')2 Fragment Goat Anti-Mouse IgG (H+L) | Jackson ImmunoResearch | Cat# 115-546-146; RRID: AB_2338868 |
| Goat anti-Mouse IgG H&L (IRDye® 680RD) | Abcam | Cat# ab216776 |
| Goat anti-Rabbit IgG H&L (IRDye® 800CW) | Abcam | Cat# ab216773 |
| Goat anti-Mouse IgG H&L (IRDye® 800CW) | Abcam | Cat# ab216772 |
| Goat Anti-Rabbit IgG H&L (IRDye® 680RD) | Abcam | Cat# ab216777 |

**Bacterial and virus strains**

| HIV-1 pNL4.3 | donated by Dr M Martin (NIH) and obtained from CFAR | Cat# 2006 |
| HIV-1 pNL4.3 ΔNef | R. Sloan (University of Edinburgh, UK) | Sloan et al., 2011 |
| HIV-1 pNL4.3 ΔVpr | R. Sloan (University of Edinburgh, UK) | Sloan et al., 2011 |
| HIV-1 pNL4.3 ΔVpu | S. Neil (King’s College London, UK) | Neil et al., 2006 |
| HIV-1 pNLENG1-IRES | D. Levy (NYU, USA) | Trinité et al., 2014 |
| HIV-1 pNL4.3 BaL | G. Towers (University College London, UK) | Cat# 100135 |
| HIV-1 pCH040.c/2625 | G. Towers (University College London, UK) | Cat# ARP-11740 |
| HIV-1 pCH077.t/2627 | G. Towers (University College London, UK) | Cat# ARP-11742 |
| HIV-1 pNL4-3unc-mut4-11 | K. Bishop (Francis Crick Institute, UK) | Wanaguru and Bishop, 2021 |
| HIV-1 pNL4-3unc | K. Bishop (Francis Crick Institute, UK) | Wanaguru and Bishop, 2021 |
| HIV-1 pNL4.3 Vpr Q65R | A. Reuschl | This study |

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Continued

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| HIV-1 pNL4.3 Vpr S79A | A. Reuschl | This study |
| HIV-1 pNL4.3 Vpr R80A | A. Reuschl | This study |

**Biological samples**

| Biological samples | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| PBMCs isolated from buffy coats from healthy donors | UK NHS Blood and Transplant Service | N/A |
| Tonsillar tissue from elective tonsillectomy | Imperial College Infectious Diseases Biobank | N/A |
| Lymph nodes obtained from field surgery of participants undergoing surgery for diagnostic purposes and/or complications of inflammatory lung disease | University of KwaZulu-Natal | N/A |
| Human Serum from human male AB plasma | Sigma-Aldrich | Cat# H4522-20ML |

**Chemicals, peptides, and recombinant proteins**

| Chemicals, peptides, and recombinant proteins | SOURCE | IDENTIFIER |
|-----------------------------------------------|--------|------------|
| Phytohemagglutinin-L (PHA-L) | Sigma | Cat# 11249738001 |
| Interleukin-2 (Human, rDNA derived) | Promega | Cat# E2691 |
| Fugene 6 Transfection Reagent | Promega | Cat# DN25-100MG |
| DNase I | Sigma | Cat# C34564 |
| CellTrace™ Far Red Cell Proliferation Kit | ThermoFisher | Cat# 65-0863-14 |
| Fixable Viability Dye eFluor™ 450 | ThermoFisher | Cat# 130-095-362 |
| Recombinant human IL-7 | Miltenyi Biotec | Cat# 200-15 |
| Recombinant Human IL-15 | Peprotech | Cat# 100-12 |
| Recombinant Human IL-12 p70 | Peprotech | Cat# 100-21C |
| T20 | CFAR | Cat# 0984 |
| Efavirenz | CFAR | Cat# 0977 |
| Raltegravir | CFAR | Cat# 0980 |
| Ruxolitinib | Selleckchem | Cat# S1378 |
| Zombie NIR™ Fixable Viability Kit | Biolegend | Cat# 423106 |
| Zombie Uv™ Fixable Viability Kit | Biolegend | Cat# 423108 |
| Zombie Aqua™ Fixable Viability Kit | Biolegend | Cat# 423102 |
| Super Bright Staining Buffer | ThermoFisher | Cat# SB-4400 |
| Brefeldin A Solution | Biolegend | Cat# 420601 |
| Phorbol 12-myristate 13-acetate (PMA) | Sigma-Aldrich | Cat# P1585-1MG |
| Ionomycin | Sigma-Aldrich | Cat# J9657-1MG |
| Intracellular Staining Permeabilization Wash Buffer | Biolegend | Cat# 421002 |
| FOXP3 Fix/Perm Buffer Set | Biolegend | Cat# 421403 |
| True-Phos™ Perm Buffer | Biolegend | Cat# 425401 |
| RLT Buffer (RNeasy Lysis Buffer) | Qiagen | Cat# 79216 |
| β-mercaptoethanol (Sigma-Aldrich) | Sigma-Aldrich | Cat# M3148 |
| Phusion® Hot Start Flex DNA Polymerase | New England Biolabs | Cat# M0535L |
| TaqMan™ Master-Mix | ThermoFisher | Cat# 4369016 |
| SuperScript™ IV Reverse Transcriptase | ThermoFisher | Cat# 18090050 |
| Fast SYBR™ Green Master Mix | Applied Biosystems | Cat# 4385612 |
| Hoechst33342 | ThermoFisher | Cat# H3570 |

**Critical commercial assays**

| Critical commercial assays | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| MojoSort™ Human CD4 T Cell Isolation Kit | Biolegend | Cat# 480010 |
| CD45RA MicroBeads, human | Miltenyi Biotec | Cat# 130-045-901 |

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact, Professor Clare Jolly (c.jolly@ucl.ac.uk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- RNA-Seq data have been deposited at ArrayExpress and are publicly available as of the date of publication. This paper analyses existing, publicly available data. Accession numbers for all datasets are listed in the key resources table.
- This paper does not report original code.
- Any additional information required to reanalyse data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells
Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors (UK NHS Blood and Transplant Service) by density centrifugation using FicollPaque Plus (GE Life Sciences) and cryopreserved in 10% DMSO (Sigma-Aldrich) in 90% FBS (LabTech). Resting CD4+ T cells were isolated from total PBMCs by negative selection using the MojoSort Human CD4+ T Cell Isolation kit (Biolegend) according to the manufacturer’s instructions. CD45RA+ naïve and CD45RA- memory populations were further separated after CD4+ T cell isolation with CD45RA MicroBeads (Biolegend). For activated CD4+ T cells, PBMCs were treated with 5 μg/mL PHA (Sigma) and 10 IU/mL IL2 (Centre For AIDS Reagents, National Institute of Biological Standards and Control, UK [CFAR]) in RPMI1640 with 20% FBS to 72 h prior to CD4+ T cell isolation. Once purified, CD4+ T cells were cultured in RPMI supplemented with 20% FBS and 10 IU/mL IL2. Jurkat T cell lines (Clone E6-1; ATCC TIB-152) were cultured in RPMI with 10% FBS and 100 U/mL penicillin/streptomycin. HEK 293 T/17 cells (ATCC, CRL-11268) were cultured in DMEM with 10% FBS and 100 U/mL penicillin/streptomycin. Tonsil tissue was obtained from an individual with primary HIV infection who underwent routine tonsillectomy (2 months after commencement of ART) or from healthy donors during routine tonsillectomy. As previously described (Thornhill et al., 2019), the tonsillar tissue from elective tonsillectomy was dissected and mechanically digested, prior to cryopreservation of the cellular suspension. This was collected under the Imperial College Infectious Diseases Biobank (REC: 15/SC/0089) and under the GI Illness Biobank Ethics (16/YH/0247). Lymph nodes were obtained from the field of surgery of participants undergoing surgery for diagnostic purposes and/or complications of inflammatory lung disease. Informed consent was obtained from each participant, and the study protocol approved by the University of KwaZulu-Natal Institutional Review Board (approval BE024/09).
METHOD DETAILS

Plasmids, virus and VLP production

The HIV-1 clone pNL4.3 was obtained from the CFAR, NIBSC (cat# 2006). HIV-1 NL4.3 ΔNef and pNL4.3 ΔVpr were provided by R. Sloan (University of Edinburgh, UK) (Sloan et al., 2011). NL4.3 ΔVpu was provided by S. Neil (King’s College London, UK) (Neil et al., 2006). NL4.3-1-RES was provided by D. Levy (NYU, USA) (Trinté et al., 2014). NL4.3 bearing the CCR5-tropic BaL Env was provided by G. Towers (UCL, UK) (Rasaiyaah et al., 2013). CCR5 tropic transmitter/founder virus plasmids CH044 and CH077 were provided by G. Towers (UCL, UK) and were originally obtained through the NIH AIDS Reagent Program [NIHARP], Division of AIDS, NIAID, NIH: pCH044.c/2625 (cat# 11740) and pCH077.t/2627 (cat# 11742) from Dr. John Kappes and Dr. Christina Ochsenbauer. Plasmids for Vpr packaging mutants pNL4-3-unc-mut4-11 (termed HIV-1 Vpr_WT) and the parental wildtype pNL4-3-unc (termed HIV-1 WT) were provided by K. Bishop (Francis Crick Institute, UK). NL4.3 Vpr Q65R, NL4.3 Vpr S79A, NL4.3 Vpr R80A were generated by site-directed mutagenesis (Promega) using the following primers:

- NL4.3 VprQ65R fw: GTGGAAGCCATAAAGAATTCTCGCACAACCTGCTTTATACCATCAG
- NL4.3 VprQ65R rv: CTGAAATGGATAAACACGAGTTGTCGACAGAATTTATATAGGCTTCAC
- NL4.3 Vpr S79A fw: GAATTGGGTGTCGACATGCGAAATAGGGCGTTACTC
- NL4.3 Vpr S79A rv: GAGTAACGCCTATTCTGCGATGTCGACACCAATC
- NL4.3 Vpr R80A fw: GTGTCGACATAGCGACATAGGGCGTTACTCG
- NL4.3 Vpr R80A rv: CAGAATACCGCTATTTCGGCTATGTCGACACC.

All virus and VLP stocks were produced by plasmid transfection of HEK 293 T cells with Fugene 6 (Promega). Supernatants were harvested at 48 h and 72 h, filtered, DNase treated, purified and concentrated by ultracentrifugation through a 25% sucrose cushion and resuspended in RPMI 1640. Trans-complementation of HIV-1 ΔVpr was performed by co-transfecting 106 HEK 293 T cells with 10 μg pNL4.3 ΔVpr and 2 μg Flag-tagged NL4.3 Vpr in pcDNA3.1 (provided by G. Towers, UCL). For Env-VLP production, 20 μg p8.91 was co-transfected with 10 μg plasmid encoding the HIV-1 T/F envelope pWEAU_d15_410_5017 HIV-1 envelope (provided by LE McCoy, UCL) and 2 μg pcDNA3.1 with or without Flag-tagged NL4.3 Vpr. Viral and VLP titres were determined by measuring reverse transcriptase activity by SG-PERT assay (Pizzato et al., 2009).

HIV-1 infection, cell-to-cell spread and Vpr delivery

For cell-to-cell spread experiments, activated primary CD4+ T cells (donor cells) were infected with 800 mU reverse transcriptase per 106 cells of HIV-1 by spinoculation at 1200x g for 2 h at room temperature and incubated in RPMI 20% FBS supplemented with 10 IU/mL IL2 for up to 72 h before analysis by flow cytometry or FACS sorting. Uninfected target CD4+ T cells were pre-stained with 1-2 nM CellTrace FarRed dye (Invitrogen) prior to co-culture. For cell-to-cell spread into tonsil-derived lymphocytes, total tonsil lymphocytes were cultured at a 4:1 ratio with HIV-1 infected or uninfected eFluor450-labelled Jurkat T cells. For FACS sorting experiments, donor cells were pre-labeled with cell dye eFluor450 (ThermoFisher). For transwell experiments, HIV-1 infected donor T cells were separated from target T cells by a 0.4 μm transwell insert (Corning). Experiments to quantify cell-to-cell versus cell-free infection in the presence and absence of a transwell were performed in equivalent volumes (600 μL). For some experiments, FACS sorted infected resting CD4+ T cells were returned into culture for up to 4 days. Infected levels were measured by intracellular Gag staining and flow cytometry, and virus release into cell culture supernatant determined by SG-PERT (Pizzato et al., 2009). At day 1 or day 4 post FACS sorting, resting CD4+ T cells were washed extensively and co-cultured at a 1:1 ratio with uninfected Env-VLP labelled Jurkat T cells for 72 h, when Jurkat T cell infection was measured by Gag-staining. Where indicated, cultures were incubated in the presence of 20 ng/mL IL-7 (Miltenyi Biotec), 20 ng/mL IL15 (provided by LE McCoy, UCL) and 20 ng/mL IL12 (Peprotech) or 50 ng/mL TGFβ (Peprotech). The following inhibitors were added 30 min before co-culture at the following concentrations: T20 (25–50 ng/mL, CFAR), Efavirenz (1 μM, CFAR), Raltegravir (5 μM, CFAR) and Ruxolitinib (50 nM, Sigma). For delivery of Vpr by spinoculation, resting CD4+ T cells were incubated with 200–800 mU of virus or Env-VLPs for 15 min at room temperature and subsequently spinoculated at 1200xg for 2 h at room temperature. Cells were then cultured as described above.

For RNAi knockdown of DCAF1, primary CD4+ T cells were activated for 4 days with 1 μg/mL plate-bound αCD3 antibody (cloneOKT3, Biolegend) in the presence of 2 μg/mL soluble αCD28 antibody (clone CD28.2, Biolegend). RNAi knockdown of DCAF1 was performed as described before (Mesner et al., 2020) using ON-TARGET plusHuman DCAF1 siRNA - SMARTpool (Dharmacon) and non-targeting siRNA (Dharmacon) was used as a control. 2 x 105 cells were electroporated with 200 pmol siRNA using a NeonTransfection System (Thermo Fisher Scientific; three pulses, 10 ms, 1600 V). After 48 h, DCAF1 knockdowns were confirmed by western blotting and cells used in cell-to-cell spread experiments as described above.

Flow cytometry and FACS

For flow cytometry analysis, cells were washed in PBS and stained with fixable Zombie UV Live/Dead dye, Aqua Live/Dead dye or NIR Live/Dead dye (Biolegend) for 5 min at 37°C. Excess stain was quenched with FBS-complemented RPMI. When tonsil and lymph
node lymphocytes were used, Live/Dead staining was performed using human AB serum (Sigma) in RPMI. Cell surface staining was performed in PBS, complemented with 20% Super Bright Staining Buffer (ThermoFisher) when appropriate, at 4°C for 30 min. Unbound antibody was washed off thoroughly and cells were fixed with 4% FA or PFA before intracellular staining. For intracellular detection of cytokines in infected target CD4+ T cells after 72 h of cell-to-cell spread, cells were treated throughout the co-culture with IL-7 and treated with Brefeldin A (Biolegend) for 6 h, if not stated differently, before surface staining and fixation. Where indicated, cells were stimulated with 100 ng/mL PMA (Sigma-Aldrich) and 100 ng/mL Ionomycin (Sigma-Aldrich) for the duration of the Brefeldin A treatment. Permeabilisation for intracellular staining was performed with IC perm buffer or FoxP3 Buffer set (Biolegend) according to the manufacturer’s instructions. For detection of intracellular P-STAT5, cells were resuspended in ice cold True-Phos Perm buffer (Biolegend) and permeabilised for 48 h at −20°C. Intracellular P-STAT5 staining was then performed in PBS with wash steps performed at 1800 rpm for 6 min at 4°C. The following antibody clones and fluorochromes were used: CD3 (UCHT1, Biolegend; BV510, BV711, FITC), CD8 (SK1, Biolegend; BV605, PE), CD4 (SK3, Biolegend; APC/Fire750); CD45RA (HI100, Biolegend; BV421, PE-Dazzle); CD45RO (UCHL1, Biolegend; PerCP-Cy5.5), CD62L (DREG-56, Biolegend; BV785), CD69 (FN50, Biolegend; APC/Fire750, PE-Dazzle); CXCR6/CD186 (K041E5, Biolegend; PE-Dazzle); MCM2 (ab4461, Abcam; was detected with a secondary anti-rabbit AlexaFluor488-tagged antibody); HLA-DR (L243, Biolegend; PerCP-Cy5.5); CD25 (M-A251, Biolegend; PE-Dazzle), CD38 (HIT2, Biolegend, PE-Cy7), CD49a (TS2/7, Biolegend; PE-Cy7); PD-1 (EH12.2H7, Biolegend; PE-Cy7); Ki67 (Ki-67, Biolegend; BV711, PE); Blimp-1 (D3N2B, Cell Signaling Technology), β-Actin (A2066, Sigma-Aldrich), z-STAT5 (Clone 47/Stat5 (pY694), BD; PE), Flag-tag (L5, Biolegend; PE), HIV-1 Gag core antigen (FH190-1-1, Beckman Coulter; PE-Dazzle); CD38 (HIT2, Biolegend, PE-Cy7), CD49a (TS2/7, Biolegend; PE-Cy7); PD-1 (EH12.2H7, Biolegend; PE-Cy7); Ki67 (Ki-67, Biolegend; BV711, PE); Blimp-1 (D3N2B, Cell Signaling Technology), β-Actin (A2066, Sigma-Aldrich), z-UNG (OTI2C12, OriGene Technologies), z-α-Tubulin (T6199, Sigma-Aldrich) and z-DCAF1 antibody (11612-1-AO, Proteintech), followed by goat anti-rabbit or goat anti-mouse IRdye 800CW or 680RD infrared secondary antibody (Abcam) and imaged using an Odyssey Infrared Imager (LI-COR Biosciences) and analysed with Image Studio Lite software.

### Quantification of HIV-1 integration

To quantify integration of HIV-1 in resting T cells, nested Alu-gag quantitative PCR was performed as previously described (Liszewski et al., 2009). Briefly, DNA was isolated from FACs sorted infected resting CD4+ memory T cells after 72 h of cell-to-cell spread using the Qiagen Blood Mini Kit.

Integrated DNA was pre-amplified using 100 nM Alu fw primer, 600 nM HIV-1 Gag rv primer, 0.2 mM dNTP, 1 U Phusion Hot Start Flex (Promega), and 45 ng DNA in 50 μL reactions. Cycling conditions were: 94°C for 30 s, followed by 40 cycles of 94°C for 10 s, 55°C for 30 s, and 70°C for 2.5 min. For quantitation of HIV-1 integration, a second round real-time quantitative PCR was performed using the pre-amplified DNA. These samples were run alongside a standard curve of known dilutions of CEM cells containing integrated HIV-1 DNA. Reactions contained 0.25 μM of RU5 fw and rv primers, and 0.2 μM probes, 1× Qiagen Multiplex Mastermix, and 10 μL pre-amplified DNA. Cyclin conditions were: 95°C for 15 min, followed by 50 cycles of 94°C for 60 s and 60°C for 60 s. 2LTR circles were measured by quantitative PCR (Apolonia et al., 2007). Reactions contained 150 ng DNA, 10μL 2LTR fw and rv primers, 10 μM probe and 1× TaqMan Gene Expression Master Mix (ThermoFisher). Cycling conditions were: 95°C for 15 min, followed by 50 cycles of 95°C for 15 s and 60°C for 90 s. Reactions were performed using 7500 Real-Time PCR System (Applied Biosystems). The following primers and probes were used:

- **Alu fw**: GCCTCCCAAAGTGCTGGGATTACAG
- **HIV-1 gag rv**: GTTCTCGCTATGTCACTTCC
- **RU5 fw**: TTAACCCCTAATAAGCTTGCC
- **RU5 rv**: GTTCGGGGCCACTGCTCGA
- **RU5-WT probe**: FAM-CCAGAGTCACACACAGACAGCCAGC-TAMRA
- **RU5-degenerate 1 probe**: FAM-CCAGAGTCACACACAGACAGCCAGC-TAMRA
- **RU5-degenerate 2 probe**: FAM-CCAGAGTCACACACAGATGGCAGC-TAMRA
- **2LTR fw**: AACTAGATCCCTAGACCCCTT
- **2LTR rv**: CTTGCTTCGTTGGGAGATGAAT
- **2LTR probe**: FAM-CTAGAGATTTCACACTGAC-TAMRA
RT-PCR
RNA was extracted from FACS sorted target memory CD4+ T cells with RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. cDNA was synthesised using SuperScript IV with random hexamer primers (Invitrogen) and qRT-PCR was performed using Fast SYBR Green Master Mix and 7500 Real-Time PCR System (Applied Biosystems). Gene expression was determined using the 2^- ΔΔCt method and normalised to GAPDH expression. The following primers were used:

\[
\text{GAPDH} \text{ fw: ACATCGCTCAGACACCAG; rv: TGTAATTGAGCTCAATGAGG;}
\]
\[
\text{CXCR6} \text{ fw: GACTATGGTTCCAGCAAGTTT; rv: GGCTCTGCAACTTATGGTAGA;}
\]
\[
\text{PRDM1} \text{ fw: ATGCCGGATATGACTCTGTGGA; rv: CTGGACCGAAGTGACCGCCATC;}
\]
\[
\text{CD69} \text{ fw: TCTGCTGGCAAATTCAAGCGA; rv: GTTGTCCTCCCTCGCTTCTGTG;}
\]
\[
\text{KLF2} \text{ fw: CTACACCAAGGTTCCAGATCG; rv: CCCTGTGCCTTCCGAGTG.}
\]

Immunofluorescence staining and image analysis
CD4+ T cells were spinoculated with HIV-1 WT or Dvpr virus and incubated in the presence or absence of 1 ng/mL IL-7. At 24 h, cells were adhered onto poly-L-lysine tissue-culture treated CellCarrier Ultra plates (Perkin Elmer) for 1 h and subsequently formaldehyde fixed. For staining, a blocking step was carried out for 1 h at room temperature with 10% goat serum/1% BSA in PBS. STAT5 and P-STAT5 detection was performed by primary incubation with rabbit α-P-STAT5 (D964) (1:47E7, Cell Signaling Technology) or rabbit α-STAT5 (D32N2, Cell Signaling Technology) for 18 h at 4°C and washed thoroughly in PBS. STAT5 or P-STAT5 staining was followed by incubation with mouse α-Gag (HIV-1 Gag core antigen (FH190-1-1, Beckman Coulter) for 18 h at 4°C. Primary antibodies were detected with secondary α-rabbit-AlexaFluor-488 and α-mouse-AlexaFluor-568 conjugates (Jackson Immuno Research) for 1 h at room temperature. All cells were labeled with Hoechst33342 (H3570, Thermo Fisher). Images were acquired using the WiScan® Hermes 7-Colour High-Content Imaging System (IDEA Bio-Medical, Rehovot, Israel) at magnification 60Χ/1.2NA. Three channel automated acquisition was carried out sequentially. Images were acquired across a well area density resulting in 350–500 FOV/well and 10–20,000 cells. For image analysis, P-STAT5 and STAT5 channels were pre-processed by applying a batch rolling ball background correction in FIJI ImageJ software package (Schindelin et al., 2012) prior to quantification. Cellular intensity of P-STAT5 or STAT5 was quantified using the Athena Image analysis software (IDEA Bio-Medical, Rehovot, Israel). Nuclei were identified as primary objects by segmentation of the Hoechst33342 channel. Cells were identified as secondary objects by nucleus dependent segmentation of the P-STAT5 or STAT5 channel. HIV-1 infected Gag+ cells were identified by segmenting Gag+ signal as primary objects followed by measuring of intracellular Gag intensity. Infected cells were identified by thresholding the population by a minimum Gag+ Average intracellular signal of 3.05 × 10^4 AU/Cell. For all populations, P-STAT5 and STAT5 intensity properties were then calculated.

Whole transcriptome profiling by RNA-Sequencing
RNA was extracted from FACS sorted target memory CD4+ T cells with RNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. For preparation of RNA-Sequencing libraries, RNA concentration was measured using the Qubit RNA High Sensitivity kit (Life Technologies) and quality checked on the 4200 Tapestation using either the High Sensitivity or standard RNA ScreenTape assay (Agilent Technologies), depending on the measured RNA concentrations. PolyA-tailed mRNA was separated for sequencing during library preparation. Libraries were prepared using KAPA’s mRNA HyperPrep kit (Roche Diagnostics) according to the manufacturer’s instructions using an input of up to 200 ng and a fragmentation incubation time of 8 min at 94°C. Samples were sequenced on Illumina’s NextSeq500 (Illumina Cambridge) using a high output 75 cycle paired-end run. 24 libraries were multiplexed in the same run. Libraries were pooled in equimolar quantities, calculated from concentrations measured using the Qubit dsDNA High Sensitivity kit (Life Technologies) and fragment analysis using the D1000 High Sensitivity assay on the 4200 Tapestation (Agilent Technologies).

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Transcriptomic comparison with published human T_{RM} cells
TPM data from previously published transcriptomes of human T_{RM} cells (GSE94964) (Kumar et al., 2017) were summed on gene level with Ensembl gene ID, gene name, and gene biotype using tximport and BioMart (Smedley et al., 2015; Soneson et al., 2016). TPM values < 0.001 were adjusted to 0.001 as a lower limit of detection. These data were aligned to the transcriptomic data from the published human T_{RM} cell lines using the methods described above.
present study using gene symbol in an integrated log2 transformed data matrix and subjected to batch correction by study using Combat (Leek et al., 2012). Expression of selected genes previously identified to be up and downregulated in TRM (Kumar et al., 2017) were used to cluster the samples in both studies using 1-Spearman rank correlation with average linkage in ClustVis (Metsalu and Vilo, 2015). A transcriptional signature score for TRM was derived from the difference between the sum of up and downregulated genes in TRM in the previously published signature. This score was used to evaluate the relative similarity of each transcriptome dataset in the present project to TRM and non-TRM data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism. Normally distributed data was analyzed for statistical significance by two-tailed t-tests (when comparing two groups) or one-way ANOVA with Bonferroni or Dunnett’s post-test (when comparing more than two groups). Data show the mean +/- the S.E.M with significance shown on the figures. Where appropriate, the median + IQR is shown and Kruskall-Wallis test was used to compare groups. Significance levels were defined as *, p < 0.05; **, p < 0.01 and ***, p < 0.001.
Supplemental information

HIV-1 Vpr drives a tissue residency-like phenotype during selective infection of resting memory T cells

Ann-Kathrin Reuschl, Dejan Mesner, Maitreyi Shivkumar, Matthew V.X. Whelan, Laura J. Pallett, José Afonso Guerra-Assunção, Rajhmun Madansein, Kaylesh J. Dullabh, Alex Sigal, John P. Thornhill, Carolina Herrera, Sarah Fidler, Mahdad Noursadeghi, Mala K. Maini, and Clare Jolly
Supplementary data Fig. 1. Related to Figure 1. (a) Experimental set-up schematic. (b) Flow cytometry gating strategy. (c) CD69, CD25, CD38 and HLA-DR expression on resting and activated CD4+ T cells (n=5). (d) Ki67 expression on resting and activated primary CD4+ T cells. Representative flow cytometry plots. (e) MCM2 expression on resting and activated primary CD4+ T cells. Representative flow cytometry plots. (f) Resting or (g) mitogenically-activated primary target CD4+ T cells cultured with HIV-1 infected Jurkat T cells separated by a 0.4μm transwell or in direct co-culture. Target cell infection levels was measured by intracellular staining for Gag. Representative flow cytometry plots are shown. (h) Infection levels of target CD4+ T cells determined by intracellular Gag staining and flow cytometry (n=2). (i) Cell-free infection of resting (open circles) or activated (closed circles) CD4+ T cells with increasing doses of HIV-1 (measured in RT units). Infection levels (%Gag+, left) and virus release (RT units, right) at 72h are shown (n=6). (j) Virus release into cultures supernatants during cell-to-cell spread at 72h of co-culture (n=10). (k) Survival of resting or activated CD4+ target T cells during cell-to-cell spread over time (n=6). (l) Survival of resting or activated CD4+ target T cells during cell-to-cell spread after 24h of αCD3/αCD28 restimulation (n=5). (m) Gating strategy for flow cytometry sorting of resting memory target T cells from co-cultures.
Supplementary data Fig. 2. Related to Figure 1. (a) Proportion of CD45RA+ naive and CD45RA- memory CD4+ T cells in unstimulated PBMCs (n=8). (b) Resting target CD4+ T cells were cultured with mock-treated or HIV-1-infected donor cells. Surface expression of CD45RA and CD62L were measured after 72h of co-culture. Representative flow cytometry plots are shown. (c) Quantification of T cell subsets in infected (Gag+) and uninfected (Gag-) resting CD4+ T cells according to CD45RA/CD62L expression (n=3). (d) Representative flow cytometry plots of CD45RA+ and CD45RA- CD4+ T cells pre- and post-isolation. (e) Expression of HIV-1 co-receptors CXCR4 (left) and CCR5 (right) receptors on naive and memory T cells at 72h of co-culture (n=4). (f) Representative histogram of intracellular Gag staining in resting naive (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread ± T20. (g) Gating strategy for (f). (h) Representative histogram of intracellular Gag levels in resting naive (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread ± Efavirenz. (i) Gating strategy for (h). (j) Representative histogram of CD4 surface levels in resting naive (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread. (k) HIV-1 infection downregulates CD4 expression. Shown are the CD4 MFI of the total CD3+ target cell population (n=6). (l) Mean post-sort population purity of T cells from (Fig. 1 n-p) was 99.92% target cells of which 99.86% were memory T cells (n=5). (m) and (n) Resting CD4+ memory T cells were isolated after 72h of cell-to-cell spread by FACS sorting and cultured for 1 or 4 days in the presence or absence of αCD3/αCD28. HIV-1 infection was measured by intracellular Gag staining at day 4 (m, n=5) and virus release measured by culture supernatant RT activity (n, n=4).
Related to Figure 2. (a and b) Representative flow cytometry plots showing (a) Ki67 and Gag, or (b) MCM2 and Gag staining of resting CD4+ T cells after co-culture with mock or HIV-1 infected primary donor CD4+ T cells. (c) FACS sorted CD69- resting naïve or memory CD4+ T cells co-cultured with HIV-1 infected primary CD4+ donor T cells and infection of targets measured by Gag staining (n=4). (d) FACS sorted CD69- CD4+ T cells co-cultured with HIV-1 infected primary CD4+ donor T cells. CD69 expression was measured on resting memory CD4+ T cells (n=3). Data are the means±SEM. Paired two-tailed t-test or one-way ANOVA with Bonferroni post-test was used. For (i), median+IQR is shown and Kruskall-Wallis test was used to compare groups *, p<0.05 ; **, p<0.01; ***, p<0.001; n.s., not significant.
Supplementary data Fig. 4. Related to Figure 2. (a) Flow cytometry gating strategy for infection of tonsil-derived T cells by cell-to-cell spread. (b) Total lymphocytes from one mediastinal lymph node sample co-cultured with HIV-1 infected activated autologous LN-derived lymphocytes. Targets gated as in (a). Infection of resting CD4+ T cells (CD3+/CD8-/Ki67- lymphocytes) shown as CD45RA vs Gag. (c) CD69 and CXCR6 co-expression on infected Gag+ and uninfected Gag- lymph node resting memory CD4+ T cells ± IL7.
**Supplementary data Fig. 5.** Related to Figure 3 and 7. (a) CD69 surface expression on resting CD45RA- memory CD4+ T cells following co-culture with primary CD4+ donor T cells infected with HIV-1 NL4-3 (WT), ΔVpr, ΔNef or ΔVpu or uninfected (mock) donors (n=9). (b) CXCR6 expression from (a) (n=9). (c) CD69/CXCR6 co-expression from (a) (n=9). (d) As for (b) but cells were incubated in the presence of IL7 (n=9). (e) CD69/CXCR6 surface co-expression from (d) (n=9). (f) Gag MFI of cell-to-cell spread of HIV-1 WT and ΔVpr to resting memory CD4+ T cells (n=10). Correlation plot of CD69 MFI with Gag MFI in (g) or presence (h) of IL7 (n=18). (i) SIPPR1 and KLF2 mRNA levels in FACS sorted resting memory CD4+ T cells from Fig. 3e. Fold change over mock is shown (n=5). (j) CD69 upregulation in response to IL7 on resting memory CD4+ T cells infected with HIV-1 WT, ΔVpr or Vpu mutants, categorised by Gag expression (n=9). (k) Infection levels of resting memory T lymphocytes from cellularised tonsils co-cultured with HIV-1 WT or ΔVpr infected Jurkat T cells (l) CD69 (left) and CD69/CXCR6 (right) expression on cells from (k) in response to IL7 (n=4). (m) Western blot showing siRNA knockdown of DCAF1 in CD3/CD28-activated CD4+ T cells 48h post transfection. Two representative samples are shown. (n) Number of live CD3+ Target cells recovered after 72h of cell-to-cell spread into control or DCAF1 siRNA-treated T cells (n=3). (o) Quantification of cell-to-cell spread of HIV-1 WT and ΔVpr to resting memory CD4+ T cells in the presence IL7 ± AC-4-130 (n=6).
Supplementary data Fig. 6. Related to Figure 3. Resting memory CD4+ T cells were co-cultured with HIV-1 infected primary CD4+ T cells infected with HIV-1 WT or mutant viruses. Representative flow cytometry plots of HIV-1 Gag and CD69 co-expression in the presence or absence of IL7 from three independent experiments are shown (a-c).
Supplementary data Fig. 7. Related to Figure 4 and 7. (a) Quantification of cell-to-cell spread of the indicated viruses to resting memory CD4+ T cells at 72h of co-culture (n=8). (b) Quantification of intracellular Gag-levels in resting memory CD4+ T cells at 72h post spinoculation with the indicated viruses (n=10). (c) Representative flow cytometry plots for (b) of virus delivery by spinoculation. (d) Western blot showing the degradation of Vpr-target UNG2 in Jurkat T cells 24h post spinoculation with Env-VLPs±Vpr. (e) Quantification of intracellular Gag-levels in resting memory CD4+ T cells at 72h post spinoculation with VLPs ± Vpr (n=5). (f) Representative flow cytometry plots for (e) of VLP-delivery of flag-tagged Vpr by spinoculation. (g) Expression of CD69 (left) and CD69/CXCR6 (right) on Gag+ resting memory CD4+ T cells at 72h post spinoculation of Env-VLPs with or without Vpr in the absence of IL7 (n=5).
Supplementary data Fig. 8. Related to Figure 5. (a) and (b) Venn diagrams showing overlap of DEGs comparing expression profiles of HIV-1 WT+IL7/Mock+IL7 with (a) HIV-1 ΔVpr+IL7/Mock+IL7 or (b) HIV-1 ΔVpr+IL7/HIV-1 WT+IL7. (c) GSEA was performed on expression profiles comparing HIV-1 WT+IL7/Mock+IL7 (black) or HIV-1 ΔVpr+IL7/HIV-1 WT+IL7 (grey). Normalised enrichment scores are shown for significantly enriched Hallmark gene sets are shown (FDR q-value<0.05 and NES>1.75). (d) and (e) top ten significantly enriched canonical pathways predicted by ingenuity pathway (IPA) analysis of DEGs (d) HIV-1 WT+IL7/Mock+IL7 or (e) HIV-1 ΔVpr+IL7/HIV-1 WT+IL7 (adjusted p-value<0.05). (f) Cytokines and (g) transcription regulators predicted to be upstream regulators by IPA of gene expression signatures HIV-1 WT+IL7/Mock+IL7 (black) or HIV-1 ΔVpr+IL7/Mock+IL7 (grey), line indicates p=0.05.