

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

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1 Human immunodeficiency virus type 1 (HIV-1) replicates in CD4+ T cells leading to profound
2 T cell loss, immunological dysfunction and AIDS. Determining how HIV-1 shapes the
3 immunological niche in which it resides to create a permissive environment is central to
4 informing efforts to limit pathogenesis, disturb viral reservoirs and achieve a cure. A key
5 roadblock in understanding HIV-T cell interactions is the requirement to activate CD4+ T cells
6 *in vitro* in order to make them permissive to infection. This dramatically alters T cell biology,
7 obscuring native virus-host interactions. Here we show that HIV-1 cell-to-cell spread permits
8 efficient and productive infection of resting CD4+ T cells without the need for prior activation.
9 Infection is preferential for resting memory T cells, is observed with both CXCR4-tropic virus
10 and CCR5-tropic transmitter-founder viruses and results in virus production and onward
11 spreading infection. Strikingly, we find that HIV-1 infection of resting memory CD4+ T cells
12 primes for induction of a tissue-resident memory (T_{RM})-like phenotype evidenced by
13 upregulation of T_{RM} markers CD69/CXCR6 alongside co-expression of CD49a, PD-1, CD101
14 as well as transcription factor Blimp-1. Furthermore, we reveal that HIV-1 initiates a
15 transcriptional program that overlaps with the core T_{RM} transcriptional signature. This
16 reprogramming depends on the HIV-1 accessory protein Vpr. We propose that HIV-1 infection

17 **drives a CD4+ T_{RM}-phenotype potentially sequestering infected cells within tissues to support**
18 **viral replication and persistence.**

19

20 **Introduction**

21 Resting primary CD4+ T cells cannot be efficiently infected by cell-free HIV-1 virions *in vitro* and
22 require robust mitogenic stimulation to support viral replication¹⁻³. This has led to the notion that T
23 cell activation is necessary for HIV-1 replication and that resting T cells are not permissive for HIV-
24 1 infection. However, mitogenic T cell activation *in vitro* results in wide-spread phenotypic and
25 functional reprogramming which dominates changes to gene and protein expression⁴⁻⁶, concealing
26 and altering authentic virus-host interactions. This presents a significant challenge for understanding
27 the cellular response to HIV-1 infection and the consequences of the virus-host interaction for HIV-
28 1 replication and persistence. While it is clear HIV-1 efficiently infects and replicates in activated T
29 cells, the outcomes of the virus-host interaction with resting T cells has been reported to be cell
30 death⁷ or latency⁸. However, previous data demonstrating that HIV-1 cell-to-cell spread is highly-
31 efficient and drives widespread changes in protein phosphorylation status in both infected and target
32 cells⁹⁻¹³ suggested that cell-to-cell spread may overcome the barrier to productive infection of resting
33 T cells. Here, we comprehensively show for the first time that HIV-1 exploits cell-to-cell spread to
34 efficiently infect resting memory CD4+ T cells and have used this to uncover a hitherto unknown
35 consequence of HIV-1 infection for T cell reprogramming driven by the accessory protein Vpr.

36

37 **Results**

38 To test whether cell-to-cell spread allows for productive infection of resting T cells, HIV-1 infected
39 Jurkat T cells (Fig. 1a) or primary CD4+ T cells (Fig. 1b) were co-cultured with uninfected resting
40 CD4+ T cells (Extended data Fig. 1a-e). We confirmed that CD4+ T cells isolated from peripheral
41 blood display a resting phenotype by staining for Ki67, CD69, HLA-DR and MCM2. (Extended data
42 Fig. 1c-e). Infection of resting target cells in the absence of mitogenic or cytokine activation was
43 measured. Direct co-culture of infected and uninfected cells resulted in significant levels of HIV-1

44 infection of resting CD4+ target T cells (Gag+) measured by intracellular flow cytometry staining (Fig.
45 1a, b and Extended data Fig. 1f). By contrast, resting CD4+T cells were not infected (<1%) when
46 cell-cell contact was prevented by separating the two cell populations by a transwell (Fig. 1a,b), a
47 condition that only allows for cell-free infection. As expected, mitogenic activation of target T cells
48 made them more permissive to cell-free HIV-1 but, as previously shown, infection was still
49 substantially boosted by direct co-culture allowing for additional cell-to-cell spread (Extended data
50 Fig.1f-h)^{9,11,12}. Infection of resting CD4+ target T cells was preferentially detected in CD45RA-ve
51 resting memory T cell populations rather than CD45RA+ naïve T cells, which are both abundant in
52 peripheral blood (Fig. 1c-e, Extended Fig. 1i). Co-staining for CD62L confirmed that the infected
53 CD45RA+ve cells were mainly naïve rather than T_{EMRA} (Extended data Fig. 1j,k)¹⁴. The preferential
54 infection of CD45RA-ve memory T cell populations rather than the CD45RA+ve naïve population, is
55 in agreement with HIV-1 being predominantly detected in memory CD4+ T cells *in vivo*¹⁵⁻¹⁷. This
56 was not due to competition between naïve and memory cells because the same effect was observed
57 when CD45RA+ve and CD45RA-ve resting CD4+ target T cells were separated prior to cell-to-cell
58 infection (Fig. 1f and Extended data Fig. 2a). Cell-to-cell infection of resting memory T cells was
59 observed with CXCR4-tropic strain NL4.3, and CCR5-tropic viruses NL4.3 BaL and two transmitter-
60 founder (T/F) primary isolates (CH040 and CH077) (Fig. 1g-i) demonstrating that selective
61 permissivity was not unique to a particular virus or receptor tropism. Preventing viral entry with fusion
62 inhibitor (T20), or blocking reverse transcription (Efavirenz), inhibited the appearance of Gag+ve or
63 GFP+ve cells (Fig. 1j-l and Extended data Fig. 2b,c) demonstrating that this signal reflects productive
64 infection and not simply virus capture^{9,11-13}. Consistent with infection, we also observed
65 downregulation of CD4 expression on target cells (Fig. 1m and Extended data Fig. 2d) that was
66 most pronounced in the resting memory T cell population. Importantly, flow-sorting and culturing
67 HIV-1 infected resting target T cells showed that these cells support viral replication by producing
68 new virus and transmitting infection to new target cells (Fig. 1n-p and Extended data Fig. 2e), further
69 demonstrating productive infection.

70 We confirmed that HIV-1+ target T cells infected by cell-to-cell spread maintained their resting
71 phenotype and did not upregulate Ki67 or MCM2, two markers of cell-cycle progression (Extended

72 data Fig. 3a,b), showing these cells were not simply being activated and driven into cell cycle, either
73 by infection or bystander effects during co-culture. Intriguingly, expression of CD69 on HIV-1 infected
74 resting memory target T cells was significantly increased compared to mock-treated target T cells
75 (Fig. 2a,b). This was not due to preferential infection of a minor pre-existing population of CD69+ve
76 CD4+ T cells in blood (Extended data Fig. 1c) because flow cytometry sorting of CD69-ve CD4+ T
77 cells (prior to co-culture with HIV-1 infected donor T cells) showed *de novo* upregulation of CD69 on
78 the newly-infected resting memory target cells (Extended data Fig.3c,d). While CD69 is classically
79 thought of as a marker of early T cell activation, expression can occur independently of cell cycle
80 progression and T cell activation^{18–20}. Consistent with this we did not detect T cell activation
81 concomitant with CD69 upregulation in HIV-1 infected resting CD4+ memory T cells since CD69+ve
82 cells remained HLA-DR-negative (Extended data Fig.3e).

83 Functionally, CD69 is crucial for T cell retention in tissues by interfering with S1P receptor
84 mediated egress and has been identified as a hallmark of tissue-resident memory (T_{RM}) T cells²¹.
85 Recently it has been demonstrated that although T_{RM} cells are largely absent in peripheral blood
86 (Extended data Fig. 3f), precursor cells poised to adopt a T_{RM} phenotype are present in
87 circulation^{22,23}. Interestingly, while HIV-1 infection alone was associated with upregulation of CD69,
88 exposure of HIV-1 infected resting memory T cells to the homeostatic T cell cytokine IL7 further
89 boosted CD69 upregulation 4-fold, as compared to HIV-1 alone or IL7 alone (Fig. 2c and Extended
90 data Fig. 3j). IL7 secreted by stromal cells is required for long-term maintenance of CD4+ T_{RM} cells^{24–}
91 ²⁶. Although IL7 can enhance HIV-1 infection of T cells²⁷ (Extended data Fig. 3g), infection of resting
92 memory T cells mediated by cell-to-cell spread does not require IL7 (Fig.1) and IL7 increased CD69
93 expression on infected cells even when added 48h post-infection (Extended data Fig. 3j). HIV-1
94 induced CD69 upregulation was completely abrogated by suppressing infection with the fusion
95 inhibitor T20 (Fig. 2c) or by treating cells with Ruxolitinib that blocks IL7-mediated JAK signalling
96 (Fig. 2d) demonstrating that the enhanced CD69 induction requires both infection and cytokine
97 signalling. Similar infection-driven enhancement of CD69 expression was observed in response to
98 γ_c-chain cytokine IL15 (Fig. 2e,f) but not IL12 or TGF-β (Extended data Fig.3h,i). Consistent with the
99 hypothesis that HIV-1 infection induces a CD4+ T_{RM}-like phenotypic signature, resting memory T

100 cells also upregulated the T_{RM} marker CXCR6²¹ during HIV-1 infection, (Fig. 2g,h and Extended data
101 Fig. 3k,n), as well as increasing the population of CD69+ve cells co-expressing CD49a, PD-1 and
102 CD101 that are also associated with the core T_{RM} signature²¹ (Fig. 2i, j and Extended data Fig. 3l).
103 As expected for T_{RM}s, we saw no upregulation of CX3CR1 expression (Extended data Fig. 3o) and
104 no transcriptional upregulation of S1PR1 or KLF2 mRNA (Extended data Fig. 5i). Critically, induction
105 of T_{RM}-associated markers did not occur in uninfected Gag-ve bystander cells (Fig. 2k and Extended
106 data Fig. 3m,n). By contrast to CD8+ T_{RM} cells, CD103 was barely detectable and not upregulated
107 (Extended data Fig. 3p,q), consistent with the observation of limited CD103 expression on CD4+ T
108 cells²¹. Induction of CD69 expression was also concomitant with upregulation of the T_{RM}-associated
109 transcription factor Blimp-1²⁸⁻³⁰ (Fig. 2l,m). Similar upregulation of T_{RM}-associated markers were also
110 observed when unstimulated CD4+ T cells from tonsil (Fig. 2n,o) or mediastinal lymph nodes (Fig.
111 2p,q) were infected with HIV-1 via cell-to-cell spread and exposed to IL7 (Fig. 2o,q), demonstrating
112 that induction of TRM-like phenotype also occurs in tissue-derived T cells following HIV-1 infection.
113 Taken together, these data suggest that HIV-1 infection of resting memory CD4+ T cells
114 reprogrammes cells by upregulating expression of T_{RM}-associated marker proteins, and thus induces
115 a phenotype characteristic of tissue residency.

116 HIV-1 expresses four accessory proteins, Vif, Vpu, Vpr and Nef which directly and indirectly
117 manipulate host cell factors to facilitate efficient viral replication *in vivo* and drive pathogenesis³¹. Co-
118 culture of resting target T cells with donor T cells infected with HIV-1 accessory protein mutants
119 showed that deletion of Vpr (HIV-1 ΔVpr) resulted in a complete abrogation of the T_{RM}-like phenotype
120 as evidenced by a lack of CD69 upregulation, and no detection of the CD69+/CXCR6+/CD49a+
121 triple-positive T_{RM}-like memory population (Fig. 3a,b,d, Extended data Fig. 4 and 5a-e). By contrast,
122 deletion of Vpu or Nef did not affect HIV-1 induction of T_{RM}-markers on infected resting T cells (Fig.
123 3a,b, Extended data Fig. 4 and Fig. 5a-e). HIV-1 ΔVif could not be tested because Vif is required to
124 antagonise APOBEC3-mediated viral restriction and allow infection³². HIV-1 Vpr is not required for
125 infection of T cells *in vitro*^{33,34} and concordantly, loss of T_{RM}-marker protein induction was not due to
126 lack of infection of target cells by HIV-1 ΔVpr virus, nor reduced Gag expression (Fig. 3c and
127 Extended data Fig. 5f-h). Critically, Vpr was also required for induction of CD69 expression observed

128 at the mRNA level, as well as induction of CXCR6 and Blimp1 (*PRDM1*) mRNA (Fig. 3e). As
129 expected, there was no upregulation of *S1PR1* or *KLF2* mRNA by either HIV-1 WT or Δ Vpr virus
130 (Extended data Fig.5i), consistent with their suppression under conditions of T_{RM} induction²¹. HIV-1
131 dependent upregulation of CD69 surface expression was inhibited by Ruxolitinib treatment (Fig. 2d)
132 in a Vpr-dependent manner (Extended data Fig. 5j). Furthermore, HIV-1 infection downregulated the
133 IL7 receptor alpha-subunit (CD127) from the cell surface (Extended data Fig 5k) and transcriptionally
134 (fold change=0.693, adjusted p-value=5.89E-08, Supplementary table 1), indicative of activation of
135 this pathway³⁵ by HIV-1. This was accompanied by a significant increase in the intracellular levels of
136 phosphorylated transcription factor STAT5 (Extended data Fig. 5j and k). These data are consistent
137 with Vpr manipulating cellular signalling pathways to drive induction of a T_{RM} -like phenotype in
138 response to IL7. Vpr-dependent T_{RM} -like induction was also accompanied by spontaneous
139 production of IFN γ by infected CD4+ memory T cells (Fig. 3f), in line with the increased capacity of
140 T_{RM} to produce this cytokine^{21,36}. Notably, Vpr-mediated induction of a T_{RM} -like phenotype was not
141 inhibited by the integrase inhibitor Raltegravir (Fig. 3g, h) that potently suppressed HIV-1 integration
142 into resting memory CD4+ T cells infected by cell-to-cell spread (Fig. 3i), demonstrating that the T_{RM} -
143 like phenotype does not require integration. Of note, the presence of integrated provirus in resting
144 CD4+ T cells (less than or equal to 1 provirus per cell Fig. 3i) further supports the observation that
145 cell-to-cell spread results in resting CD4+ T cell infection (Fig. 1). Collectively, these data identify
146 Vpr as the viral determinant required for upregulation of T_{RM} -associated proteins on HIV-1 infected
147 resting CD4+ T cells.

148 Vpr is a multifunctional protein that is packaged into viral particles and is present during the
149 early stages of infection where it plays an important, but as yet poorly-defined role in HIV-1
150 pathogenesis. Amongst the best-defined functions of Vpr are its ability to a) bind the Cul4A-DDB1
151 (DCAF1) complex (leading to an interaction with the ubiquitinylation and proteasomal machinery); b)
152 induce G2/M cell-cycle arrest and; c) drive apoptosis in infected cells³⁷⁻⁴¹. We abrogated these
153 functions individually by introducing the mutations Q65R, S79A or R80A into Vpr in the context of
154 virus and confirmed that each Vpr mutant is packaged into virions (Fig. 3j). Co-culture of resting
155 memory target T cells with HIV-1+ T cells infected with different Vpr mutants revealed that the Vpr

156 mutants S79A or R80A (inhibits cell cycle arrest) behaved similarly to WT virus and did not induce
157 CD69 and CXCR6 upregulation (Fig. 3k-m). By contrast, Q65R (that is most closely associated with
158 loss of DCAF1 binding) was unable to induce a T_{RM}-like phenotype following infection of target cells,
159 behaving like ΔVpr virus in these experiments (Fig. 3k-m).

160 Next we performed transcriptional profiling by RNA-Seq analysis of flow-sorted cells infected
161 with HIV-1 WT or ΔVpr virus by cell-to-cell spread. Figure 4 shows that HIV-1 infection induced
162 widespread changes to gene expression in resting memory T cells when compared to uninfected
163 cells (232 differentially expressed genes (DEG), fold change >1.2, adjusted p-value<0.01) (Fig. 4).
164 Hierarchical clustering and principal component analysis revealed that the gene expression patterns
165 in response to HIV-1 WT infection were clearly distinct from those induced following infection with
166 ΔVpr virus (Fig. 4 a-c, e,f) demonstrating that Vpr-deletion suppresses the global transcriptional
167 response to HIV-1 infection. Specifically, infection with ΔVpr virus resulted in only 13 genes showing
168 statistically significant changes compared to uninfected cells, by contrast to 232 genes for HIV-1
169 WT virus (Supplementary table 1 and 3, Fig. 4e). In fact, much of the transcriptional response to
170 HIV-1 infection was regulated by Vpr, as evidenced by changes in DEG in the presence and absence
171 of Vpr (Fig. 4f). The requirement for Vpr in driving many of the changes to DEG was also observed
172 in response to IL7 (Fig. 4d, Supplementary table 2 and 4, and Extended data Fig. 6a). Consistent
173 with Vpr manipulating the T cell response to HIV-1 infection (Supplementary table 5 and 6), gene-
174 set enrichment analysis (GSEA) (Fig. 4g) and Ingenuity Pathway Analysis (IPA) (Fig. 4h and i)
175 revealed enrichment of numerous cellular signalling pathways following HIV-1 infection that
176 appeared Vpr-dependent, most notably pathways associated with cytokine and inflammatory
177 responses as well as immune signalling (Fig. 4g, Extended data Fig. 6 and Supplementary table 7
178 and 8). This was further evidenced by Upstream Regulator analysis that showed significant
179 enrichment for genes associated with cytokine signalling and transcriptional regulators that were
180 again largely Vpr-dependent (Fig. 4j and k, and Extended data Fig. 6). Taken together, these data
181 reveal that HIV-1 induces dramatic reprogramming during infection of resting memory CD4+ T cells
182 that is driven largely by Vpr.

183 Tissue residency of T cells has been associated with a 31-gene core transcriptional
184 signature²¹. We took advantage of this dataset and our RNAseq analysis to determine whether this
185 core T_{RM} signature was enriched in our transcriptome of HIV-1 infected resting memory T cells.
186 Hierarchical clustering of our data, compared to the core signature of T_{RM} cells (CD69+ve T cells
187 isolated from human lung and spleen)²¹ from Kumar et al, showed that HIV-1 infected memory T
188 cells exposed to IL7 grouped distinctly and clustered with CD69+ve T_{RM} cells (Fig. 5a,
189 Supplementary table 9) distinct from non-T_{RM} T cells (CD69-ve T cells isolated from tissue and blood
190 from Kumar et al). We further corroborated this finding by calculating a T_{RM} enrichment score based
191 on the published core transcriptional signature, which showed that HIV-1 infected resting memory T
192 cells (both +/-IL7) harbour a higher T_{RM} signature score, significantly different from non-T_{RM} T cells,
193 approximating more closely to that of *bona fide* T_{RM} cells (Fig. 5b). Critically, this was Vpr-dependent,
194 with mock and ΔVpr infected cells showing an enrichment score that was not statistically different to
195 non-T_{RM}. Taken together, these data demonstrate that HIV-1 induces both a phenotypic and
196 transcriptional T_{RM}-like signature in resting T cells via the accessory protein Vpr.

197

198 **Discussion**

199 Our discovery that resting CD4+ T cells can be productively infected by cell-to-cell spread, allowing
200 for viral integration, replication and dissemination, transforms our ability to determine how T cells
201 respond to and support HIV-1 replication without confounding activation-induced changes.
202 Recently, it has been reported that cell-to-cell spread can also facilitate latent infection of resting T
203 cells without productive infection⁸. Together, these two studies highlight the distinct advantages of
204 co-culture models (that do not require mitogenic, experimental stimulation of T cells to drive infection)
205 to study native HIV-1-host cell interactions and the cellular response to infection.

206 Here we have employed our co-culture model to show that HIV-1 infection of resting memory
207 T cells induces a T_{RM}-like phenotype evidenced by upregulation and co-expression of T_{RM}-associated
208 markers on infected cells and induction of a core T_{RM} transcriptional signature. HIV-1 establishes
209 cellular and tissue reservoirs (both active and latent) that ultimately prevent cure with antiretroviral

210 therapy. Importantly, T_{RM} cells are long-lived and are thought to be largely confined to tissue⁴²
211 providing an alternate model for a tissue-associated reservoir driven by the virus itself. Our results
212 suggest that HIV-1 persistence and the establishment of tissue reservoirs may be driven, in part,
213 through direct viral induction of a T_{RM} -like phenotype via transcriptional reprogramming. Recently,
214 TRM in cervical tissue were found to be preferentially infected by HIV-1 and can harbour an HIV-1
215 reservoir *in vivo*⁴³. The relative contribution of pre-existing versus HIV-1 induced T_{RM} cells to viral
216 reservoirs and their relative abundance in different anatomical compartments *in vivo* remains to be
217 quantified, but we expect T_{RM} cells harbouring virus to be important contributors to viral persistence.
218 In light of these findings it is possible that HIV-1 infected cells circulating in peripheral blood may in
219 fact represent cells that have failed to become part of the tissue reservoir, leading to an
220 underestimation of the true viral burden. Having shown that HIV-1 infection of resting T cells by cell-
221 to-cell spread results in productive infection, we hypothesise that induction of a T_{RM} -like phenotype
222 in infected cells may also play additional roles in establishing and maintaining viral reservoirs by
223 sequestering infected cells in tissue sites where susceptible target T cells are in abundance, thus
224 supporting localised viral replication. Indeed we have shown that infected resting memory T cells
225 support spreading infection to disseminate virus. Given the importance of T_{RM} cells as a population
226 that are increasingly recognised to be critical in providing localised immunity and
227 immunosurveillance^{20,30}, future work should focus on understanding the contribution of HIV-1
228 induced T_{RM} -like cells in pathogenesis and persistence.

229 It is now emerging that committed T_{RM} precursors, imprinted with the capacity to become
230 mature T_{RM} pre-exist in blood and that when exposed to the appropriate cues in tissues or *ex vivo*
231 can become tissue-resident^{22,23}. Thus the ontogeny, derivation and maintenance of T_{RM} cells
232 appears more complex than initially appreciated. Our discovery that HIV-1 induces a T_{RM} -like
233 phenotype in CD4+ T cells provides an opportunity to gain new understanding of mechanisms behind
234 CD4+ T_{RM} induction and maintenance.

235 We found that HIV-1 infection of resting memory T cells was associated with striking
236 transcriptional reprogramming that was licensed by Vpr, thus identifying a novel function for this
237 enigmatic HIV accessory protein. Notably, HIV-1 induction of a T_{RM} -like phenotype via Vpr was

238 accompanied by induction of a T_{RM} transcriptional signature that aligned closely with a published
239 core T_{RM} signature²¹. Vpr-deletion not only abolished induction of this T_{RM} signature, but also many
240 HIV-1 induced changes to gene expression following infection of resting T cells. This is in keeping
241 with wide-spread proteome remodelling by Vpr in activated T cells⁴⁴, but suggests that these
242 changes may be driven in part by a hitherto unappreciated role for Vpr in modulating the host cell
243 gene expression profile. Whether this reflects wide-spread epigenetic changes mediated by Vpr or
244 manipulation of key upstream regulators remains to be determined. Our data show HIV-1 Vpr
245 modulates T cell responsiveness to external stimuli by manipulation of immune signalling pathways,
246 including innate and inflammatory responses. This is particularly intriguing and suggests HIV-1
247 manipulates immune signalling pathways to benefit the virus, in this case by priming resting memory
248 T cells for T_{RM} -like induction. Vpr-mediated induction of T_{RM} -like phenotype was dependent on
249 residue Q65 and Vpr is reported to drive wide-spread remodelling of the cellular proteome via its
250 recruitment of DCAF1 through Q65⁴⁴. Whether this requirement for Q65 in induction of a T_{RM} -like
251 phenotype is DCAF1-dependent or not remains unclear because DCAF1 knockdown in primary T
252 cells made cells hyper-responsive to HIV-1 induced cell death (Extended data Fig. 7). Thus we
253 cannot at present formally exclude other functions of Vpr Q65 in the process of T_{RM} -induction.

254 Notably, a rare case of laboratory-derived infection with Vpr-defective HIV-1 was
255 characterised by markedly delayed seroconversion, suppressed viremia and normal CD4+ T cell
256 counts⁴⁵, consistent with reduced pathogenesis and failure to establish and maintain a significantly
257 large tissue reservoir. We envisage therapeutic targeting of Vpr to manipulate persistence and
258 pathogenesis. In order to achieve an HIV-1 cure it is essential to understand the nature and
259 establishment of HIV-1 reservoirs and how to manipulate them. By demonstrating that HIV-1
260 infection drives a T_{RM} -like phenotype during infection of resting memory T cells we have taken a
261 significant step towards this to help accelerate the quest for an HIV-1 cure.

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370

371 **Methods**

372 **Cells**

373 Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats from healthy donors (UK
374 NHS Blood and Transplant Service) by density centrifugation using FicollPaque Plus (GE Life
375 Sciences) and cryopreserved in 10% DMSO (Sigma-Aldrich) in 90% FBS (LabTech). Resting CD4+
376 T cells were isolated from total PBMCs by negative selection using the MojoSort Human CD4+ T
377 Cell Isolation kit (Biolegend) according to the manufacturer's instructions. CD45RA+ve naïve and
378 CD45RA-ve memory populations were further separated after CD4+ T cell isolation with CD45RA
379 MicroBeads (Biolegend). For activated CD4+ T cells, PBMCs were treated with 5µg/ml PHA (Sigma)
380 and 10IU/ml IL2 (Centre For AIDS Reagents, National Institute of Biological Standards and Control,
381 UK [CFAR]) in RPMI1640 with 20% FBS for 72h prior to CD4+ T cell isolation. Once purified, CD4+
382 T cells were cultured in RPMI supplemented with 20% FBS and 10IU/ml IL2. Jurkat T cell lines
383 (Clone E6-1; ATCC TIB-152) were cultured in RPMI with 10% FBS and 100U/ml
384 penicillin/streptomycin. HEK 293T/17 cells (ATCC, CRL-11268) were cultured in DMEM with 10%
385 FBS and 100U/ml penicillin/streptomycin. Tonsil tissue was obtained from an individual with primary
386 HIV infection who underwent routine tonsillectomy (2 months after commencement of ART). As
387 previously described⁴⁶, the tonsillar tissue from elective tonsillectomy was dissected and
388 mechanically digested, prior to cryopreservation of the cellular suspension. This was collected under
389 the Imperial College Infectious Diseases Biobank (REC: 15/SC/0089). Lymph nodes were obtained
390 from the field of surgery of participants undergoing surgery for diagnostic purposes and/or
391 complications of inflammatory lung disease. Informed consent was obtained from each participant,

392 and the study protocol approved by the University of KwaZulu-Natal Institutional Review Board
393 (approval BE024/09).

394

395

396 **Plasmids and virus production**

397 The HIV-1 clone pNL4.3 was obtained from the CFAR, NIBSC (cat# 2006). HIV-1 NL4.3 ΔNef and
398 pNL4.3 ΔVpr were provided by R. Sloan (University of Edinburgh, UK)⁴⁷. NL4.3 ΔVpu was provided
399 by S. Neil (King's College London, UK)⁴⁸. NLENG1-IRES was provided by D. Levy (NYU, USA)⁴⁹.
400 NL4.3 bearing the CCR5-tropic BaL *Env* was provided by G. Towers (UCL, UK)⁵⁰. CCR5 tropic
401 transmitter/founder virus plasmids CH044 and CH077 were provided by G. Towers (UCL, UK) and
402 were originally obtained through the NIH AIDS Reagent Program [NIHARP], Division of AIDS, NIAID,
403 NIH: pCH040.c/2625 (cat# 11740) and pCH077.t/2627 (cat# 11742) from Dr. John Kappes and Dr.
404 Christina Ochsenbauer. NL4.3 Vpr Q65R, NL4.3 Vpr S79A, NL4.3 Vpr R80A were generated by site-
405 directed mutagenesis (Promega) using the following primers:

406 NL4.3 VprQ65R fw:GTGGAAGCCATAATAAGAATTCTGCGACAACTGCTGTTATCCATTCAG
407 NL4.3 VprQ65R rv:CTGAAATGGATAAACACAGCAGTGTGCGAGAATTCTTATTATGGCTTCCAC
408 NL4.3 Vpr S79A fw: GAATTGGGTGTCGACATGCCAGAATAGGCGTTACTC
409 NL4.3 Vpr S79A rv:GAGTAACGCCTATTCTGGCATGTCGACACCCAATT
410 NL4.3 Vpr R80A fw: GGTGTCGACATAGCGCAATAGGCGTTACTCG
411 NL4.3 Vpr R80A rv: CGAGTAACGCCTATTGCGCTATGTCGACACC.

412 All virus stocks were produced by plasmid transfection of HEK 293T cells with Fugene 6 (Promega).
413 Supernatants were harvested at 48h and 72h, filtered, DNase treated, purified and concentrated by
414 ultracentrifugation through a 25% sucrose cushion and resuspended in RPMI1640 with 10% FBS.
415 Viral titres were determined by measuring reverse transcriptase activity by SG-PERT assay⁵¹.

416

417 **HIV-1 infection and cell-to-cell spread**

418 For cell-to-cell spread experiments, activated primary CD4+ T cells (donor cells) were infected with
419 800mU reverse transcriptase per 10⁶ cells of HIV-1 by spinoculation at 1200xg for 2h at room
420 temperature and incubated in RPMI 20% FBS supplemented with 10IU/ml IL2 for 72h. HIV-1+ donor

421 CD4+ T cells were washed with medium, counted and cultured with primary CD4+ target T cells at
422 a 1:1 ratio in RPMI 20% FBS supplemented with 10IU/ml IL2 for up to 72h before analysis by flow
423 cytometry or FACS sorting. Uninfected target CD4+ T cells were pre-stained with 1-2nM CellTrace
424 FarRed dye (Invitrogen) prior to co-culture. For cell-to-cell spread into tonsil-derived lymphocytes,
425 total tonsil lymphocytes were cultured at a 4:1 ratio with HIV-1 infected or uninfected eFluor450-
426 labelled Jurkat T cells. For FACS sorting experiments, donor cells were pre-labeled with cell dye
427 eFluor450 (ThermoFisher). For transwell experiments, HIV-1 infected donor T cells were separated
428 from target T cells by a 0.4µm transwell insert (Corning). Experiments to quantify cell-to-cell versus
429 cell-free infection in the presence and absence of a transwell were performed in equivalent volumes
430 (600µl). For some experiments, FACS sorted infected resting CD4+ target T cells were returned into
431 cultured for up to 4 days. Infection levels were measured by intracellular Gag staining and flow
432 cytometry, and virus release into cell culture supernatant determined by SG-PERT⁵¹. At day 1 or day
433 4 post FACS sorting, resting CD4+ T cells were washed extensively and co-cultured at a 1:1 ratio
434 with uninfected eFluor450-labelled Jurkat T cells for 72h, when Jurkat T cell infection was measured
435 by Gag-staining. Where indicated, cultures were incubated in the presence of 20ng/ml IL7 (Miltenyi
436 Biotec), 20ng/ml IL15 (Peprotech), 20ng/ml IL12 (Peprotech) or 50ng/ml TGFβ (Peprotech). The
437 following inhibitors were added 30min before co-culture at the following concentrations: T20 (25-
438 50ng/ml, CFAR), Efavirenz (1µM, CFAR), Raltegravir (5µM, CFAR) and Ruxolitinib (50nM, Sigma).
439 For RNAi knockdown of DCAF1, primary CD4+T cells were activated for 4 days with 1µg/ml plate-
440 bound αCD3 antibody (cloneOKT3, Biolegend) in the presence of 2 µg/mL soluble αCD28
441 antibody(clone CD28.2, Biolegend). RNAi knockdown of DCAF1 was performed as described
442 before⁵² using ON-TARGET plusHuman DCAF1 siRNA - SMARTpool (Dharmacon) and non-
443 targeting siRNA (Dharmacon) was used as a control. 2×10⁶cells were electroporated with 200 pmol
444 siRNA using a NeonTransfection System (Thermo Fisher Scientific; three pulses, 10 ms, 1,600 V).
445 After 48h, DCAF1 knockdowns were confirmed by western blotting and cells used in cell-to-cell
446 spread experiments as described above.

447

448

449 **Flow cytometry and FACS**

450 For flow cytometry analysis, cells were washed in PBS and stained with fixable Zombie UV
451 Live/Dead dye, Aqua Live/Dead dye or NIR Live/Dead dye (Biolegend) for 5 min at 37°C. Excess
452 stain was quenched with FBS-complemented RPMI. When tonsil and lymph node lymphocytes were
453 used, Live/Dead staining was quenched using human AB serum (Sigma) in RPMI. Cell surface
454 staining was performed in PBS, complemented with 20% Super Bright Staining Buffer
455 (ThermoFisher) when appropriate, at 4°C for 30min. Unbound antibody was washed off thoroughly
456 and cells were fixed with 4% FA or PFA before intracellular staining. For intracellular detection of
457 IFN γ in infected target CD4+ T cells after 72h of cell-to-cell spread, cells were treated throughout the
458 co-culture with IL7 and Brefeldin A (Biolegend) treated for 6h before surface staining and fixation.
459 Permeabilisation for intracellular staining was performed with IC perm buffer or FoxP3 Buffer set
460 (Biolegend) according to the manufacturer's instructions. For detection of intracellular P-STAT5,
461 cells were resuspended in ice cold True-Phos Perm buffer (Biolegend) and permeabilised for 48h at
462 -20°C. Intracellular P-STAT5 staining was then performed in PBS with wash steps performed at 1800
463 rpm for 6 min at 4°C. The following antibody clones and fluorochromes were used: CD3 (UCHT1,
464 Biolegend; Bv510, Bv711, FITC), CD8 (SK1, Biolegend; Bv605, PE), CD4 (SK3, Biolegend;
465 APC/Fire750); CD45RA (HI100, Biolegend; Bv421, PE-Dazzle); CD45RO (UCHL1, Biolegend;
466 PerCp-Cy5.5), CD69 (FN50, Biolegend; APC/Fire750, PE-Dazzle); CXCR6/CD186 (K041ES,
467 Biolegend; PE-Dazzle); MCM2 (ab4461, ABCAM; was detected with a secondary anti-rabbit
468 AlexaFluor488-tagged antibody); HLA-DR (L234, Biolegend; PerCp-Cy5.5); CD49a (TS2/7,
469 Biolegend; PE-Cy7); PD-1 (EH12.2H7, Biolegend; PE-Cy7); Ki67 (Ki-67, Biolegend; Bv711, PE);
470 Blimp-1 (6D3, BD Pharmingen; PE); CD101 (BB27, Biolegend; PE-Cy7); CX3CR1 (2A9-1,
471 Biolegend; PE-Dazzle); CD103 (Ber-ACT8, Biolegend; Bv711); CD127 (AO19D5, Biolegend; PE-
472 Cy7), IFN γ (B27, Biolegend; PE), Phospho-STAT5 (Clone 47/Stat5 (pY694), BD; PE) and HIV-1
473 Gag core antigen (FH190-1-1, Beckman Coulter; PE, FITC). All samples were acquired on either an
474 BD Fortessa X20 or LSR II using BD FACSDiva software and analyzed using FlowJo v10 (Tree
475 Star). FACS sorting was performed with a BD FACSaria III or BD FACSaria IIu Cell Sorter. Cells
476 were either lysed immediately in RLT lysis buffer (Qiagen) with 1% β -mercaptoethanol (Sigma) and
477 stored at -80C for later RNA extraction or resuspended in RPMI supplemented with 20% FBS and
478 10IU/ml IL2 and used immediately.

479 **Western blotting**

480 Virus-containing supernatants (normalised for equal loading by measuring RT activity) or fifteen
481 micrograms of total CD4+ T cell protein lysate were separated by SDS-PAGE, transferred onto
482 nitrocellulose and blocked in PBS with 0.05% Tween 20 (v/v) and 5% skimmed milk (w/v). Blots
483 were probed with rabbit antisera raised against HIV-1 Gag p24 (cat# 0432 donated by Dr G. Reid
484 and obtained from the CFAR), Vpr anti-serum (cat# 3951, NIH ARP), α -alpha-Tubulin (T6199,
485 Sigma-Aldrich) and α -DCAF1 antibody (11612-1-AO, Proteintech), followed by goat anti-rabbit or
486 goat anti-mouse IRdye 800CW or 680RD infrared secondary antibody (Abcam) and imaged using
487 an Odyssey Infrared Imager (LI-COR Biosciences) and analysed with Image Studio Lite software.

488

489 **Quantification of HIV-1 integration**

490 To quantify integration of HIV-1 in resting T cells, nested Alu-gag quantitative PCR was performed
491 as previously described⁵³. Briefly, DNA was isolated from FACS sorted infected resting CD4+
492 memory T cells after 72h of cell-to-cell spread using the Qiagen Blood Mini Kit.
493 Integrated DNA was pre-amplified using 100nM Alu fw primer, 600nM HIV-1 Gag rv primer, 0.2mM
494 dNTP, 1U Phusion Hot Start Flex (Promega), and 45ng DNA in 50 μ l reactions. Cycling conditions
495 were: 94°C for 30s, followed by 40 cycles of 94°C for 10s, 55°C for 30s, and 70°C for 2.5min. For
496 quantitation of HIV-1 integration, a second round real-time quantitative PCR was performed using
497 the pre-amplified DNA. These samples were run alongside a standard curve of known dilutions of
498 CEM cells containing integrated HIV-1 DNA. Reactions contained 0.25 μ M of RU5 fw and rv primers,
499 and 0.2 μ M probes, 1x Qiagen Multiplex Mastermix, and 10 μ l pre-amplified DNA. Cycling conditions
500 were: 95°C for 15min, followed by 50 cycles of 94°C for 60s and 60°C for 60s. 2LTR circles were
501 measured by quantitative PCR⁵⁴. Reactions contained 150ng DNA, 10 μ 2LTR fw and rv primers,
502 10 μ M probe and 1x TaqMan Gene Expression Master Mix (ThermoFisher). Cycling conditions were:
503 95°C for 15min, followed by 50 cycles of 95°C for 15s and 60°C for 90s. Reactions were performed
504 using 7500 Real-Time PCR System (Applied Biosystems). The following primers and probes were
505 used:

506 Alu fw: GCCTCCCAAAGTGCTGGGATTACAG

507 HIV-1 Gag rv: GTTCCTGCTATGTCACCTTCC

508 RU5 fw: TTAAGCCTCAATAAAGCTTGCC
509 RU5 rv: GTTCGGGCGCCACTGCTAGA
510 RU5-WT probe: FAM-CCAGAGTCACACAACAGACGGGCACA-TAMRA
511 RU5-degenerate 1 probe: FAM-CCAGAGTCACATAACAGACGGGCACA-TAMRA
512 RU5-degenerate 2 probe: FAM-CCAGAGTCACACAACAGATGGGCACA-TAMRA
513 2LTR fw: AACTAGAGATCCCTCAGACCCTTT
514 2LTR rv: CTTGTCTCGTTGGGAGTGAAT
515 2LTR probe: FAM-CTAGAGATTTCCACACTGAC-TAMRA
516

517 **qRT-PCR**

518 RNA was extracted from FACS sorted target memory CD4+ T cells with RNeasy Micro Kit (Qiagen)
519 according to the manufacturer's instructions. cDNA was synthesised using SuperScript IV with
520 random hexamer primers (Invitrogen) and qRT-PCR was performed using Fast SYBR Green Master
521 Mix and 7500 Real-Time PCR System (Applied Biosystems). Gene expression was determined
522 using the $2^{-\Delta\Delta Ct}$ method and normalised to GAPDH expression. The following primers were used:
523 *GAPDH* fw: ACATCGCTCAGACACCATG, rv: TGTAGTTGAGGTCAATGAAGGG;
524 *CXCR6* fw: GACTATGGGTTCAGCAGTTCA, rv: GGCTCTGCAACTATGGTAGAAG;
525 *PRDM1* fw: ATGCGGATATGACTCTGTGGA, rv: CTGAACCGAAGTACCGCCATC;
526 *CD69* fw: ATTGTCCAGGCCAATACACATT, rv: CCTCTCTACCTGCGTATCGTTT;
527 *S1PR1* fw: TCTGCTGGCAAATTCAAGCGA, rv: GTTGTCCCCTTCGTCTTCTG;
528 *KLF2* fw: CTACACCAAGAGTCGCATCTG; rv: CCGTGTGCTTCGGTAGTG.
529
530

531 **Whole transcriptome profiling by RNA-Sequencing**

532 RNA was extracted from FACS sorted target memory CD4+ T cells with RNeasy Micro Kit (Qiagen)
533 according to the manufacturer's instructions. For preparation of RNA-Sequencing libraries, RNA
534 concentration was measured using the Qubit RNA High Sensitivity kit (Life Technologies) and quality
535 checked on the 4200 Tapestation using either the High Sensitivity or standard RNA ScreenTape
536 assay (Agilent Technologies), depending on the measured RNA concentrations. PolyA-tailed mRNA

537 was separated for sequencing during library preparation. Libraries were prepared using KAPA's
538 mRNA HyperPrep kit (Roche Diagnostics) according to the manufacturer's instructions using an
539 input of up to 200ng and a fragmentation incubation time of 8 minutes at 94°C. Samples were
540 sequenced on Illumina's NextSeq500 (Illumina Cambridge) using a high output 75 cycle paired-end
541 run. 24 libraries were multiplexed in the same run. Libraries were pooled in equimolar quantities,
542 calculated from concentrations measured using the Qubit dsDNA High Sensitivity kit (Life
543 Technologies) and fragment analysis using the D1000 High Sensitivity assay on the 4200
544 Tapestation (Agilent Technologies).

545 RNA sequencing data was quality assessed using FASTQC
546 (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) before and after low-quality and
547 adapter trimming using Trimmomatic⁵⁵. Filtered reads were then pseudo-mapped using Kallisto⁵⁶ to
548 the transcriptome available in Ensembl v.101 (<http://aug2020.archive.ensembl.org/index.html>). Per-
549 transcript counts were imported and aggregated per gene using the TXimport R package⁵⁷. The
550 DESeq2 package⁵⁸ was used for data normalisation, outlier detection and differential gene
551 expression analysis between biological groups. The DESeq2 results were ranked based on the log2
552 transformation of the adjusted p-values, to provide a pre-ranked list for Gene Set Enrichment
553 Analysis (GSEA)⁵⁹ as described in the GSEA documentation. Pathway enrichment and upstream
554 regulator analysis was performed using Gene Set Enrichment Analysis (GSEA)⁵⁹ and Ingenuity
555 Pathway Analysis (IPA,) respectively. Heatmaps were generated using ClustVis
556 (<https://biit.cs.ut.ee/clustvis/>)⁶⁰

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560 **Transcriptomic comparison with published human T_{RM} cells**

561 TPM data from previously published transcriptomes of human T_{RM} cells (GSE94964)²¹ were summed
562 on gene level with Ensembl gene ID, gene name, and gene biotype using tximport and BioMart^{57,61}.
563 TPM values <0.001 were adjusted to 0.001 as a lower limit of detection. These data were aligned to
564 the transcriptomic data from the present study using gene symbol in an integrated Log₂ transformed
565 data matrix and subjected to batch correction by study using Combat⁶². Expression of selected genes

566 previously identified to be up and downregulated in T_{RM}²¹ were used to cluster the samples in both
567 studies using 1-Spearman rank correlation with average linkage in ClustVis⁶⁰. A transcriptional
568 signature score for T_{RM} was derived from the difference between the sum of up and down-regulated
569 genes in T_{RM} in the previously published signature. This score was used to evaluate the relative
570 similarity of each transcriptome data set in the present project to T_{RM} and non-T_{RM} data.

571

572

573 **Statistical analysis**

574 Statistical analysis was performed using GraphPad Prism. Normally distributed data was analysed
575 for statistical significance by two-tailed *t*-tests (when comparing two groups) or one-way ANOVA
576 with Bonferroni or Dunnett's post-test (when comparing more than two groups). Data show the mean
577 +/- the S.E.M with significance shown on the figures. Where appropriate, the median+IQR is shown
578 and Kruskall-Wallis test was used to compare groups. Significance levels were defined as *, P <
579 0.05; **, P < 0.01 and ***, P < 0.001.

580

581 **Methods References**

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624

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634

635 **Author contributions**

636 A.K.R., and C.J. conceived the project. A.K.R., and C.J. designed the experiments. A.K.R., M.S. and
637 D.M performed the experiments. A.K.R., C.J., L.P., M.K.M., M.S., D.M., A.G-A and M.N. analysed
638 the data. L.P. and M.K.M provided reagents. J.P.T., C.H., S.F., R.M, K.J.D., and A.S. provided
639 lymphoid tissue samples. A.G-A and M.N. performed the core T_{RM} transcriptional mapping analysis.
640 A.K.R., and C.J. prepared the manuscript. All authors provided critical review of the manuscript.

641

642 **Competing interest**

643 The authors declare they have no competing interests.

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646

647 **Figure legends:**

648 **Fig. 1 HIV-1 exploits cell-to-cell spread to preferentially infect resting memory CD4+ T cells.**
649 HIV-1 NL4.3 infected (**a**) Jurkat or (**b**) mitogenically-activated primary CD4+ donor T cells co-cultured
650 with resting primary CD4+ target T cells separated by a 0.4 μ m transwell (cell-free) or direct co-culture
651 (cell-cell). Target cell infection was measured by intracellular staining for HIV-1 Gag protein.
652 Representative flow cytometry plots are shown. Bar graphs show mean of independent experiments
653 (n=4). (**c,d**) Cell-to-cell spread from activated primary donor CD4+ T cells to resting primary target
654 CD4+ T cells preferentially infects CD45RA- memory CD4+ T cells. A representative flow cytometry
655 plot and quantification is shown (n=4). (**e**) Quantification of infection performed as in (**c**) (n=11). (**f**)
656 HIV-1 infection of target CD4+ T cells as part of the total resting CD4+ T cell population (total)
657 compared to pre-isolated naive and memory CD4+ target T cells (isolated) (n=9). (**g**) Representative
658 flow cytometry plots of cell-to-cell infection of resting CD4+ T cells with CCR5-tropic HIV-1 NL4.3
659 BaL and transmitter founder viruses HIV-1 CH040 and CH077 as performed in (**c**). (**h**) Quantification
660 of infection of CXCR4 (X4) and CCR5 (R5)-tropic viruses (n=4) and (**i**) transmitter/founder viruses
661 HIV-1 CH040 and CH077 (n=7). (**j**) Cell-to-cell infection of resting CD4+ T cells is reduced by the
662 HIV-1 fusion-inhibitor T20 (n=6) and (**k**) the reverse transcriptase inhibitor Efavirenz (n=6) measured
663 by intracellular Gag staining (MFI) or (**l**) HIV-1 LTR-driven GFP-reporter gene expression (n=4). (**m**)
664 HIV-1 infection downregulates CD4 expression. Shown are the percentage of CD4+ cells of the total
665 CD3+ target cell population (n=6). Resting CD4+ memory T cells were isolated after 72h of cell-to-
666 cell spread by FACS sorting and cultured for 4 days. HIV-1 infection was measured by intracellular
667 Gag staining (**n**) and virus release measured by culture supernatant RT activity (**o**) (n=5-7). T cells
668 from (**n**) were recovered at day 1 or 4 post isolation and cultured with uninfected eFluor450+ target
669 Jurkat T cells. Infection of Jurkat T cells was measured after 72h (**p**) (n=3). Data are the mean \pm SEM.
670 Paired two-tailed *t*-test or one-way ANOVA with Bonferroni post-test were used. For (**m**),
671 median+IQR is shown and Friedman test with Dunn's post-test was used. For (**o**), unpaired one-
672 tailed *t*-test was used. *, p<0.05 ; **, p<0.01; ***, p<0.001; n.s., not significant.

673

674 **Fig. 2 HIV-1 infection induces a T_{RM}-like phenotype in resting memory CD4+ T cells. (a)** CD69
675 expression on resting memory CD4+ target T cells following co-culture with HIV-1 infected primary
676 donor T cells or uninfected donor T cells (mock) (n=17). **(b)** Representative flow cytometry plots from
677 **(a)**. **(c)** CD69 expression on infected resting memory CD4+ T cells ± IL7 and T20 (n=7). **(d)** CD69
678 expression on infected resting memory CD4+ T cells ± IL7 and Ruxolitinib (n=8). **(e)** CD69 expression
679 on infected resting memory CD4+ T cells in response to IL7 and IL15 (n=11). **(f)** CD69 expression
680 on infected Gag+ resting memory CD4+ T cells and uninfected Gag- bystander cells in response to
681 IL7 and IL15 (n=11). **(g)** CXCR6 surface expression from **(f)** (n=11). **(h)** Representative flow
682 cytometry plots of CD69 and CXCR6 co-expression. **(i)** Co-expression of CD69 with CXCR6, CD49A
683 or PD-1 on infected resting memory CD4+ T cells (n=5-7). **(j)** As for **(i)** in the presence of IL7 (n=4-
684 7). **(k)** As for **(i)** comparing infected Gag+ memory CD4+ T cells and uninfected Gag- bystander
685 cells. **(l)** and **(m)** Blimp-1 expression in CD69+ infected resting memory CD4+ T cells and infected
686 CD69- cells in response to IL7 (n=8). **(n)** Total lymphocytes from cellularised tonsils co-cultured with
687 HIV-1 infected Jurkat T cells. Infection of resting CD4+ T cells (live CD3+/CD8-/Ki67- lymphocytes)
688 shown as CD45RO vs Gag. **(o)** CD69 and CXCR6 co-expression on infected Gag+ and uninfected
689 Gag- tonsil resting memory CD4+ T cells ± IL7. **(p)** Total lymphocytes from mediastinal lymph nodes
690 co-cultured with HIV-1 infected activated autologous LN-derived lymphocytes. Infection of resting
691 CD4+ T cells (live CD3+/CD8-/Ki67- lymphocytes) shown as CD45RA vs Gag. **(q)** CD69 and CXCR6
692 co-expression on infected Gag+ and uninfected Gag- lymph node resting memory CD4+ T cells ± IL7.
693 Data are the mean±SEM. Paired two-tailed *t*-test or one-way ANOVA with Bonferroni or Dunnett's
694 post-test were used. *p<0.05 ; **, p<0.01; ***, p<0.001; n.s., not significant.
695

696 **Fig. 3 Vpr drives HIV-1-induced T_{RM}-induction in resting memory CD4+ T cells.** Resting memory
697 CD4+ T cells were co-cultured with HIV-1 infected primary CD4+ T cells infected with HIV-1 WT or
698 mutant viruses, or uninfected donor cells (mock). **(a)** CD69 upregulation in response to IL7 compared
699 to mock (n=9). **(b)** CD69 expression on HIV-1 infected Gag+ resting memory CD4+ T cells compared
700 to uninfected Gag- bystander cells (n=9). **(c)** Quantification of cell-to-cell spread of HIV-1 WT and
701 ΔVpr to resting memory CD4+ T cells (n=9). **(d)** CD69/CXCR6/CD49a co-expression on resting

702 memory CD4+ T cells infected with HIV-1 WT or Δ Vpr (n=9). (e) CD69, CXCR6 and PRDM1 (Blimp1)
703 mRNA levels from FACS sorted infected resting memory CD4+ T cells. Fold change relative to
704 uninfected (mock) is shown (n=5). (f) IFNg expression by HIV-1 infected resting memory CD4+ T cells
705 at 72h in response to IL7 (n=3). (g) CD69 and (h) CD69/CXCR6 co-expression in response to IL7 in
706 the presence of integrase inhibitor Raltegravir (n=6). (i) Quantification of integrated provirus and
707 2LTR circles in FACS sorted target CD4+ memory T cells after 72h of cell-to-cell spread in the
708 presence or absence of Raltegravir. (j) Western blot showing Vpr packaging into HIV-1 WT and Vpr-
709 mutant virions. (k) CD69 upregulation in response to IL7 on resting memory CD4+ T cells infected
710 with HIV-1 WT, Δ Vpr or Vpr mutants (n=9). (l) as for (k) showing CD69 expression on HIV-1 infected
711 Gag+ memory T cells compared to uninfected Gag- bystander cells. (m) Co-expression of CD69 and
712 CXCR6 from (k) (n=9). Data are the mean \pm SEM. Paired two-tailed *t*-test or one-way ANOVA with
713 Bonferroni or Dunnett's post-test were used. 2LTR circles (i) were compared by unpaired one-tailed
714 *t*-test. *, p<0.05 ; **, p<0.01; ***, p<0.001; n.s., not significant.

715

716 **Fig. 4 Transcriptional profiling of HIV-1 infected resting memory CD4+ T cells.** (a) Heatmap
717 showing hierarchical clustering of 226 differentially expressed genes (DEG) of infected (HIV-1 WT)
718 over uninfected (Mock) resting memory CD4+ T cells (adjusted p-value < 0.01, Fold change +/-
719 1.2). Mean log2 TPM of 4 biological repeats are shown. Cytokine indicates presence or absence of
720 IL7. Virus indicates infection with HIV-1 WT, HIV-1 Δ Vpr or uninfected (Mock) conditions. (b)
721 Principal component analysis (PCA) of (a), with ellipses indicating 95% CI. (c) and (d) show scatter
722 plots of mean log2 TPMs of DEGs from HIV-1 WT/Mock (grey circles) or HIV-1 Δ Vpr/Mock (orange
723 circles) in the absence (c) or presence (d) of IL7 (adjusted p-value < 0.01, Fold change +/- 1.2).
724 Lines indicate line of identity (LOD). Genes above or below LOD are up or downregulated,
725 respectively. (e) and (f) Venn diagrams showing overlap of DEGs comparing expression profiles of
726 HIV-1 WT/Mock with (e) HIV-1 Δ Vpr/Mock or (f) HIV-1 Δ Vpr/HIV-1 WT. (g) GSEA was performed
727 on expression profiles comparing HIV-1 WT / Mock (black) or HIV-1 Δ Vpr/HIV-1 WT (grey).
728 Normalised enrichment scores are shown for significantly enriched Hallmark gene sets are shown
729 (FDR q-value<0.05 and NES>1.75). (h) and (i) top ten significantly enriched canonical pathways

730 predicted by ingenuity pathway (IPA) analysis of DEGs (h) HIV-1 WT/Mock or (i) HIV-1 Δ Vpr/HIV-1
731 WT (adjusted p-value<0.05). (j) Cytokines and (k) transcription regulators predicted to be upstream
732 regulators by IPA of gene expression signatures HIV-1 WT/Mock (black) or HIV-1 Δ Vpr/Mock
733 (grey), line indicates p=0.05.

734

735 **Fig. 5 Vpr drives a T_{RM}-like transcriptomic program in HIV-1 infected resting memory CD4+
736 T cells.** (a) Heatmap showing hierarchical clustering based on a T_{RM} core gene expression
737 signature²¹ that was performed to compare transcriptional profiles of HIV-1 infected resting
738 memory CD4 T cells (Reuschl et al, 2021) (Mock, HIV-1 WT, HIV-1 Δ Vpr) with previously
739 described gene expression profiles (Kumar et al, 2017) of T_{RM} (CD69 POS), non-T_{RM} (CD69 NEG)
740 tissue-derived T cells (lung and spleen) and blood-derived CD69- (CD69 NEG) T cells. Cytokine
741 indicates presence or absence of IL7. Virus indicates infection with HIV-1 WT, HIV-1 Δ Vpr or
742 uninfected (Mock) conditions. n/a, not applicable. (b) shows the T_{RM} signature score for the
743 indicated conditions calculated based on (a). Subsets from²¹ are indicated in red, shown are CD4+
744 or CD8+ T cells from lungs or spleens. T_{RM}+, CD69+ T cells; T_{RM}-, CD69- T cells. T_{RM} signature
745 scores for resting CD4 memory T cells infected or uninfected are shown in the presence or
746 absence of IL7. Means are shown.

747

748

749 **Extended data Fig. 1.** (a) Experimental set-up schematic. (b) Flow cytometry gating strategy. (c)
750 Ki67, CD69 and HLA-DR expression of CD3+CD4+ T cells from unstimulated PBMCs (n=8). (d)
751 Ki67 expression on resting and activated primary CD4+ T cells. Representative flow cytometry plots.
752 (e) MCM2 expression on resting and activated primary CD4+ T cells. Representative flow cytometry
753 plots. (f) Resting or (g) mitogenically-activated primary target CD4+ T cells cultured with HIV-1
754 infected Jurkat T cells separated by a 0.4 μ m transwell or in direct co-culture. Target cell infection
755 levels was measured by intracellular staining for Gag. Representative flow cytometry plots are

756 shown. (h) Infection levels of target CD4+ T cells determined by intracellular Gag staining and flow
757 cytometry (n=2). (i) Proportion of CD45RA+ naïve and CD45RA- memory CD4+ T cells in
758 unstimulated PBMCs (n=8). (j) Resting target CD4+ T cells were cultured with mock-treated or HIV-
759 1-infected donor cells. Surface expression of CD45RA and CCR7 were measured after 72h of co-
760 culture. Representative flow cytometry plots are shown. (k) Quantification of T cell subsets in infected
761 (Gag+) and uninfected (Gag-) resting CD4+ T cells according to CD45RA/CD62L expression (n=3).
762 $T_{\text{naive}} = \text{CD45RA+}/\text{CD62L+}$, $T_{\text{EMRA}} = \text{CD45RA+}/\text{CD62L-}$, $T_{\text{EM}} = \text{CD45RA-}/\text{CD62L-}$, $T_{\text{CM}} = \text{CD45RA-}$
763 $/\text{CD62L+}$. Data are shown as mean \pm SEM.

764

765 **Extended data Fig. 2.** (a) Representative flow cytometry plots of CD45RA+ and CD45RA- CD4+ T
766 cells pre- and post-isolation. (b) Representative histogram of intracellular Gag staining in resting
767 naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread \pm T20. (c)
768 Representative histogram of intracellular Gag-levels in resting naïve (CD45RA+) and memory
769 (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread \pm Efavirenz. (d) Representative histogram
770 of CD4 surface levels in resting naïve (CD45RA+) and memory (CD45RA-) CD3+ T cells after 72h
771 of cell-to-cell spread. (e) Mean post-sort population purity of T cells from (Fig. 1 n-p) was 99.92%
772 target cells of which 99.86% were memory T cells (n=5).

773

774 **Extended data Fig. 3** (a and b) Representative flow cytometry plots showing (a) Ki67 and Gag, or
775 (b) MCM2 and Gag staining of resting CD4+ T cells after co-culture with mock or HIV-1 infected
776 primary donor CD4+ T cells. (c) FACS sorted CD69- resting naïve or memory CD4+ T cells co-
777 cultured with HIV-1 infected primary CD4+ donor T cells and infection of targets measured by Gag
778 staining (n=4). (d) FACS sorted CD69- CD4+ T cells co-cultured with HIV-1 infected primary CD4+
779 donor T cells. CD69 expression was measured on resting memory CD4+ T cells (n=4). (e) Total
780 CD69 expression alongside CD69 with or without HLA-DR co-expression on infected resting memory
781 T cells (n=6). (f) Expression of T_{RM}-markers CXCR6, CD49A and CD69 on resting CD4+ memory T
782 cells from unstimulated PBMCs (n=8). (g) Quantification of cell-to-cell spread of HIV-1 WT to resting
783 memory CD4+ T cells in the presence or absence of IL7 (n=10). (h and i) CD69 expression on

784 infected resting memory CD4+ target T cells in the presence or absence of (h) IL12 (n=4) or (i) TFG β
785 (n=4). (j) CD69 expression on infected resting memory CD4+ T cells with IL7 added at the indicated
786 times post cell-mixing (n=5). (k) CD69/CXCR6 co-expression from (j) (n=5). (l) CD69/CD101 co-
787 expression on infected resting memory CD4+ T cells (n=3) (m) CD69 upregulation in response to
788 IL7 on resting memory CD4+ T cells infected HIV-1 NL4.3, or transmitter-founder viruses CH040
789 and CH077 comparing infected Gag+ and uninfected Gag- bystander cells (n=7). (n) CD69/CXCR6
790 co-expression on resting memory CD4+ T cells from (i) (n=7). (o) CD69/CX3CR1 co-expression on
791 infected resting memory CD4+ T cells (n=3). (p) CD103 expression and (q) CD69/CD103 co-
792 expression on infected resting memory CD4+ T cells (n=4). Data are the mean \pm SEM. Paired two-
793 tailed *t*-test or one-way ANOVA with Bonferroni post-test was used. For (i), median+IQR is shown
794 and Kruskall-Wallis test was used to compare groups *, p<0.05 ; **, p<0.01; ***, p<0.001; n.s., not
795 significant.

796

797 **Extended data Fig. 4** Resting memory CD4+ T cells were co-cultured with HIV-1 infected primary
798 CD4+ T cells infected with HIV-1 WT or mutant viruses. Representative flow cytometry plots of HIV-
799 1 Gag and CD69 co-expression in the presence or absence of IL7 from three independent
800 experiments are shown (a-c).

801

802 **Extended data Fig. 5** (a) CD69 surface expression on resting CD45RA- memory CD4+ T cells
803 following co-culture with primary CD4+ donor T cells infected with HIV-1 NL4.3 (WT), Δ Vpr, Δ Nef or
804 Δ Vpu or uninfected (mock) donors (n=9). (b) CXCR6 expression from (a) (n=9). (c) CD69/CXCR6
805 co-expression from (a) (n=9). (d) As for (b) but cells were incubated in the presence of IL7 (n=9). (e)
806 CD69/CXCR6 surface co-expression from (d) (n=9). (f) Gag MFI of cell-to-cell spread of HIV-1 WT
807 and Δ Vpr to resting memory CD4+ T cells (n=10). Correlation plot of CD69 MFI with Gag MFI in
808 absence (g) or presence (h) of IL7 (n=18). (i) S1PR1 and KLF2 mRNA levels in FACS sorted resting
809 memory CD4+ T cells from Fig. 3e. Fold change over mock is shown (n=5). (j) CD69 expression on
810 infected resting memory CD4+ T cells \pm Ruxilitinib (n=4). (k) CD127 MFI on infected resting memory
811 CD4+ T cells \pm IL7 (n=7). (l) Representative histogram of intracellular STAT5-phosphorylation in

812 infected resting memory T cells. (m) Quantification of (l) (n=10). Data are the mean \pm SEM. One-way
813 ANOVA with Dunnet's post-test was used. Statistical significance is shown relative to mock treated
814 cells (no HIV-1). R² in (g) and (h) was determined by simple linear regression. *, p<0.05 ; **, p<0.01;
815 ***, p<0.001; n.s., not significant.

816

817 **Extended data Fig. 6 (a)** and **(b)** Venn diagrams showing overlap of DEGs comparing expression
818 profiles of HIV-1 WT+IL7/Mock+IL7 with **(e)** HIV-1 Δ Vpr+IL7/Mock+IL7 or **(f)** HIV-1 Δ Vpr+IL7/HIV-1
819 WT+IL7. **(g)** GSEA was performed on expression profiles comparing HIV-1 WT+IL7/Mock+IL7
820 (black) or HIV-1 Δ Vpr+IL7/HIV-1 WT+IL7 (grey). Normalised enrichment scores are shown for
821 significantly enriched Hallmark gene sets are shown (FDR q-value<0.05 and NES>1.75). **(h)** and **(i)**
822 top ten significantly enriched canonical pathways predicted by ingenuity pathway (IPA) analysis of
823 DEGs **(h)** HIV-1 WT+IL7/Mock+IL7 or **(i)** HIV-1 Δ Vpr+IL7/HIV-1 WT+IL7 (adjusted p-value<0.05). **(j)**
824 Cytokines and **(k)** transcription regulators predicted to be upstream regulators by IPA of gene
825 expression signatures HIV-1 WT+IL7/Mock+IL7 (black) or HIV-1 Δ Vpr+IL7/Mock+IL7 (grey), line
826 indicates p=0.05.

827

828 **Extended data Fig. 7 (a)** Western blot showing siRNA knockdown of DCAF1 in CD3/CD28-activated
829 CD4+ T cells 48h post transfection. Two representative samples are shown. **(b)** Number of live CD3+
830 Target T cells recovered after 72h of cell-to-cell spread into control or DCAF1 siRNA-treated T cells
831 (n=3).

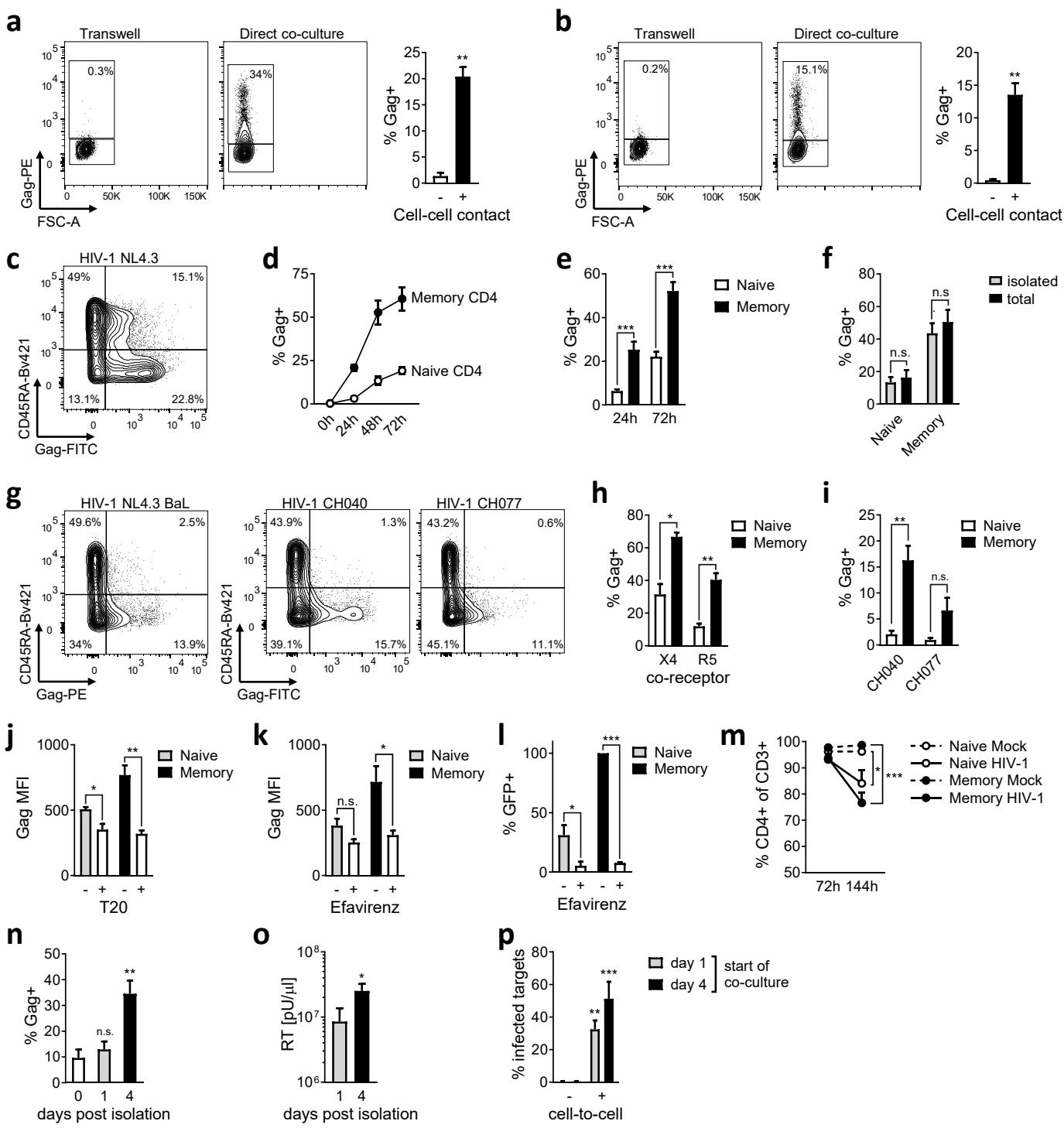


Fig. 1 HIV-1 exploits cell-to-cell spread to preferentially infect resting memory CD4+ T cells. HIV-1 NL4.3 infected (a) Jurkat or (b) mitogenically-activated primary CD4+ donor T cells co-cultured with resting primary CD4+ target T cells separated by a 0.4 μ m transwell (cell-free) or direct co-culture (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). (c,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 is 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 to pre-isolated naive and memory CD4+ target T cells (isolated) (n=9). (g) 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). (h) Quantification of infection of CXCR4 (X4) and CCR5 (R5)-tropic viruses (n=4) and (i) transmitter/founder viruses HIV-1 CH040 and CH077 (n=7). (j) Cell-to-cell infection of resting CD4+ T cells is reduced by the HIV-1 fusion-inhibitor T20 (n=6) and (k) the reverse transcriptase inhibitor Efavirenz (n=6) measured by intracellular Gag staining (MFI) or (l) HIV-1 LTR-driven GFP-reporter gene expression (n=4). (m) HIV-1 infection downregulates CD4 expression. Shown are the percentage of CD4+ cells of the total CD3+ target cell population (n=6). Resting CD4+ memory T cells were isolated after 72h of cell-to-cell spread by FACS sorting and cultured for 4 days. HIV-1 infection was measured by intracellular Gag staining (n) and virus release measured by culture supernatant RT activity (o) (n=5-7). T cells from (n) were recovered at day 1 or 4 post isolation and cultured with uninfected eFluor450+ target Jurkat T cells. Infection of Jurkat T cells was measured after 72h (p) (n=3). Data are the mean \pm SEM. Paired two-tailed t-test or one-way ANOVA with Bonferroni post-test were used. For (m), 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.

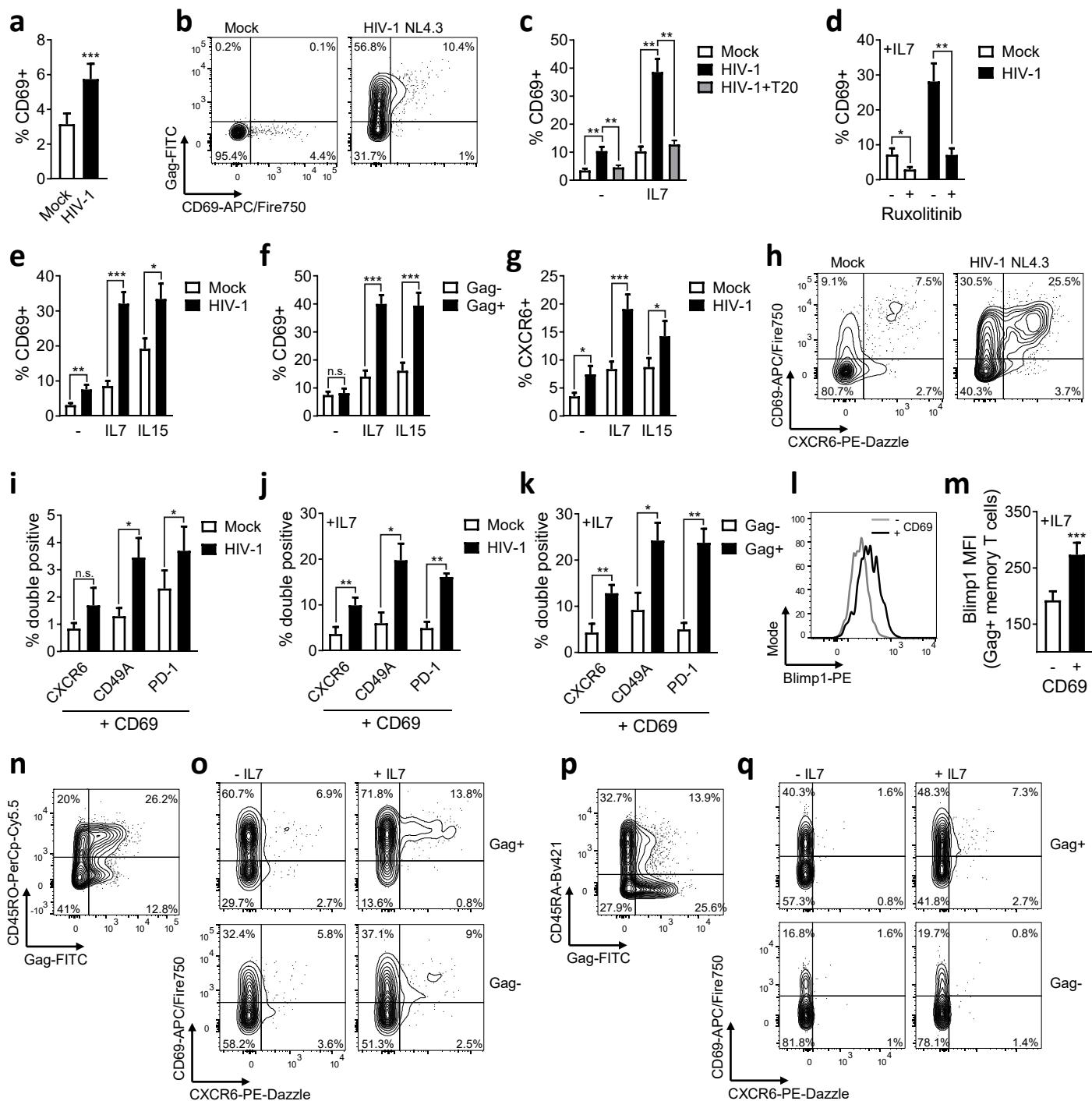


Fig. 2 HIV-1 infection induces a T_{RM} -like phenotype in resting memory CD4+ T cells. (a) CD69 expression on resting memory CD4+ target T cells following co-culture with HIV-1 infected primary donor T cells or uninfected donor T cells (mock) (n=17). (b) Representative flow cytometry plots from (a). (c) CD69 expression on infected resting memory CD4+ T cells \pm IL7 and T20 (n=7). (d) CD69 expression on infected resting memory CD4+ T cells \pm IL7 and Ruxolitinib (n=8). (e) CD69 expression on infected resting memory CD4+ T cells in response to IL7 and IL15 (n=11). (f) CD69 expression on infected Gag+ resting memory CD4+ T cells and uninfected Gag- bystander cells in response to IL7 and IL15 (n=11). (g) CXCR6 surface expression from (f) (n=11). (h) Representative flow cytometry plots of CD69 and CXCR6 co-expression. (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 IL7 (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+ infected resting memory CD4+ T cells and infected CD69- cells in response to IL7 (n=8). (n) Total lymphocytes from cellularised tonsils co-cultured with HIV-1 infected Jurkat T cells. Infection of resting CD4+ T cells (live CD3+/CD8-/Ki67- lymphocytes) shown as CD45RO vs Gag. (o) CD69 and CXCR6 co-expression on infected Gag+ and uninfected Gag- tonsil resting memory CD4+ T cells \pm IL7. (p) Total lymphocytes from mediastinal lymph nodes co-cultured with HIV-1 infected activated autologous LN-derived lymphocytes. Infection of resting CD4+ T cells (live CD3+/CD8-/Ki67- lymphocytes) shown as CD45RA vs Gag. (q) CD69 and CXCR6 co-expression on infected Gag+ and uninfected Gag- lymph node resting memory CD4+ T cells \pm IL7. Data are the mean \pm SEM. Paired two-tailed t-test or one-way ANOVA with Bonferroni or Dunnett's post-test were used. *p<0.05; **, p<0.01; ***, p<0.001; n.s., not significant.

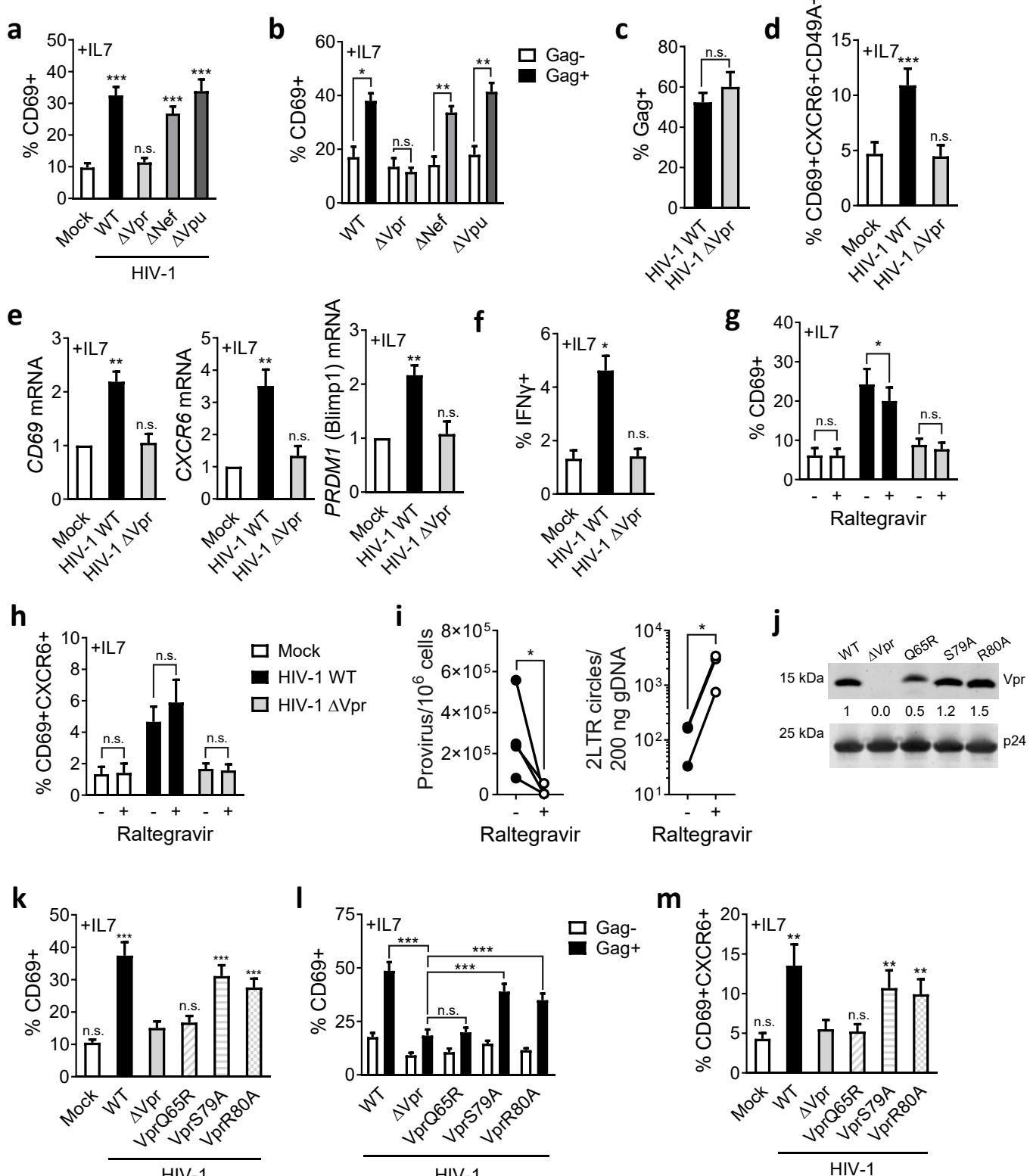


Fig. 3 Vpr drives HIV-1-induced T_{RM}-induction in resting memory CD4+ T cells. Resting memory CD4+ T cells were co-cultured with HIV-1 infected primary CD4+ T cells infected with HIV-1 WT or mutant viruses, or uninfected donor cells (mock). (a) CD69 upregulation in response to IL7 compared to mock (n=9). (b) CD69 expression on HIV-1 infected Gag+ resting memory CD4+ T cells compared to uninfected Gag- bystander cells (n=9). (c) Quantification of cell-to-cell spread of HIV-1 WT and Δ Vpr to resting memory CD4+ T cells (n=9). (d) CD69/CXCR6/CD49a co-expression on resting memory CD4+ T cells infected with HIV-1 WT or Δ Vpr (n=9). (e) CD69, CXCR6 and PRDM1 (Blimp1) mRNA levels from FACS sorted infected resting memory CD4+ T cells. Fold change relative to uninfected (mock) is shown (n=5). (f) IFN γ expression by HIV-1 infected resting memory CD4+ T cells at 72h in response to IL7 (n=3). (g) CD69 and (h) CD69/CXCR6 co-expression in response to IL7 in the presence of integrase inhibitor Raltegravir (n=6). (i) Quantification of integrated provirus and 2LTR circles in FACS sorted target CD4+ memory T cells after 72h of cell-to-cell spread in the presence or absence of Raltegravir. (j) Western blot showing Vpr packaging into HIV-1 WT and Vpr-mutant virions. (k) CD69 upregulation in response to IL7 on resting memory CD4+ T cells infected with HIV-1 WT, Δ Vpr or Vpr mutants (n=9). (l) as for (k) showing CD69 expression on HIV-1 infected Gag+ memory T cells compared to uninfected Gag- bystander cells. (m) Co-expression of CD69 and CXCR6 from (k) (n=9). Data are the mean \pm SEM. Paired two-tailed t-test or one-way ANOVA with Bonferroni or Dunnett's post-test were used. 2LTR circles (i) were compared by unpaired one-tailed t-test. *, p<0.05; **, p<0.01; ***, p<0.001; n.s., not significant.

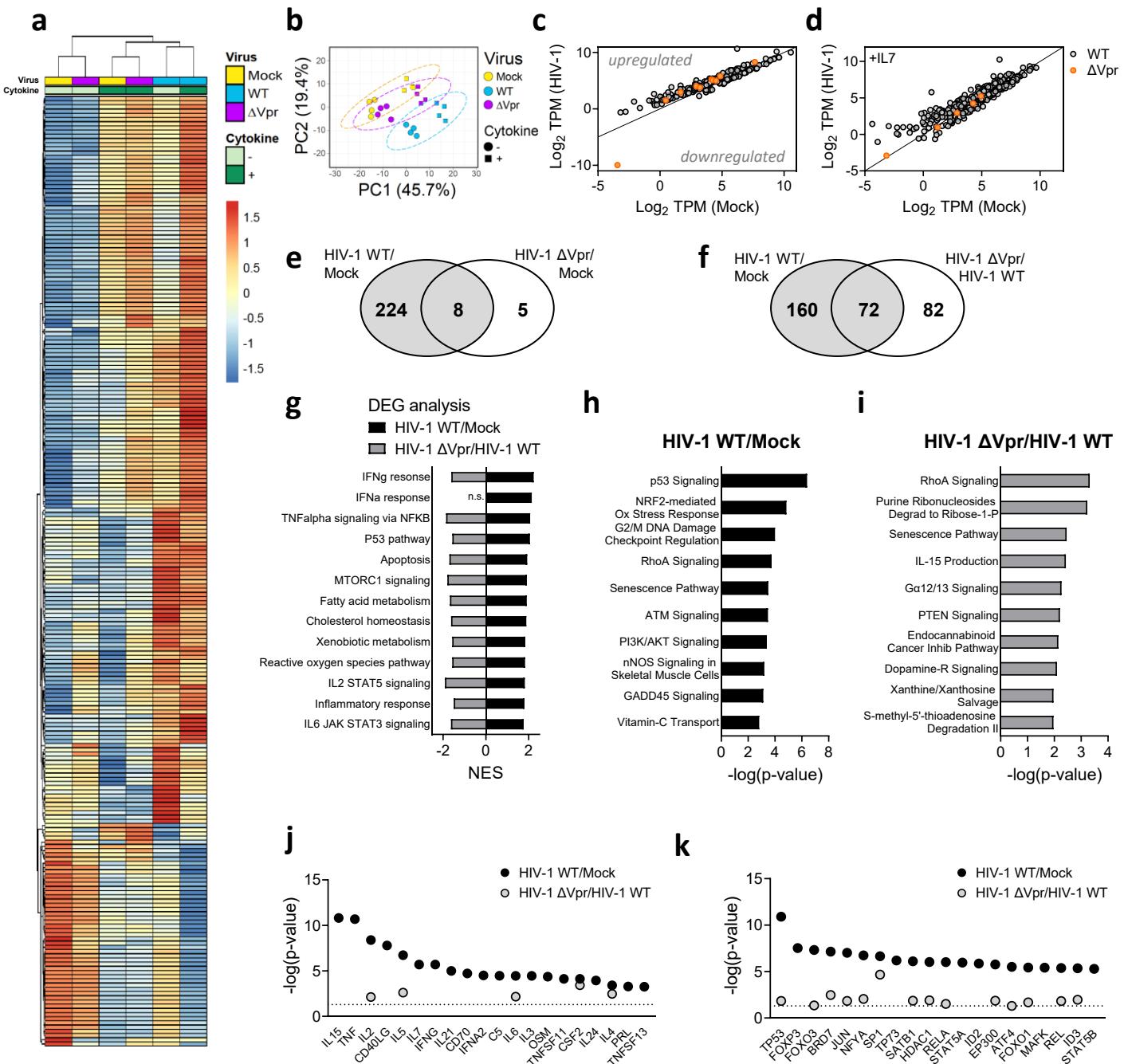


Fig. 4 Transcriptional profiling of HIV-1 infected resting memory CD4+ T cells. (a) Heatmap showing hierarchical clustering of 226 differentially expressed genes (DEG) of infected (HIV-1 WT) over uninfected (Mock) resting memory CD4+ T cells (adjusted p-value < 0.01, Fold change +/- 1.2). Mean log2 TPM of 4 biological repeats are shown. Cytokine indicates presence or absence of IL7. Virus indicates infection with HIV-1 WT, HIV-1 ΔVpr or uninfected (Mock) conditions. (b) Principal component analysis (PCA) of (a), with ellipses indicating 95% CI. (c) and (d) show scatter plots of mean log2 TPMs of DEGs from HIV-1 WT/Mock (grey circles) or HIV-1 ΔVpr/Mock (orange circles) in the absence (c) or presence (d) of IL7 (adjusted p-value < 0.01, Fold change +/- 1.2). Lines indicate line of identity (LOD). Genes above or below LOD are up or downregulated, respectively. (e) and (f) Venn diagrams showing overlap of DEGs comparing expression profiles of HIV-1 WT/Mock with (e) HIV-1 ΔVpr/Mock or (f) HIV-1 ΔVpr/HIV-1 WT. (g) GSEA was performed on expression profiles comparing HIV-1 WT / Mock (black) or HIV-1 ΔVpr/HIV-1 WT (grey). Normalised enrichment scores are shown for significantly enriched Hallmark gene sets are shown (FDR q-value<0.05 and NES>1.75). (h) and (i) top ten significantly enriched canonical pathways predicted by ingenuity pathway (IPA) analysis of DEGs (h) HIV-1 WT/Mock or (i) HIV-1 ΔVpr/HIV-1 WT (adjusted p-value<0.05). (j) Cytokines and (k) transcription regulators predicted to be upstream regulators by IPA of gene expression signatures HIV-1 WT/Mock (black) or HIV-1 ΔVpr/Mock (grey), line indicates p=0.05.

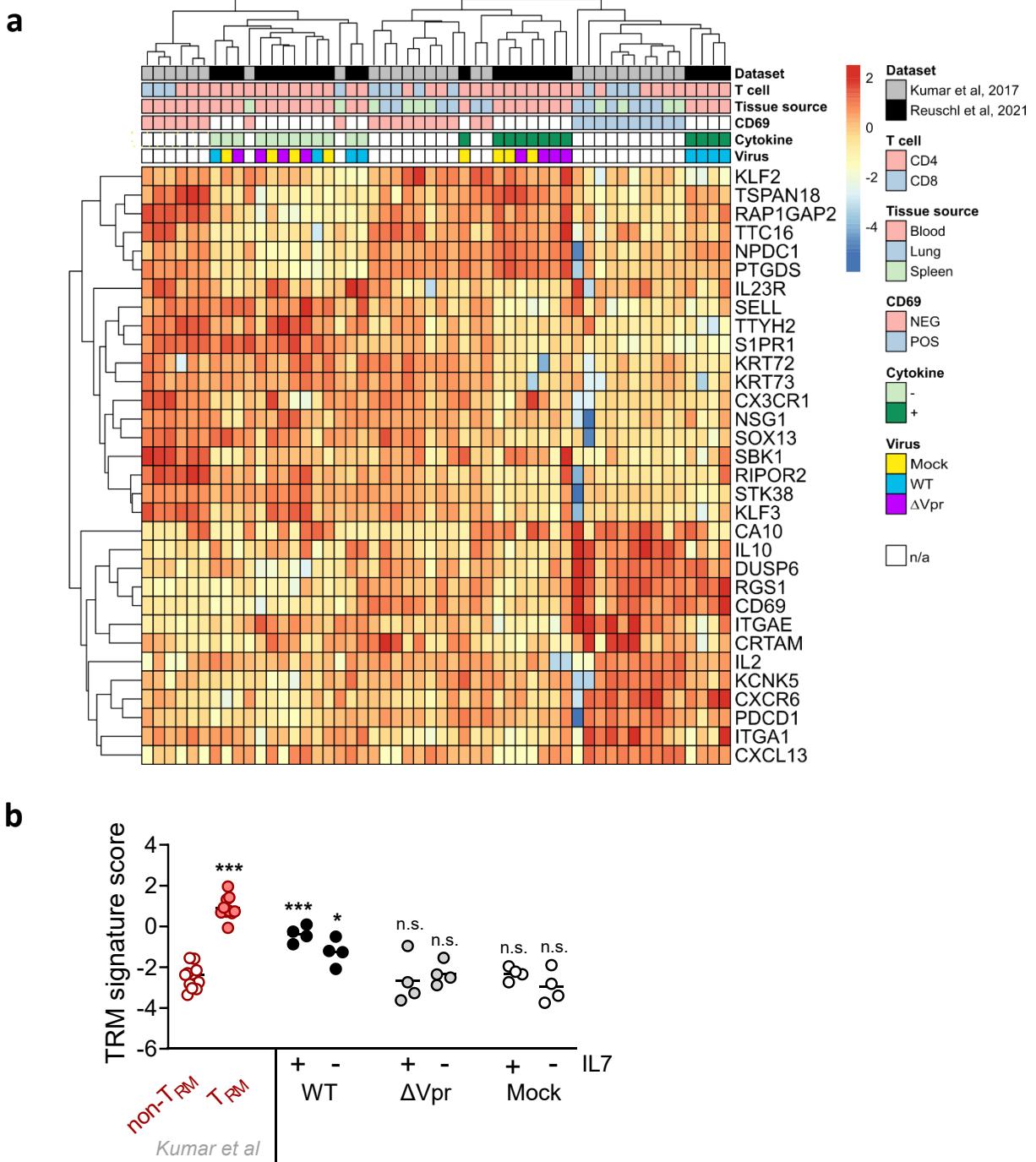
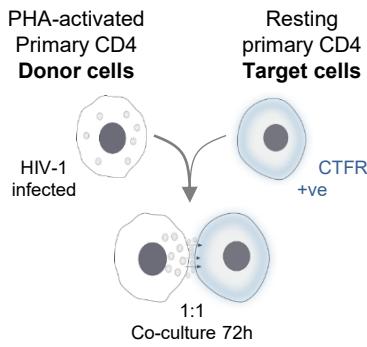
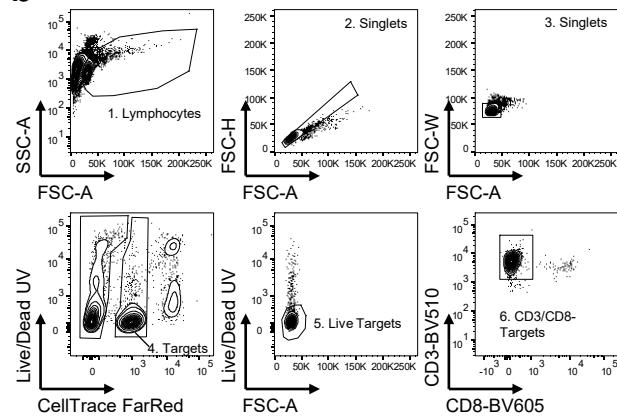
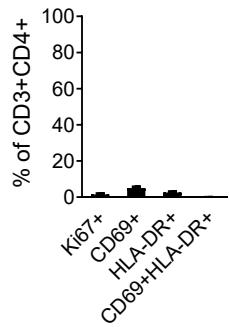
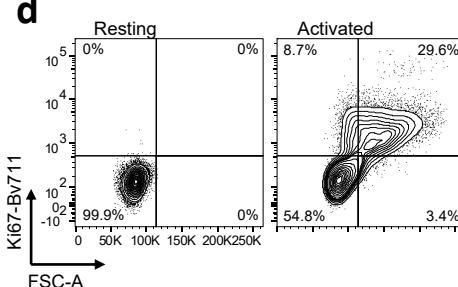
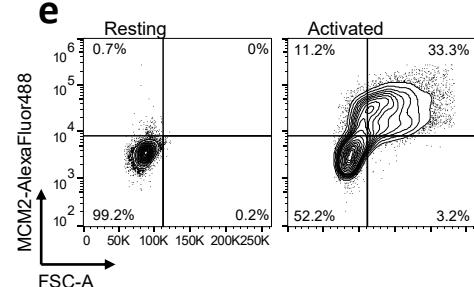
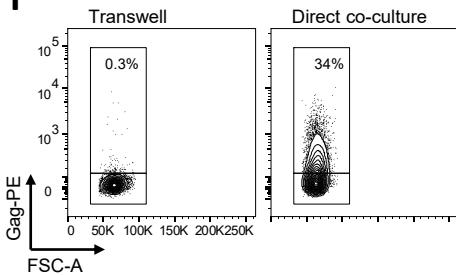
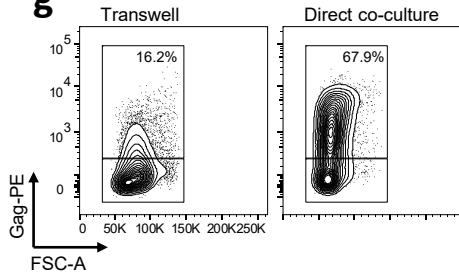
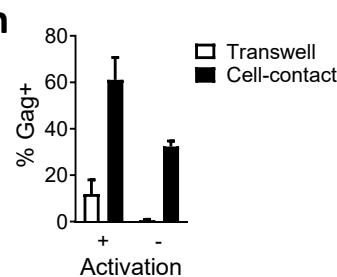
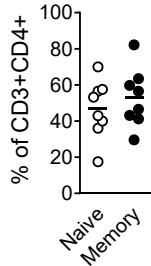
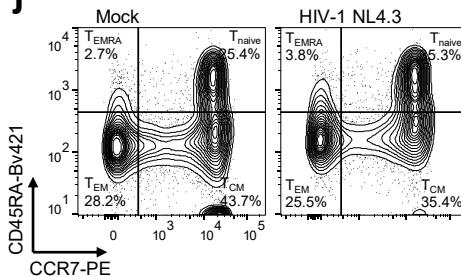
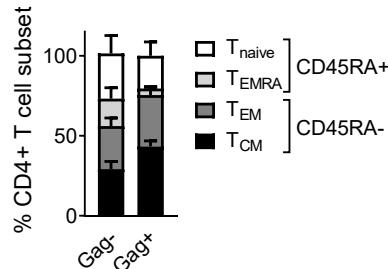
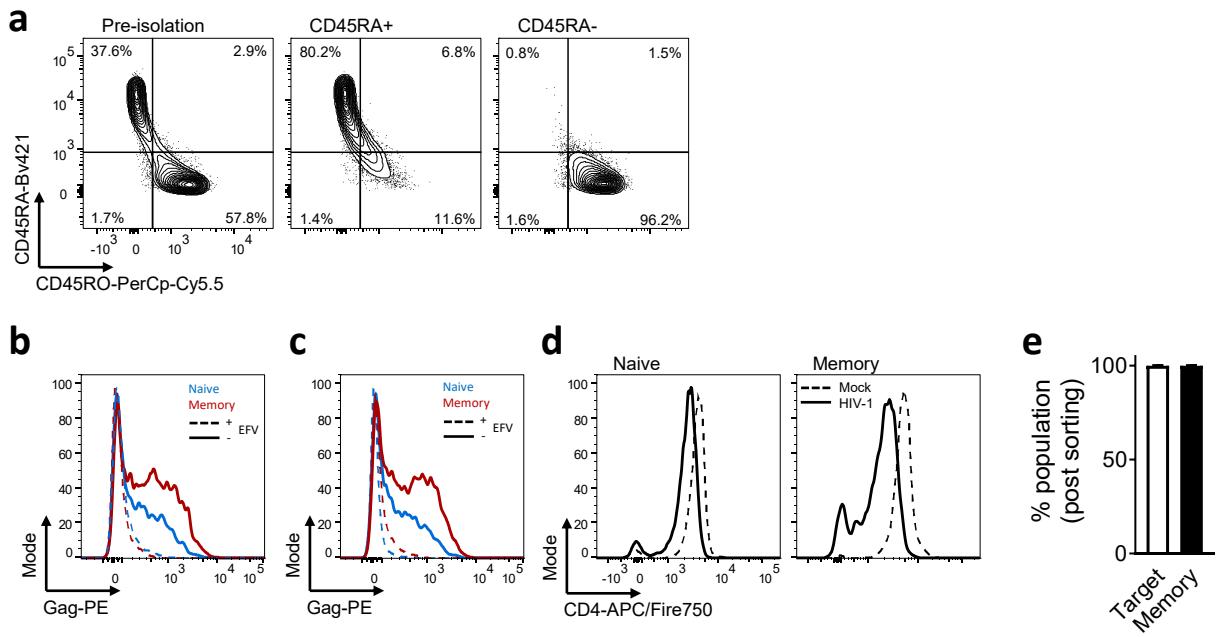


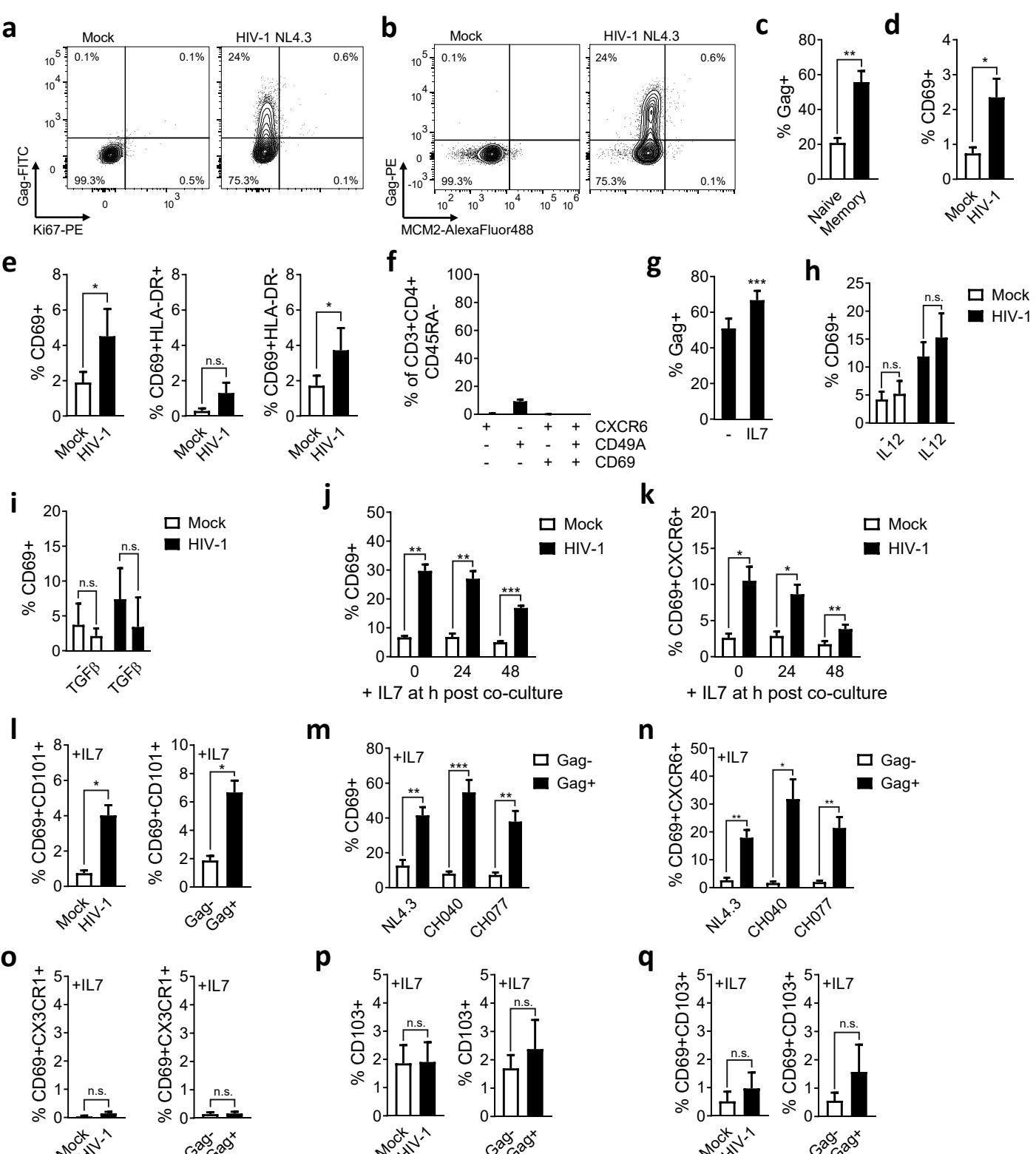
Fig. 5 Vpr drives a T_{RM} -like transcriptomic programme in HIV-1 infected resting memory CD4+ T cells. (a) Heatmap showing hierarchical clustering based on a T_{RM} core gene expression signature²¹ that was performed to compare transcriptional profiles of HIV-1 infected resting memory CD4 T cells (Reuschl et al, 2021) (Mock, HIV-1 WT, HIV-1 Δ Vpr) with previously described gene expression profiles (Kumar et al, 2017) of T_{RM} (CD69 POS), non- T_{RM} (CD69 NEG) tissue-derived T cells (lung and spleen) and blood-derived CD69- (CD69 NEG) T cells. Cytokine indicates presence or absence of IL7. Virus indicates infection with HIV-1 WT, HIV-1 Δ Vpr or uninfected (Mock) conditions. n/a, not applicable. **(b)** shows the T_{RM} signature score for the indicated conditions calculated based on (a). Subsets from²¹ are indicated in red, shown are CD4+ or CD8+ T cells from lungs or spleens. T_{RM}^+ , CD69+ T cells; T_{RM}^- , CD69- T cells. T_{RM} signature scores for resting CD4 memory T cells infected or uninfected are shown in the presence or absence of IL7. Means are shown.

a**b****c****d****e****f****g****h****i****j****k**

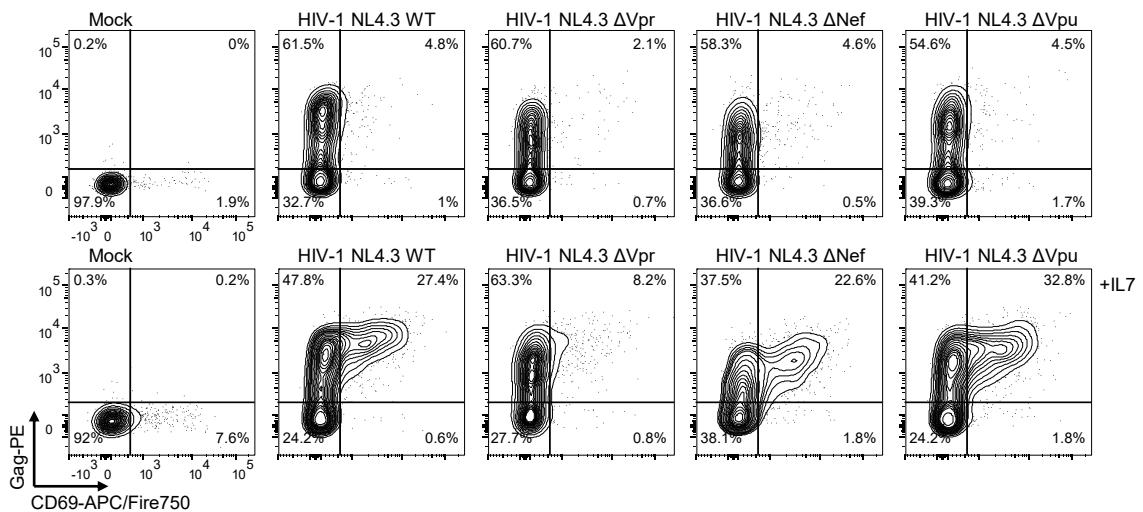
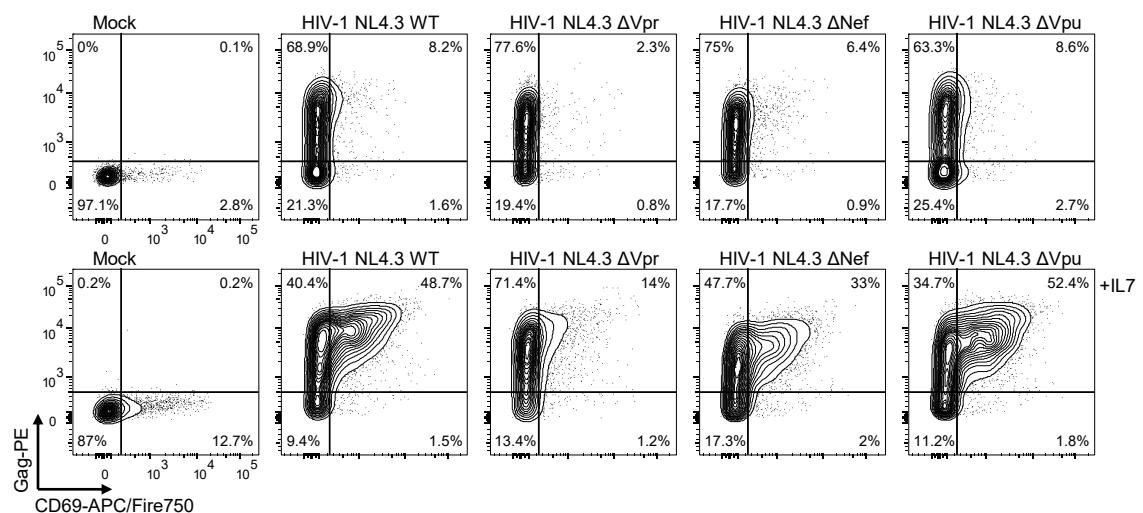
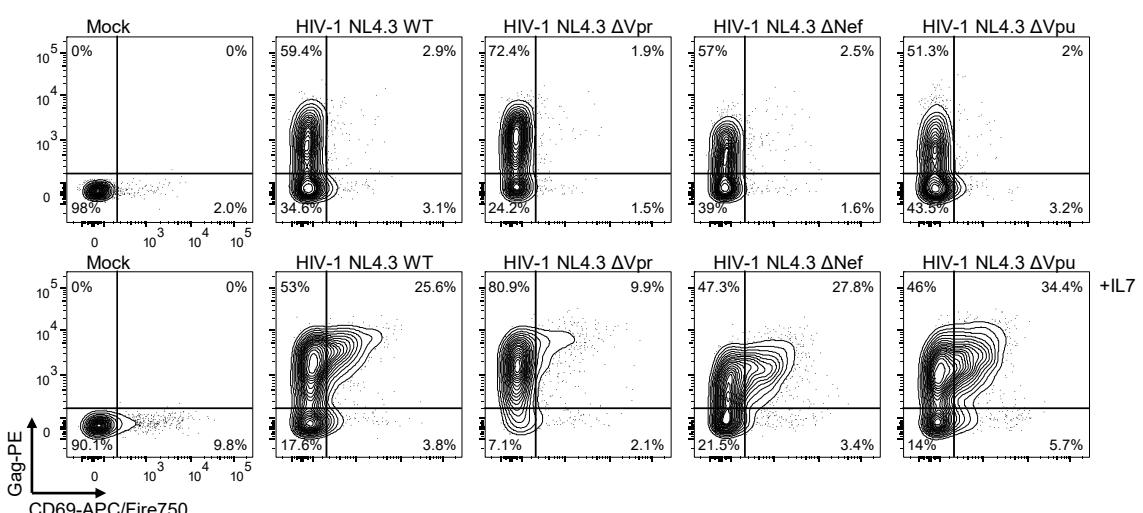
Extended data Fig. 1. (a) Experimental set-up schematic. (b) Flow cytometry gating strategy. (c) Ki67, CD69 and HLA-DR expression of CD3+CD4+ T cells from unstimulated PBMCs (n=8). (d) Ki67 expression on resting and activated primary CD4+ T cells. Representative flow cytometry plots. (e) MCM2 expression 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 0.4 μ m 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) Proportion of CD45RA+ naive and CD45RA- memory CD4+ T cells in unstimulated PBMCs (n=8). (j) Resting target CD4+ T cells were cultured with mock-treated or HIV-1-infected donor cells. Surface expression of CD45RA and CCR7 were measured after 72h of co-culture. Representative flow cytometry plots are shown. (k) Quantification of T cell subsets in infected (Gag+) and uninfected (Gag-) resting CD4+ T cells according to CD45RA/CD62L expression (n=3). $T_{naive} = CD45RA+/CD62L+$, $T_{EMRA} = CD45RA+/CD62L-$, $T_{EM} = CD45RA-/CD62L-$, $T_{CM} = CD45RA-/CD62L+$. Data are shown as mean \pm SEM.



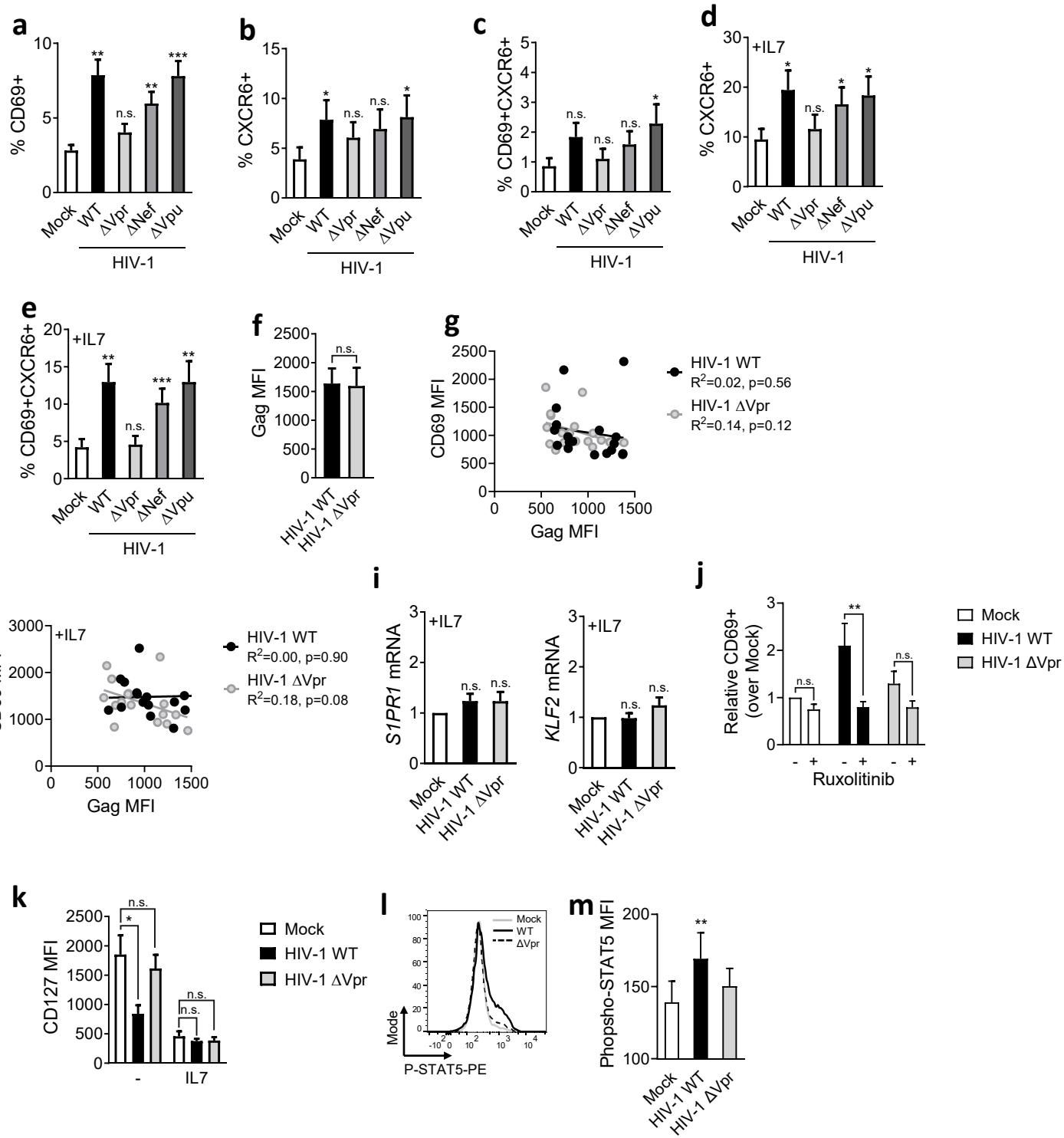
Extended data Fig. 2. (a) Representative flow cytometry plots of CD45RA+ and CD45RA- CD4+ T cells pre- and post-isolation. (b) Representative histogram of intracellular Gag staining in resting naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread \pm T20. (c) Representative histogram of intracellular Gag-levels in resting naïve (CD45RA+) and memory (CD45RA-) CD4+ T cells after 72h of cell-to-cell spread \pm Efavirenz. (d) Representative histogram of CD4 surface levels in resting naïve (CD45RA+) and memory (CD45RA-) CD3+ T cells after 72h of cell-to-cell spread. (e) 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).



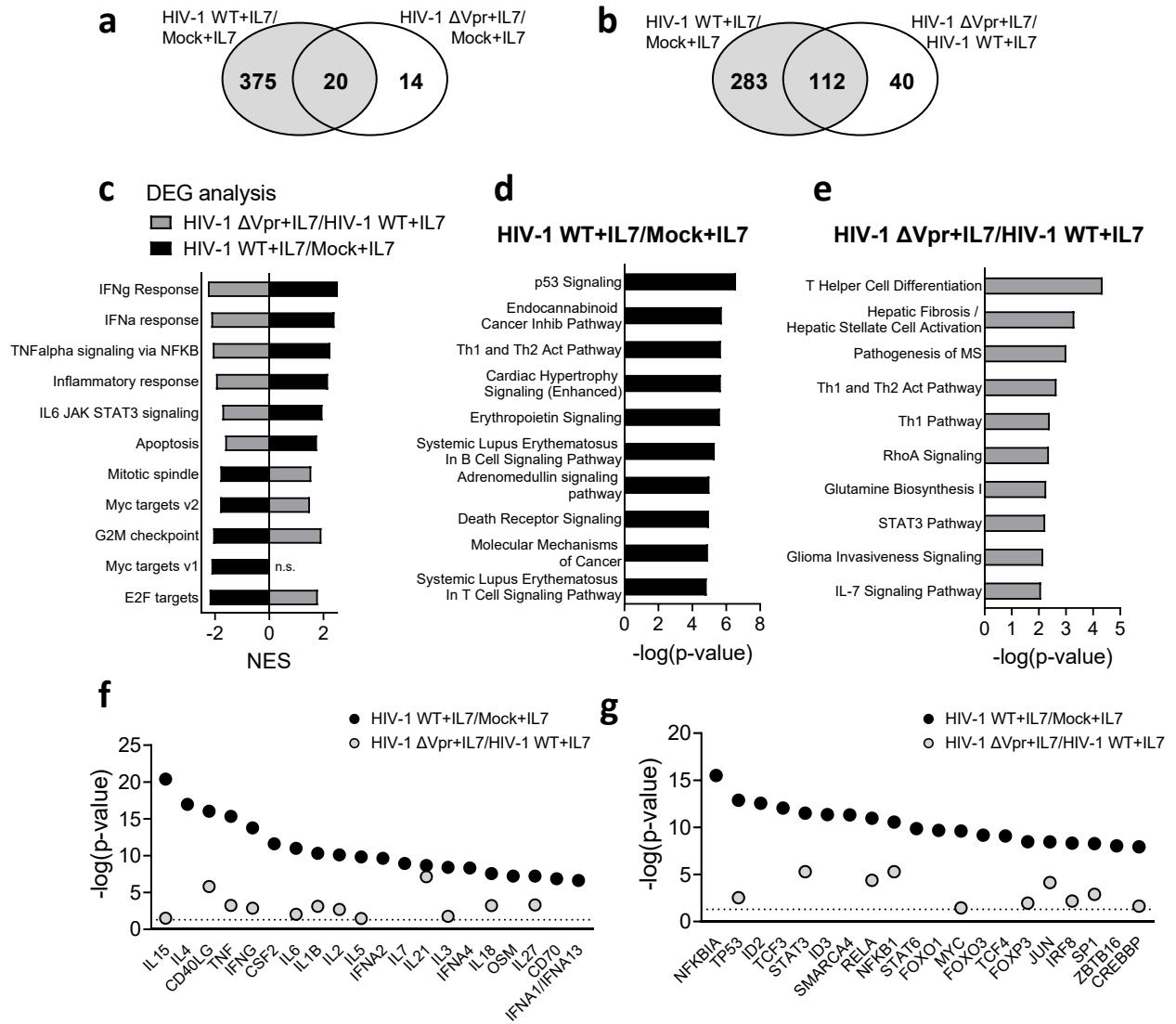
Extended data Fig. 3 (a and b) Representative flow cytometry plots showing (a) Ki67 and Gag, or (b) MCM2 and Gag staining of resting CD4+ T cells after co-culture with mock or HIV-1 infected primary donor CD4+ T cells. (c) FACS sorted CD69- resting naïve or memory CD4+ T cells co-cultured with HIV-1 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=4). (e) Total CD69 expression alongside CD69 with or without HLA-DR co-expression on infected resting memory T cells (n=6). (f) Expression of T_{RM} -markers CXCR6, CD49A and CD69 on resting CD4+ memory T cells from unstimulated PBMCs (n=8). (g) Quantification of cell-to-cell spread of HIV-1 WT to resting memory CD4+ T cells in the presence or absence of IL7 (n=10). (h and i) CD69 expression on infected resting memory CD4+ target T cells in the presence or absence of (h) IL12 (n=4) or (i) TGF β (n=4). (j) CD69 expression on infected resting memory CD4+ T cells with IL7 added at the indicated times post cell-mixing (n=5). (k) CD69/CXCR6 co-expression from (j) (n=5). (l) CD69/CD101 co-expression on infected resting memory CD4+ T cells (n=3). (m) CD69 upregulation in response to IL7 on resting memory CD4+ T cells infected HIV-1 NL4.3, or transmitter-founder viruses CH040 and CH077 comparing infected Gag+ and uninfected Gag- bystander cells (n=7). (n) CD69/CXCR6 co-expression on resting memory CD4+ T cells from (i) (n=7). (o) CD69/CX3CR1 co-expression on infected resting memory CD4+ T cells (n=3). (p) CD103 expression and (q) CD69/CD103 co-expression on infected resting memory CD4+ T cells (n=4). Data are the mean \pm SEM. Paired two-tailed t-test or one-way ANOVA with Bonferroni post-test was used. For (i), median \pm 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.

a**b****c**

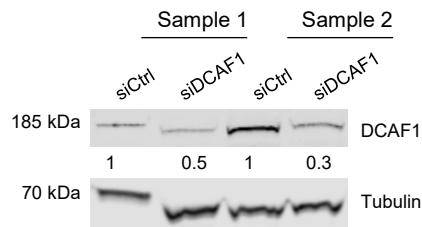
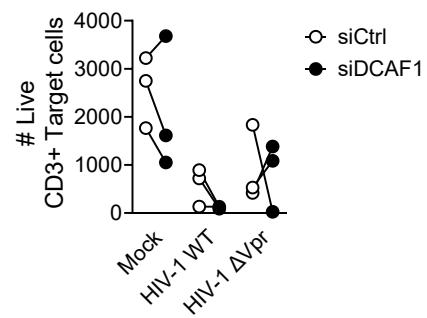
Extended data Fig. 4 Resting memory CD4+ T cells were co-cultured with HIV-1 infected primary CD4+ T cells infected with HIV-1 WT or mutant viruses. Representative flow cytometry plots of HIV-1 Gag and CD69 co-expression in the presence or absence of IL7 from three independent experiments are shown (a-c).



Extended data Fig. 5 (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), Δ Vpr, Δ Nef or Δ Vpu 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 Δ Vpr to resting memory CD4+ T cells (n=10). Correlation plot of CD69 MFI with Gag MFI in absence (g) or presence (h) of IL7 (n=18). (i) S1PR1 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 expression on infected resting memory CD4+ T cells \pm Ruxolitinib (n=4). (k) CD127 MFI on infected resting memory CD4+ T cells \pm IL7 (n=7). (l) Representative histogram of intracellular STAT5-phosphorylation in infected resting memory T cells. (m) Quantification of (l) (n=10). Data are the mean \pm SEM. One-way ANOVA with Dunnett's post-test was used. Statistical significance is shown relative to mock treated cells (no HIV-1). R^2 in (g) and (h) was determined by simple linear regression. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; n.s., not significant.



Extended data Fig. 6 (a) and (b) Venn diagrams showing overlap of DEGs comparing expression profiles of HIV-1 WT+IL7/Mock+IL7 with (e) HIV-1 ΔVpr+IL7/Mock+IL7 or (f) HIV-1 ΔVpr+IL7/HIV-1 WT+IL7. (g) 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 (FDR q-value<0.05 and NES>1.75). (h) and (i) top ten significantly enriched canonical pathways predicted by Ingenuity pathway (IPA) analysis of DEGs (h) HIV-1 WT+IL7/Mock+IL7 or (i) HIV-1 ΔVpr+IL7/HIV-1 WT+IL7 (adjusted p-value<0.05). (j) Cytokines and (k) 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.

a**b**

Extended data Fig. 7 (a) Western blot showing siRNA knockdown of DCAF1 in CD3/CD28-activated CD4+ T cells 48h post transfection. Two representative samples are shown. (b) Number of live CD3+ Target T cells recovered after 72h of cell-to-cell spread into control or DCAF1 siRNA-treated T cells (n=3).