

1 **HIV disrupts CD34⁺ progenitors involved in T-cell differentiation**

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3 *Tetsuo Tsukamoto^{1, 2, 3}

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5 ¹ The Kirby Institute for infection and immunity in society, University of New South
6 Wales, Sydney, Australia

7 ² Center for AIDS Research, Kumamoto University, Kumamoto, Japan

8 ³ Department of Immunology, Kindai University Faculty of Medicine, Osaka, Japan

9

10 *Corresponding author mailing address: Department of Immunology, Kindai

11 University Faculty of Medicine, 377-2 Onohigashi, Osakasayama-shi, 589-8511

12 Osaka, Japan

13 Telephone: +81 72 366 0221

14 Fax: +81 72 367 7660

15 Email: ttsukamoto@med.kindai.ac.jp

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Abstract

HIV-1 causes the loss of CD4⁺ T cells via depletion or impairment of their production. The latter involves infection of thymocytes, but the involvement of other cells including haematopoietic CD34⁺ cells remains unclear even though HIV-positive patients frequently manifest myelosuppression. This study utilised the OP9-DL1 coculture system, which supports in vitro T-lineage differentiation of human haematopoietic stem/progenitor cells. Cord-derived CD34⁺ cells were infected with CXCR4-tropic HIV-1_{NL4-3} and cocultured. HIV-infected cocultures exhibited sustained viral replication for 5 weeks, as well as reduced CD4⁺ T-cell growth at weeks 3–5. It was further revealed that CD34⁺CD7⁺CXCR4⁺ cells can be quickly depleted as early as in 1 week after infection of the subset, and this was accompanied by the emergence of CD34⁺CD7⁺CD4⁺ cells. These results indicate that CXCR4-tropic HIV-1 strains may disrupt CD34⁺CD7⁺ lymphoid progenitor cell pools, presumably leading to impaired T-cell production potential.

Keywords

human immunodeficiency virus, acquired immunodeficiency syndrome, haematopoietic stem/progenitor cells, C-X-C chemokine receptor type 4

Introduction

Human immunodeficiency virus (HIV) infection is associated with haematological changes¹. Antiretroviral therapy is effective in controlling viremia and treating acquired immunodeficiency syndrome (AIDS). However, some patients do not experience sufficient T-cell immune restoration despite being aviremic during treatment². HIV-infected patients may display decreased thymopoiesis³. Thymic dysfunction occurs during HIV infection, and it is associated with rapid progression in infants with prenatal HIV infection⁴. A previous report tested coculture of CD34⁺ and fetal thymic epithelial cells in the presence or absence of HIV-1, observing that infection led to the inhibition of thymocyte maturation at early stages (CD44⁺CD25⁻CD3⁻)⁵. Pancytopenia may occur as a result of bone marrow abnormalities in HIV-infected individuals^{6,7}. A previous study revealed depletion of CD34⁺CD4⁺ cells in bone marrow from HIV-infected patients. However, the study failed to uncover evidence of HIV infection of these cells⁸.

The thymopoiesis environment can be mimicked in vitro using the OP9-DL1 or OP9-DL4 cell line. These cell lines were derived from the OP9 mouse stromal cell line via transduction with a notch ligand called delta-like 1 (DL1) or DL4^{9,10}. The ability of OP9-DL1 cells to support thymopoiesis in vitro was first demonstrated in coculture with mouse cells⁹. The cell line is also known to support the differentiation of human CD34⁺ haematopoietic stem/progenitor cells (HSPCs) into thymocytes and T cells¹¹. There is evidence that OP9-DL1 cells produce stromal derived factor-1 (SDF-1, also known as CXCL12), a ligand for CXCR4¹². Although OP9-DL4 cells can induce differentiation into both specific myeloid cells and T-lineage cells, OP9-DL1 cells only permit the differentiation of T-lineage cells while inhibiting B cells

and myeloid cells ¹³. Thus, the OP9-DL1 coculture system is used to investigate events associated with T-lineage differentiation.

The chemokine receptors CXCR4 and CCR5 are common coreceptors for HIV-1 ¹⁴. Control of CCR5-tropic strains of HIV-1 is usually considered a better correlate of good clinical outcomes than control of CXCR4-tropic HIV-1 strains ¹⁵. This is due to the fact that memory CD4⁺ T cells express higher levels of CCR5, thus making them susceptible to CCR5-tropic HIV infection and subsequent depletion ¹⁶. Consequently, CCR5 may appear to be more closely involved in the immunopathogenesis of HIV infection than CXCR4 ¹⁷. Conversely, the biological functions of CXCR4 have been well documented in fields such as developmental biology and haematology. CXCR4 interacts with SDF-1 and allows CXCR4-expressing cells to home to loci in which SDF-1 is highly expressed ¹⁸. SDF-1 and CXCR4 are essential in human stem cell homing and repopulation of the host with differentiated haematopoietic cells ^{19,20}. SDF-1 is also produced by thymic epithelial cells, and it plays an important role in migration of immature progenitors in the thymus ²¹.

Therefore, it may be important to better understand the influence of CXCR4-tropic HIV-1 infection on CXCR4-associated biological events including haematopoiesis and T-cell development ²². A prior study sought to clarify the effect of CXCR4-tropic simian-human immunodeficiency virus infection on T-lineage cell production in the thymi of newborn rhesus macaques ²³. However, it is not realistic to closely follow in vivo bone marrow/thymus events in HIV-infected individuals. Instead, humanised mouse models can be beneficial for this purpose ^{24,25}. Moreover, an easy-to-use ex-vivo model may be helpful for closely monitoring the differentiation of HSPCs into T-lineage cells in the presence of HIV-1. The present

- 86 study aimed to determine the in vitro fate of CD34⁺ progenitor cells and derivatives
- 87 exposed to HIV-1 using the OP9-DL1 coculture system.

Results

Persistent HIV-1 infection observed in OP9-DL1 cocultures with cord-derived CD34⁺ cells

Two separate in vitro experiments were performed in the study. The data for experiment 1 (long coculture) are shown in Figs. 1–3 and Extended Data Figs. 1–5. To follow the in vitro fate of HIV–pre-exposed CD34⁺ cells and derivatives for several weeks, primary human umbilical cord-derived CD34⁺ cells were infected with HIV-1. These cells were partially CD4⁺ and/or CXCR4⁺ before infection (Extended Data Fig. 1A). Cells were seeded in 48-well plates and exposed to 200 ng (p24) of HIV-1_{NL4-3}. Following centrifugation and overnight incubation in the presence of HIV-1, the cells were cocultured with OP9-DL1 cells (Fig. 1A). After a week of coculture, intracellular HIV-1 p24 was detected in both CD34⁺ and CD34[–] cells (Fig. 1B). HIV infection was further examined via magnetic bead separation of CD34⁺ and CD34[–] cells followed by DNA extraction and detection of HIV-1 *gag* DNA using PCR (Extended Data Fig. 1B). The HIV-infected samples displayed persistent HIV-1 p24 expression in 0.1–1.7% of the total cocultured human cells (Fig. 1C, Extended Data Fig. 1C–G). A correlation was found between intracellular HIV-1 p24⁺ cell counts and supernatant HIV-1 p24 concentrations (Extended Fig. 1E).

The dynamics of CD4⁺ cells in OP9-DL1 cocultures with HIV–pre-exposed CD34⁺ cells

The influence of HIV pre-exposure of CD34⁺ cells on post-coculture events was analysed by flow cytometry weekly until week 5. CD19⁺, CD20⁺ or CD33⁺ cells were not detected in the samples tested (data not shown). HIV-infected cocultures had

significantly lower whole cell counts at weeks 3, 4 and 5 post-coculture than uninfected cocultures (Extended Data Fig. 2A), demonstrating reduced cell growth in the presence of HIV-1. Similarly, the CD4⁺CD8⁺ and CD4⁺CD8⁺CXCR4⁺ cell counts were significantly smaller in HIV⁺ cocultures than in HIV⁻ cocultures at week 3 (Extended Data Fig. 2B–D). When CD34⁻ cells were gated and analysed for CD7 and CD4 expression (Fig. 2A), the CD34⁻CD7⁺CD4⁺ cell counts were not significantly different between HIV-infected and uninfected samples (Fig. 2B). By contrast, there was a notable decline in CD34⁻CD7⁻CD4⁺ cell counts in HIV⁺ samples compared to those in HIV⁻ samples at week 3 (Fig. 2C). Further analysis of CD34⁻ cells revealed that the CD7⁺CD4⁺ cells contained CD4/CD8 double-positive cells and CD3⁺CD4⁺ cells, whereas the CD7⁻CD4⁺ cells were mostly CD3⁺CD4⁺ cells (Extended Data Fig. 3). In the analysis of CD34⁻CXCR4⁺, CD34⁻CD7⁺CXCR4⁺ and CD34⁻CD7⁻CXCR4⁺ cells, the CD34⁻CD7⁻CXCR4⁺ cells exhibited the greatest growth impairment following HIV-1 infection (Extended Data Fig. 4).

The dynamics of CD34⁺ cells in OP9-DL1 cocultures with HIV-pre-exposed CD34⁺ cells

HIV infection of CD34⁺ cells was detected by intracellular p24⁺ staining followed by flow cytometric analysis (Fig. 3A–B, Extended Data Fig. 5A–D). p24⁺CD34⁺ cells failed to grow by week 2 (Fig. 3A), in contrast to the rapid growth of p24⁺ cells at this time point (Fig. 1C). p24⁺CD34⁺CD7⁺ cell counts increased at weeks 2 and 3 (Fig. 3B) in association with a low average CD34⁺CD7⁺ cell count in week 1 after infection and a rapid increase in week 2 (Fig. 3C). Then, CD34⁺ cell dynamics in HIV⁺ samples were compared with those in HIV⁻ samples (Fig. 3C–D, Extended Data Fig. 5E–G). There were nonsignificant differences in CD34⁺CD7⁺ cell counts

between HIV⁺ and HIV⁻ cocultures (Fig. 3C). The difference in the average frequencies of CD34⁺CD7⁺ cells was greatest at week 3 (Fig. 3C). However, the average CD34⁺CD7⁺CXCR4⁺ cell count in HIV⁺ cocultures versus that in HIV⁻ cocultures exhibited the greatest difference at week 2 (Fig. 3D). Therefore, CD34⁺CD7⁺CXCR4⁺ cells may be affected by HIV-1 earlier than the entire CD34⁺CD7⁺ cell population or cells of other phenotypes.

HIV-1_{NL4-3} infection of HSPC-derived cells after 4–6 weeks of coculture with OP9-DL1 cells

To better describe the effect of HIV infection on the short-term dynamics of CD34⁺ cells, experiment 2 was performed (short coculture, n = 12, Fig. 4A). The experiment 2 results are shown in Figs. 4–6, Extended Data Figs. 6–8 and Extended Data Tables 1–2. Briefly, coculture of OP9-DL1 cells with HIV-uninfected cord-derived CD34⁺ cells for 4–6 weeks produced a mixture of cells with different phenotypes as determined by the expression of CD34, CD4, CD8, CD7 and CXCR4 (Figs. 1–3, Extended Data Figs. 1–5). CD4 expression levels in CD34⁺ cells before infection were lower than those in CD34⁻ cells (Fig. 4B). Cells were then harvested and infected with HIV-1_{NL4-3} following the procedures for HIV infection of CD34⁺ cells (Fig. 1A). The infected cells were cocultured again with a new OP9-DL1 monolayer and further incubated for 1 week (Fig. 4A). One week after infection, HIV replication was detected in all HIV-infected samples (Fig. 4C). The majority of HIV-1 p24⁺ cells were CD34⁻ (Fig. 4C, Extended Data Fig. 6). The majority of p24⁺CD34⁺ cells were CD7⁺ (Fig. 4C, Extended Data Table 1).

Partial loss of CD34⁺CD7⁺CXCR4⁺ cells after HIV-1_{NL4-3} infection of OP9-DL1–cocultured cells

The phenotypes of the cells in experiment 2 (short coculture) were analysed 1 week after infection (Fig. 5, Extended Data Fig. 7, Extended Data Table 2). The whole cell counts were obtained using parts of the tested batches, with no significant differences noted between HIV⁺ and HIV[−] samples (n = 4, p = 0.5000, Extended Data Table 2). The frequencies of CD4⁺CD8⁺, CD34⁺ and CD34⁺CD7⁺ cells were not significantly different between HIV⁺ and HIV[−] cocultures (Extended Data Fig. 7). Regarding CXCR4 expression levels in different subsets, the frequencies of CD34⁺CD7⁺CXCR4⁺ cells in HIV⁺ samples were significantly reduced compared to those in HIV[−] samples (Fig. 5A and C middle). This may be in accordance with the results in Fig. 3D in which the growth of CD34⁺CD7⁺CXCR4⁺ cells slowed earlier (at week 2) than those of other phenotypes (at week 3).

Increased number of CD34⁺CD7⁺CD4⁺ cells after HIV-1_{NL4-3} infection of OP9-DL1–cocultured cells

Figure 5 indicates the distinct dynamics of CD34⁺CD7⁺CXCR4⁺ cells in the presence of HIV-1. For increased clarity, CD34⁺CD7⁺ cells were further analysed for CD4 expression (Fig. 6, Extended Data Fig. 8). Surprisingly, HIV-infected samples had higher frequencies of CD34⁺CD7⁺CD4⁺CXCR4⁺ cells than uninfected samples (Fig. 6A and B). These cells were found to be partially HIV p24⁺ after carefully adjusting the compensation matrices of the flow cytometry data (Fig. 6A and C). One of nine batches was selected for further analyses of CD34⁺CD7⁺CD4⁺ cells. Both the frequencies and CD4 fluorescence intensities of CD34⁺CD7⁺CD4⁺ cells were higher in HIV⁺ samples than in HIV[−] samples (Extended Fig. 8B), implying the emergence

187 of CD34⁺CD7⁺CD4^{hi} cells. In addition, the frequencies of CD34⁺CD7⁺CD4⁺CXCR4⁺
 188 cells were correlated with those of p24⁺ cells (Fig. 6D). A separate staining of an
 189 experiment 2 sample with annexin V at week 1 after infection didn't result in
 190 increased annexin V reactivity of CD34⁺CD4⁺ cells in HIV⁺ samples compared to
 191 those in HIV⁻ samples (data not shown).

Discussion

Regarding the dynamics of CD34⁺ cells in HIV-infected individuals, there has been a debate regarding whether HSPCs comprise an unignorable viral reservoir²⁶. There is also a possibility of modified turnover of HSPCs through HIV infection and depletion of CD4⁺ cells. For example, HIV infection commonly manifests as bone marrow abnormalities⁷. Some patients fail to exhibit CD4⁺ T-cell recovery even after effective antiretroviral therapy, thus being termed immunological nonresponders. Such immunological nonresponsiveness can be associated with immune activation and/or bone marrow impairment^{27,28}. To approach these problems, the present study revealed a method for utilising the OP9-DL1 coculture system, which enables in vitro follow-up of the early events in T-cell differentiation such as lymphoid progenitor cell generation that normally occur in the bone marrow as well as CD4⁺ thymocyte differentiation in the thymus. The events observed in the cocultures of OP9-DL1 and human CD34⁺ cells are likely to involve interactions between SDF-1 and CXCR4, as mouse SDF-1 expressed by OP9-DL1 cells has high amino acid sequence identity with human SDF-1²⁹. The observation is in accordance with previous reports illustrating that the SDF-1/CXCR4 pair is crucially involved in the homing and repopulation of HSPCs in specific bone marrow niches³⁰ and the entire T-cell developmental process in the thymus^{21,31}. It has been reported that HSPCs and thymocytes express CXCR4, but their CCR5 expression is limited³². Another report indicated that HIV utilises CXCR4 when it infects multipotent progenitor cells³³. CXCR4-tropic HIV strains might contribute to pathogenesis by interfering with haematopoiesis and/or lymphopoiesis³⁴⁻³⁶, although the underlying mechanisms are yet to be unveiled.

The present results provide insights into the impact of HIV-1 on T-lineage differentiation of haematopoietic progenitor cells. To be more physiological than the previous studies analysing the influence of HIV on CD34⁺ cells, the present study didn't include strong stimuli such as 50–100 ng/mL of stem cell factor, thrombopoietin, FMS-like tyrosine kinase 3 ligand or interleukin 3^{33,37}. In experiment 1 (long coculture), HIV-infected samples exhibited similar CD4⁺ cell production rates as uninfected samples at weeks 1 and 2 but reduced CD4⁺ cell production rates from week 3 to week 5 (Fig. 2). This was most clearly observed with the dynamics of CD34⁺CD7⁺CD4⁺ cells (Fig. 2C) that were mostly CD3⁺ T cells (Extended Data Fig. 3). It is unclear how the reduction of cell growth at week 3 after infection was so accurately reproduced in the 12 samples tested (Fig. 2C, individual data not shown). However, in the subsequent analyses of CD34⁺ cells, the growth rates of CD34⁺CD7⁺CXCR4⁺ cells tended to fall as early as week 2 (Fig. 3D). This was observed in 7/12 samples tested (data not shown), and the decline occurred earlier than the reduction in CD34⁺CD7⁺CD4⁺ cell counts (week 3). Because CD34⁺CD7⁺ cells represent lymphoid progenitor cells^{38,39}, their involvement in defining the production rates of CD4⁺ cells may be speculated.

Subsequently, experiment 2 (short coculture, Figs. 4–6) was conducted to examine the effects of HIV-1 on the short-term dynamics of T-lineage cells including CD34⁺ progenitors. HIV-1 infection resulted in significant decreases of the frequency of CD34⁺CD7⁺CXCR4⁺ cells 1 week after infection (Fig. 5B right), which may be consistent with the results of experiment 1 (Fig. 3D). Because of the limitation of the present study, the fate of the lost CD34⁺CD7⁺CXCR4⁺ cells in the HIV⁺ cocultures is unknown. The differentiation capacity of the remaining CD34⁺CD7⁺CXCR4⁺ cells also remains untested. Interestingly, a recent article reported that bone marrow CD34⁺

progenitor cells from HIV-infected patients exhibit impaired T-cell differentiation potential⁴⁰, which was related to proinflammatory cytokines. Cytokine analyses may be applicable to the coculture assays established in the present study.

The CD34⁺CD7⁺ progenitors were further analysed to better understand their association with HIV-1 infection. Interestingly, the frequencies of CD34⁺CD7⁺CD4⁺ cells were elevated in HIV-infected samples compared to those in uninfected samples (Fig. 6, Extended Data Fig. 8). In addition, CD34⁺CD7⁺CD4⁺ cells were found to be partially HIV p24⁺ (Fig. 6A and C). It may be fascinating to interpret the results as CD4 upregulation following HIV infection, thereby driving the differentiation of T-lineage precursor cells. However, caution may be necessary because exclusion of possible CD34 upregulation in the infected CD4⁺ cells was not confirmed in this study (Extended Data Fig. 8), although the flow cytometry data did not indicate correlations between HIV p24 expression and CD34 upregulation in CD34^{low} cells (data not shown). Further studies may be designed to test shorter coculture periods than 1 week and/or investigate the association of HIV-1 infection with factors that regulate the expression of CD3, CD4, CD7 and CD34. Such analyses will also help better clarify the debate on the issues of CD34⁺ viral reservoirs²⁶.

The findings in this article highlight the potential of anti-HIV treatments such as gene therapy using CD34⁺ HSPCs followed by transplantation because in this manner, all haematopoietic events in the host can be protected against HIV infection by the gene products even in the absence of effective immune responses⁴¹. The problem is that although CCR5 may be considered a reasonable target for knockout or knockdown to prevent infection by CCR5-tropic HIV strains, CXCR4 expression should not be altered in HSPCs because of the essential biological functions of the molecule⁴². Therefore, instead of modulating CXCR4 expression, anti-HIV

modalities targeting an HIV gene or component may be desirable for protecting haematopoietic cells including T-lineage cells from CXCR4-tropic HIV infection⁴³.

In summary, the results of the long/short coculture of human CD34⁺ cells and derivatives with OP9-DL1 cells in the presence of HIV-1 indicate that the dynamics of CD34⁺CD7⁺ lymphoid progenitors may be affected more quickly by HIV-1 than CD34⁺CD4⁺ thymocytes and T cells despite the lower susceptibility of CD34⁺CD7⁺ cells to HIV-1 infection as suggested by their lower CD4 expression levels. Further studies may be designed to elucidate the underlying mechanisms. In addition, the preceding reduction of CD34⁺CD7⁺CXCR4⁺ cell growth at week 2 followed by the reduction of CD34⁺CD4⁺ cell growth at week 3 in the HIV⁺ cocultures may illustrate the mechanism by which reduced sizes of progenitor cell pools may decelerate the production of T cells in HIV-infected patients (Extended Data Fig. 9). This may occur in combination with different mechanisms of CD4⁺ T-cell depletion including direct cytopathic effects, apoptosis and antigen-specific immunological mechanisms⁴⁴⁻⁴⁶.

Methods

Virus stocks. Stocks of HIV-1_{NL4-3} were produced via transfection of 293T cells with the molecular clone DNA pNL4-3⁴⁷. After transfection, the culture supernatant was collected, and viral loads were determined using an HIV p24 enzyme-linked immunosorbent assay (ELISA) kit (ZeptoMetrix, NY, USA).

Cells. Umbilical cord blood samples were collected at Fukuda Hospital, Kumamoto, Japan after obtaining informed consent. Cord blood mononuclear cells were isolated using Pancoll (PAN-Biotech GmbH, Aidenbach, Germany) and centrifugation at 800 rpm for 20 min. CD34⁺ cells were labelled and selected using human CD34 microbeads and LS columns (Miltenyi Biotec, NSW, Australia). The purity constantly exceeded 92%. The OP9-DL1 cell line was generated via retroviral transduction of the OP9 cell line (RCB1124, Riken, Tsukuba, Japan) with human DL1. The cell line was maintained in α -MEM medium (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10% heat inactivated fetal bovine serum (FBS, GE Healthcare, Tokyo, Japan).

Antibodies. Anti-human CD8 Brilliant Violet (BV) 510 (clone RPA-T8), anti-human CD3 PE-Cy7 (clone UCHT1) and anti-human CD34 APC (clone 8G12) were purchased from BD Biosciences (NSW, Australia). Anti-human CD4 PE-Cy7 (clone OKT4), anti-human CD4 PerCP-Cy5.5 (clone OKT4) and anti-human CXCR4 BV421 (clone 12G5) were purchased from BioLegend (CA, USA). Anti-human CD3 ECD (clone UCHT1) and anti-HIV-1 p24 PE (clone FH190-1-1, also known as KC57

RD1) were purchased from Beckman Coulter (NSW, Australia). Anti-human CD7 FITC (clone CD7-6B7) was purchased from CALTAG Laboratories (CA, USA).

Coculture of human cells with OP9-DL1. The OP9-DL1 coculture experiment was performed following a previously published protocol with modifications⁴⁸. Briefly, 2×10^5 HIV-infected or uninfected cord-derived CD34⁺ cells were seeded in a 6-well plate (Corning, VIC, Australia) containing a OP9-DL1 monolayer. The coculture was maintained for 5 weeks in α -MEM medium supplemented with 20% heat inactivated FBS, 5 ng/mL recombinant human FMS-like tyrosine kinase 3 ligand (R&D Systems, MN, USA) and 5 ng/mL recombinant human interleukin 7 (Miltenyi Biotec). Cells were passaged weekly with vigorous pipetting and filtering through a 70- μ m membrane and cocultured again with a fresh monolayer of OP9-DL1 cells. A portion of the collected cells was analysed by flow cytometry.

HIV infection. The method for in vitro HIV infection of CD34⁺ cells was described previously⁴⁹. To infect primary cord-derived CD34⁺ cells with HIV-1_{NL4-3}, a 48-well plate (Corning) was treated overnight with RetroNectin (Takara Bio, Kusatsu, Japan) at a concentration of 10 μ g/mL. CD34⁺ cells were re-suspended in the OP9-DL1 medium, seeded at 2×10^5 per well in the coated plate and infected with 200 ng (p24) of HIV-1_{NL4-3} using spinoculation at $1200 \times g$ at 34°C for 30 min. Cells were further cultured overnight and cocultured in a 6-well plate with a fresh monolayer of OP9-DL1 cells. For HIV infection of OP9-cocultured human cells, the cell concentration was modified to 5×10^5 per well.

Flow cytometry. Surface and intracellular antigen expression was analysed using a FACS LSR II (BD Biosciences), FACS Diva v6.0 software (BD Biosciences) and FlowJo v10.3 software (FlowJo, OR, USA). Dead cells were discriminated using Live/Dead Fixable Near-IR Dead Cell Stains (Thermo Fisher Scientific, VIC, Australia). Live cells were further gated to exclude doublets from the analysis by plotting FSC-H and FSC-A.

PCR analysis of HIV DNA. Cellular DNA was extracted using a Kaneka Easy DNA Extraction Kit (Kaneka, Takasago, Japan). DNA extraction was followed by the PCR analysis using an HIV *gag* primer set (sense: 5'-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3', antisense: 5'-TACTAGTAGTTCCTGCTATGTCACTTCC-3') as described previously⁵⁰. The oligo DNAs were purchased from Sigma-Aldrich (Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad Software, CA, USA). Statistical significance was defined as $P < 0.05$. Comparisons between HIV-infected and uninfected samples were performed using Wilcoxon's matched-pairs signed rank test, unless otherwise stated. Multiple comparison analyses were done, if necessary, using Dunn's method. Spearman's correlation coefficients were calculated for correlation analyses.

Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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499 induced by siRNAs targeted to the HIV-1 promoter region. *J RNAi Gene Silencing* **1**,
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Author information

The author declares no competing financial interests associated with the present work. Correspondence and requests for materials should be addressed to ttsukamoto@med.kindai.ac.jp.

Figures

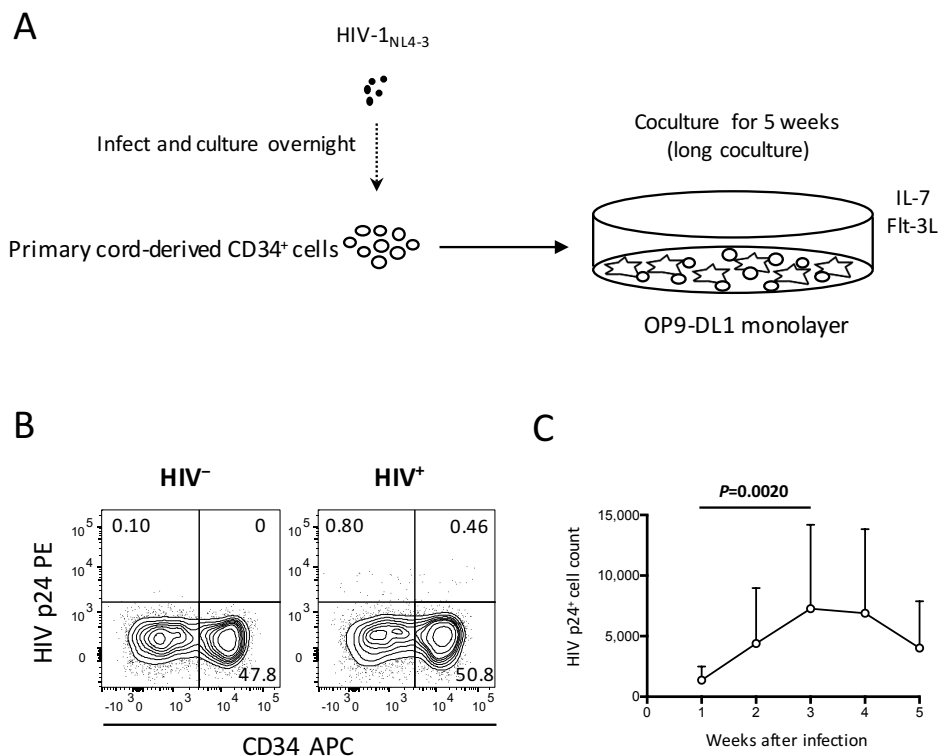


Figure 1. Primary umbilical cord-derived CD34⁺ cells are susceptible to HIV infection. Coculture of HIV-infected primary cord-derived CD34⁺ cells with OP9-DL1 cells resulted in persistent viral replication for 5 weeks (n = 12). (A) Schematic representation of experiment 1 (long coculture). Cord-derived CD34⁺ cells were infected with HIV-1_{NL4-3} and cocultured with OP9-DL1 cells for 5 weeks. (B) Intracellular HIV p24 expression was tested 1 week after infection. HIV p24⁺ cells were found in both the CD34⁺ and CD34⁻ fractions of HIV-infected samples. Representative plots are shown. (C) HIV p24⁺ cell counts at weeks 1–5 post-infection (n = 12). Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test.

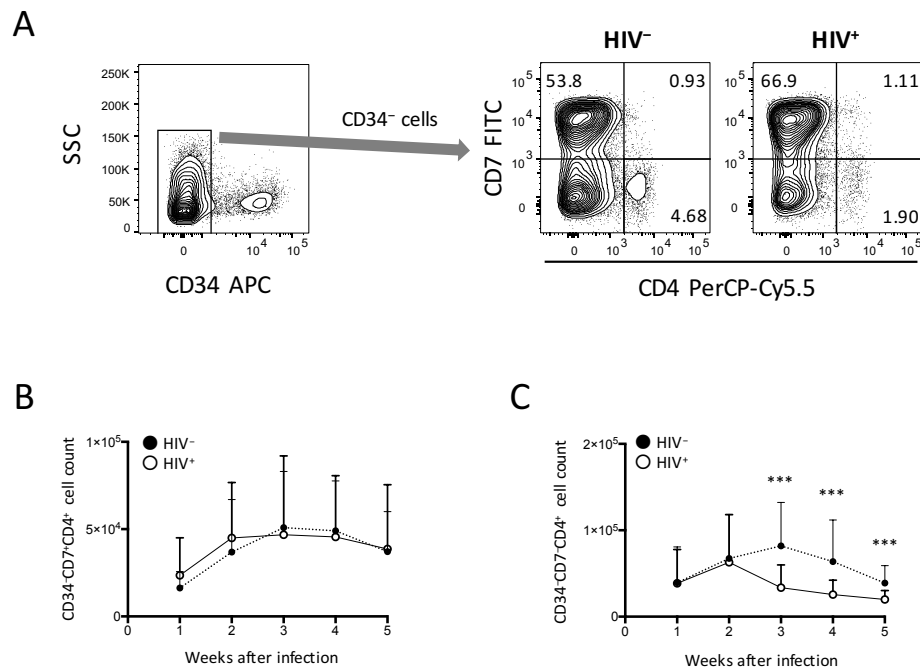


Figure 2. Pre-exposure of primary cord-derived CD34⁺ cells to HIV-1 affected the dynamics of OP9-DL1 cocultured cells (n = 12). (A) Representative plots showing the loss of CD34⁻CD7⁻CD4⁺ cells in the HIV-infected cocultures 3–5 weeks after infection. (B-C) Cell counts were compared between HIV⁺ and HIV⁻ cocultures for (B) CD34⁻CD7⁺CD4⁺ and (C) CD34⁻CD7⁻CD4⁺ cells. Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test. ***: $P < 0.001$.

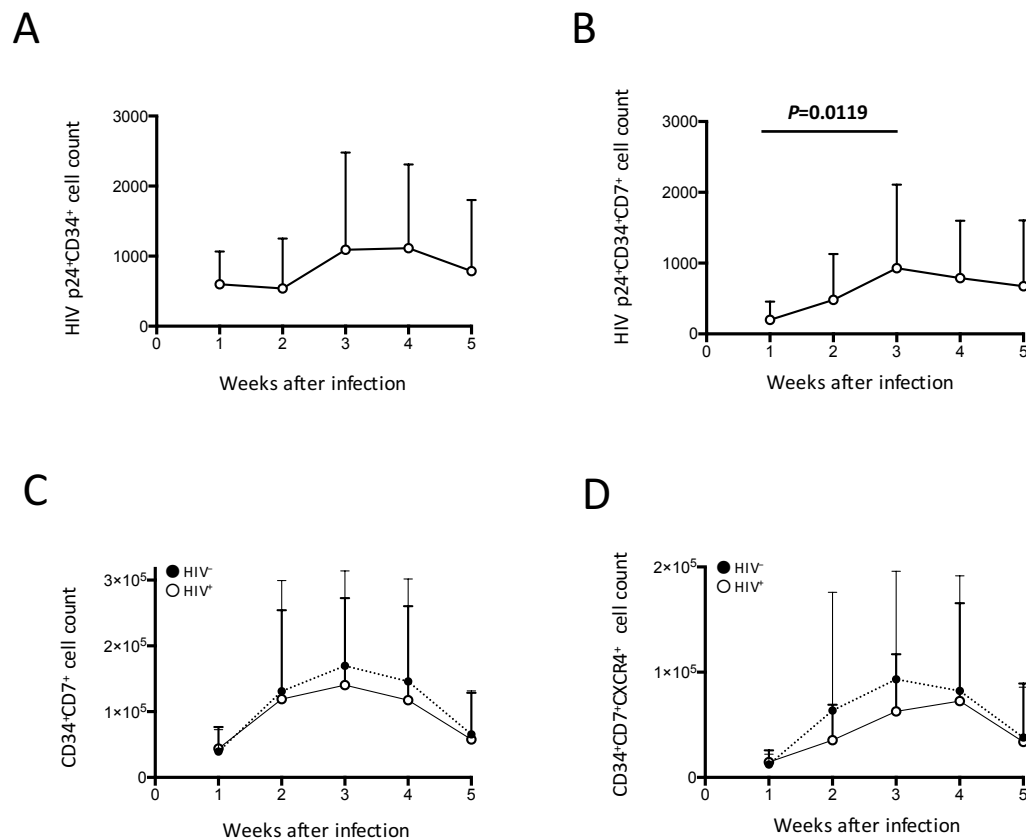


Figure 3. Pre-exposure of primary cord-derived CD34⁺ cells to HIV-1 affected the dynamics of CD34⁺ cells in the subsequent OP9-DL1 cocultures (n = 12). (A) HIV p24⁺CD34⁺ cell counts in HIV⁺ OP9-DL1 cocultures. (B) HIV p24⁺CD34⁺CD7⁺ cell counts in HIV⁺ OP9-DL1 cocultures. (C–D) Cell counts were compared between HIV⁺ and HIV⁻ cocultures for (C) CD34⁺CD7⁺ and (D) CD34⁺CD7⁺CXCR4⁺ cells. Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test.

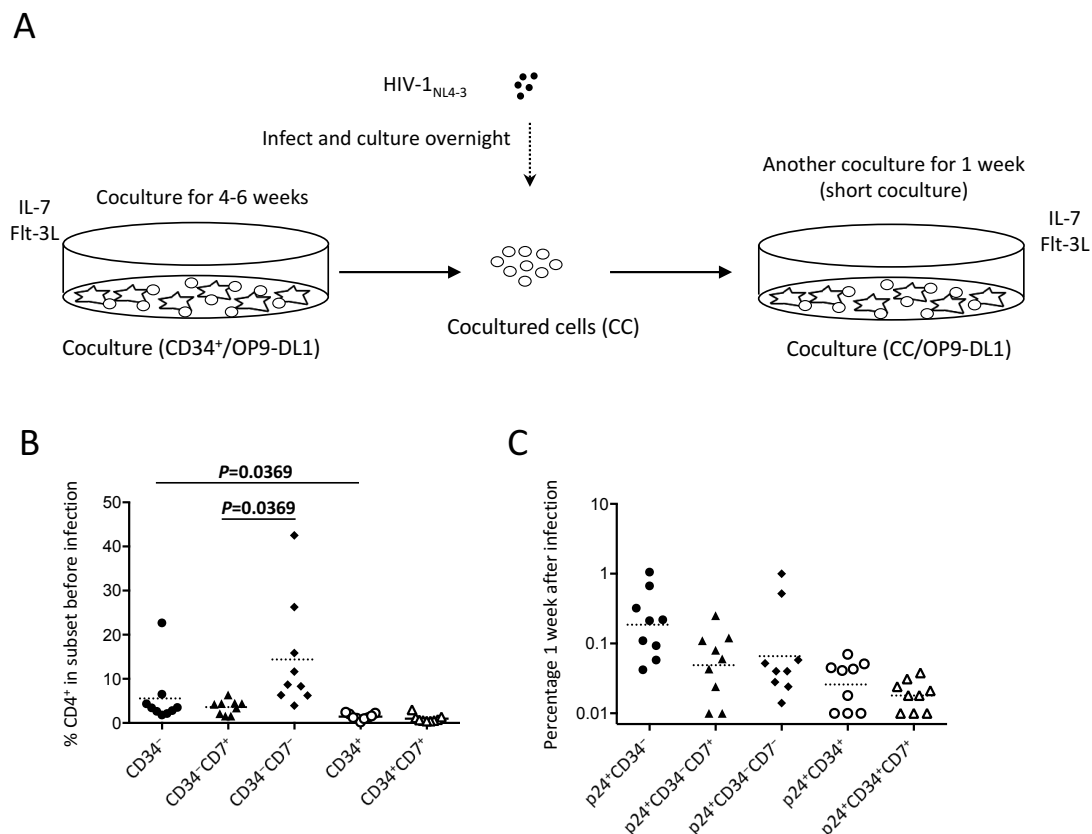
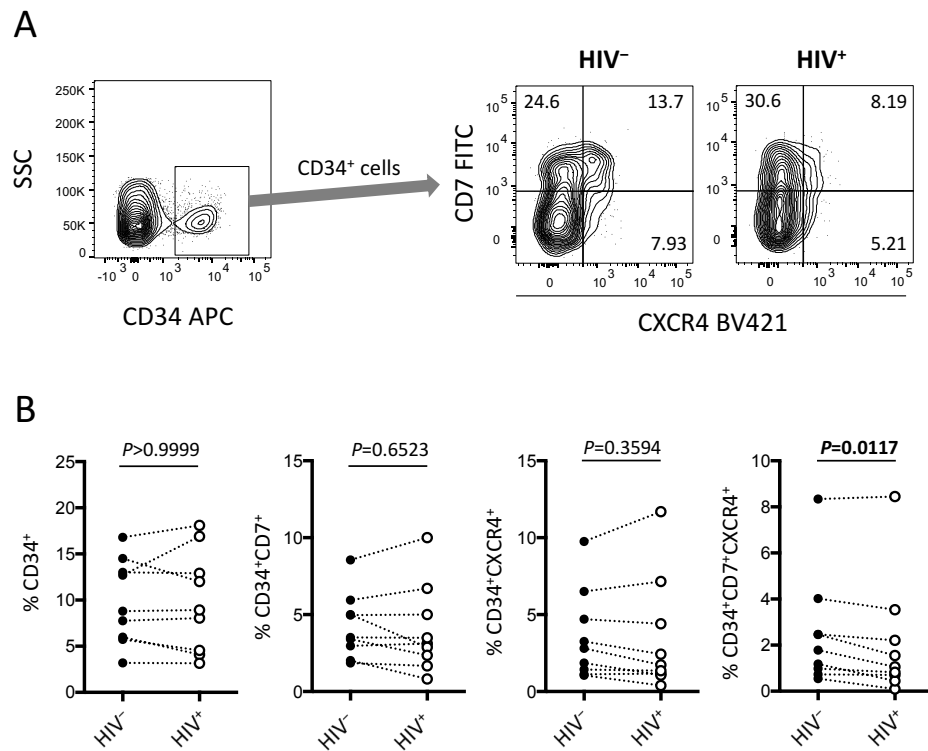


Figure 4. Experiment 2 (short coculture, $n = 9$) was performed to further analyse the dynamics of $CD34^+$ cells in the presence of HIV-1. (A) Schematic representation of experiment 2 (short coculture). Primary cord-derived $CD34^+$ cells were cocultured with OP9-DL1 cells for 4–6 weeks. Cells were then collected and infected with HIV-1_{NL4-3}. The infected cells were cocultured again with OP9-DL1 cells for 1 week, collected and analysed. (B) Percentages of $CD4^+$ cells in $CD34^-$, $CD34^-CD7^+$, $CD34^-CD7^-$, $CD34^+$ and $CD34^+CD7^+$ subsets before infection. A multiple comparison test was performed to compare $CD34^-$ and $CD34^+$ cells or $CD34^-CD7^+$ and $CD34^-CD7^-$ cells. (C) Frequencies of HIV $p24^+$, $p24^+CD34^-CD7^+$, $p24^+CD34^-CD7^-$, $p24^+CD34^+$ and $p24^+CD34^+CD7^+$ cells measured 1 week after the second post-infection coculture ($n = 9$). Statistical analysis was performed using the multiple comparison test with Dunn's method.



561

562 **Figure 5.** CD34⁺ cells in experiment 2 (short coculture, n = 9) were analysed 1 week
563 after the second post-infection coculture. (A) Representative plots showing
564 CD7/CXCR4 expression levels in CD34⁺ cells. An HIV⁺ sample is compared to its
565 autologous uninfected counterpart. (B) Frequencies of CD34⁺ (left), CD34⁺CD7⁺
566 (middle left), CD34⁺CXCR4⁺ (middle right) and CD34⁺CD7⁺CXCR4⁺ (right) cells
567 were compared between HIV⁺ and HIV⁻ samples. Statistical analyses were performed
568 using the Wilcoxon matched-pairs signed rank test.

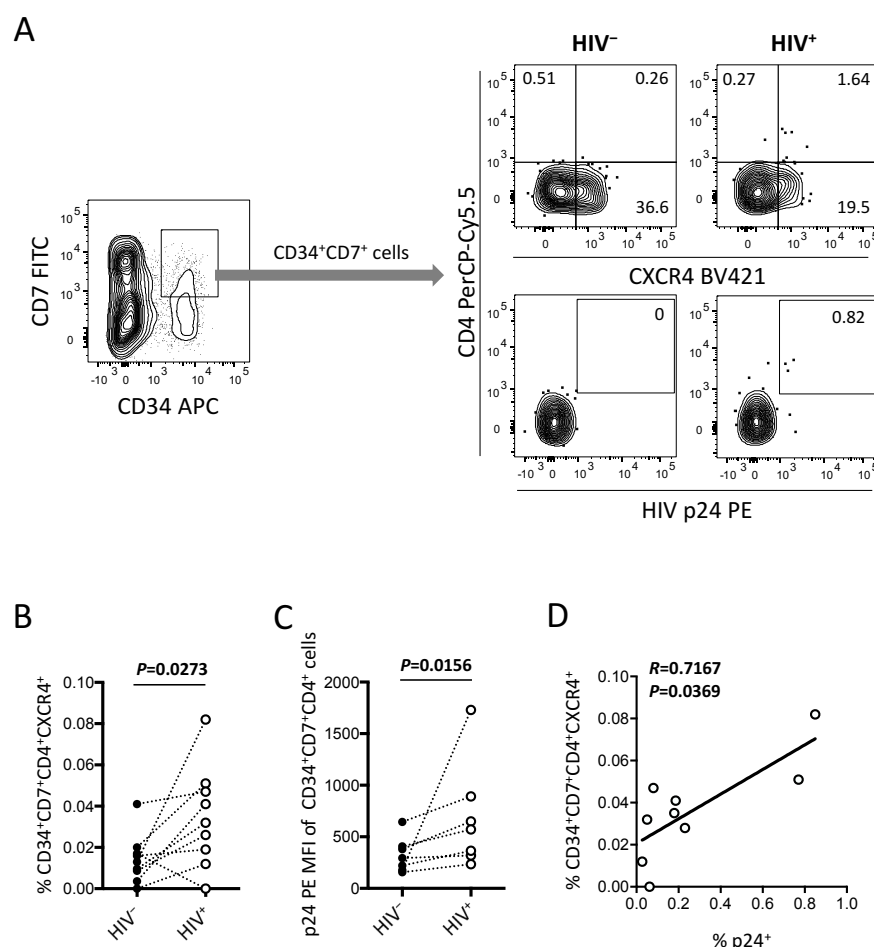
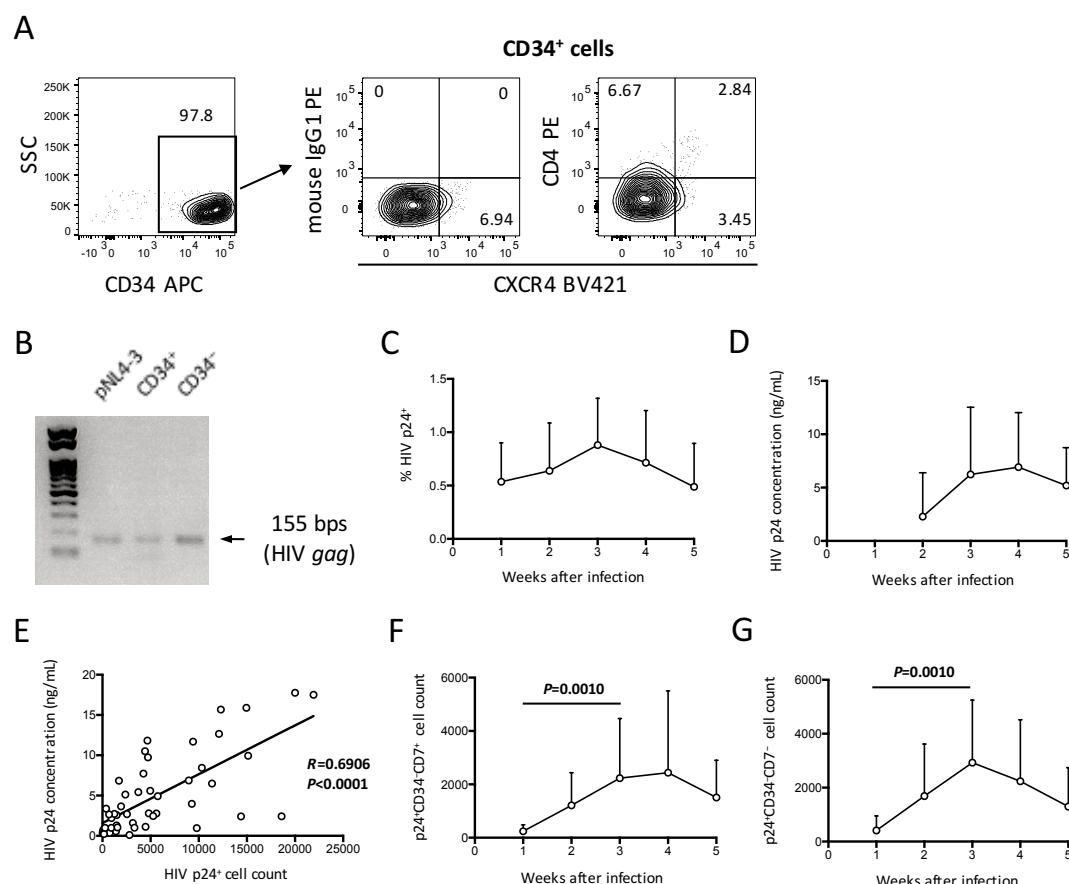
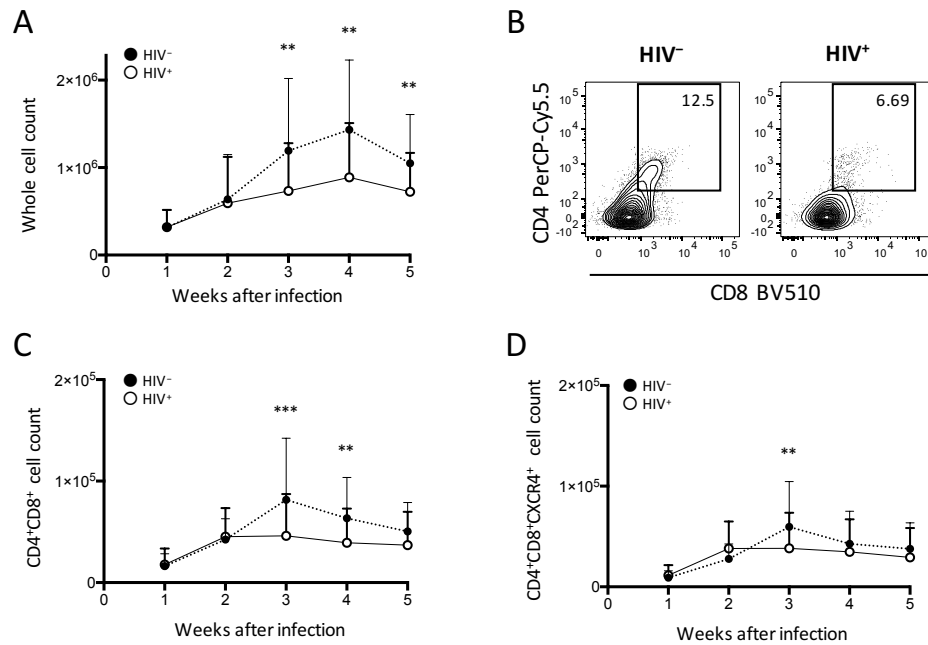


Figure 6. CD34⁺CD7⁺ cells in experiment 2 (short coculture, n = 9) were analysed 1 week after the second post-infection coculture. For these analyses, compensation matrices were checked carefully to exclude false-positive events. (A) Representative plots showing CD4/CXCR4 and CD4/p24 expression levels in CD34⁺CD7⁺ cells. An HIV⁺ sample is compared to its autologous uninfected counterpart. (B) Comparison of the frequencies of CD34⁺CD7⁺CD4⁺CXCR4⁺ cells between HIV⁺ and HIV⁻ samples. (C) p24 mean fluorescence intensities (MFIs) of CD34⁺CD7⁺CD4⁺ cells were compared between HIV⁺ and HIV⁻ samples (n = 7). (D) The frequencies of CD34⁺CD7⁺CD4⁺CXCR4⁺ cells were correlated with those of p24⁺ cells. Comparisons were performed using the Wilcoxon matched-pairs signed rank test. Spearman's correlation coefficient was calculated for the correlation analysis.

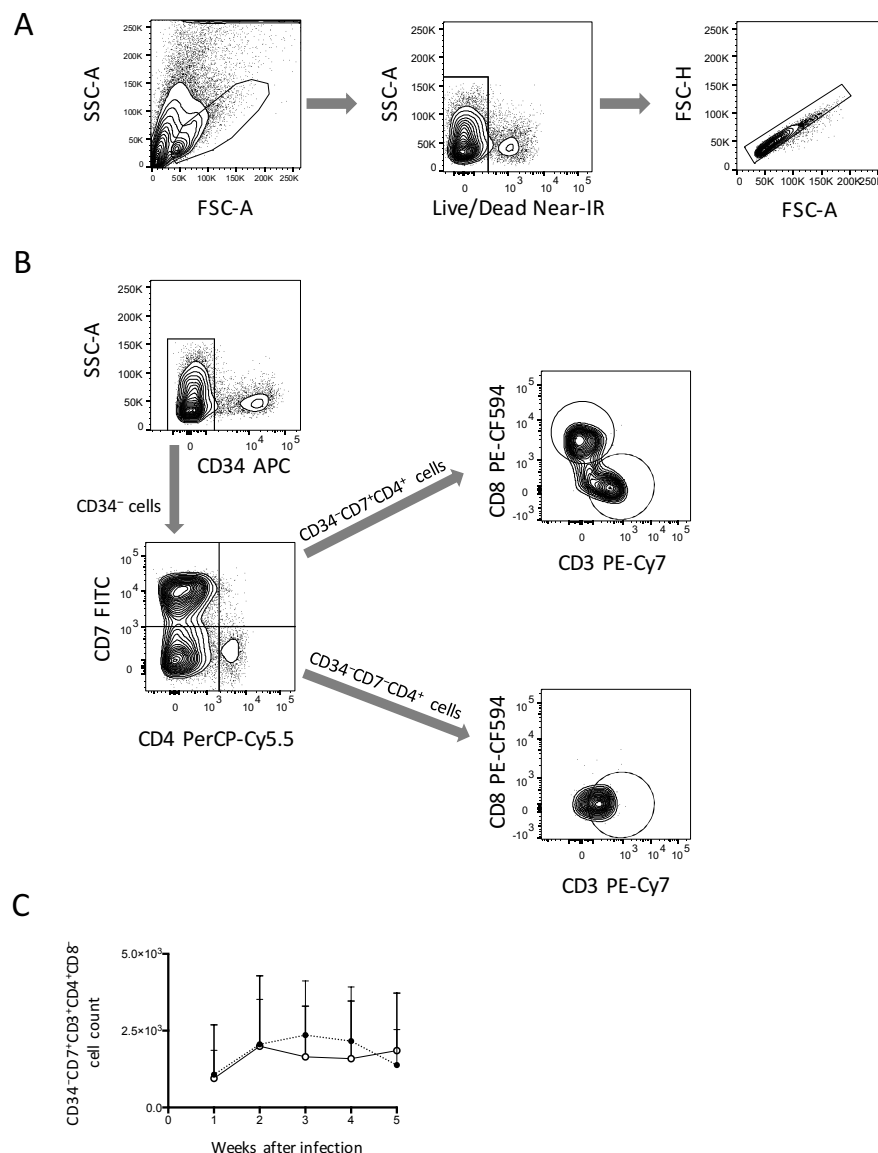
Extended Data figures and tables



Extended Data Figure 1. HIV infection of cord-derived CD34⁺ cells and derivatives were evaluated. (A) Primary cord-derived CD34⁺ cells were gated and tested for CD4 and CXCR4 expression by flow cytometry. Representative plots are shown. The CD4 signal levels were compared to the signal levels of a PE-conjugated mouse isotype control antibody. (B) The CD34⁺ and CD34⁻ fractions of an HIV-infected sample were separated using the CD34 microbead method. Total DNA was isolated and analysed by PCR. HIV gag DNA was detected in both the CD34⁺ and CD34⁻ fractions. A PCR sample using the HIV molecular plasmid pNL4-3 was used as a control. (C) Percentage of HIV p24⁺ cells in HIV⁺ OP9-DL1 coculture samples tested weekly by intracellular staining and flow cytometry. (D) Coculture supernatants of HIV-infected samples were tested for HIV p24 concentrations by ELISA at weeks 2–5. (E) Correlation analysis between the frequencies of HIV p24⁺ cells and HIV p24 concentrations. The weeks 2–5 data in F and G are included ($n = 48$). Spearman's correlation coefficients are shown. (F) p24⁺CD34⁻CD7⁻ cell counts. (G) p24⁺CD34⁻CD7⁻ cell counts. Comparisons were performed using the Wilcoxon matched-pairs signed rank test.

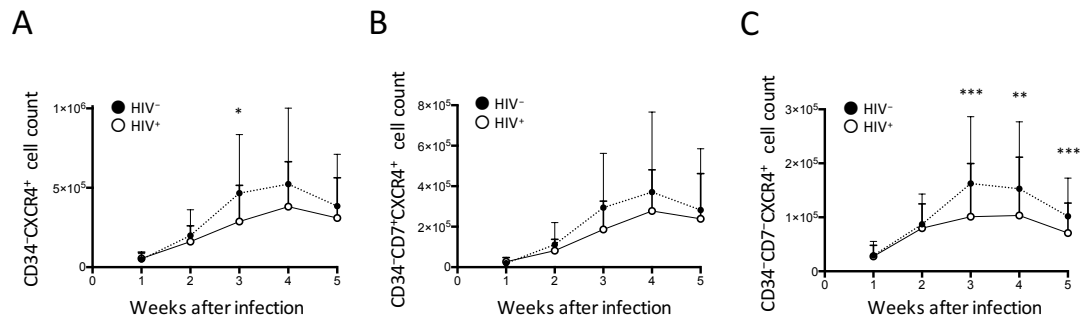


Extended Data Figure 2. Pre-exposure of primary cord-derived CD34⁺ cells to HIV-1 affected the dynamics of OP9-DL1 cocultured cells. (A) Whole cell counts were compared between HIV⁺ and HIV⁻ cocultures. (B) Representative plots for samples displaying reduced frequencies of CD4⁺CD8⁺ cells 3–5 weeks after HIV pre-exposure of primary CD34⁺ cells and coculture. The plots were selected from week 4 samples. (C–D) Cell counts were compared between HIV⁺ and HIV⁻ cocultures for (C) CD4⁺CD8⁺ and (D) CD4⁺CD8⁺CXCR4⁺ cells. Statistical analyses were performed using the Wilcoxon matched-pairs signed rank test. **: $P < 0.01$, ***: $P < 0.001$.



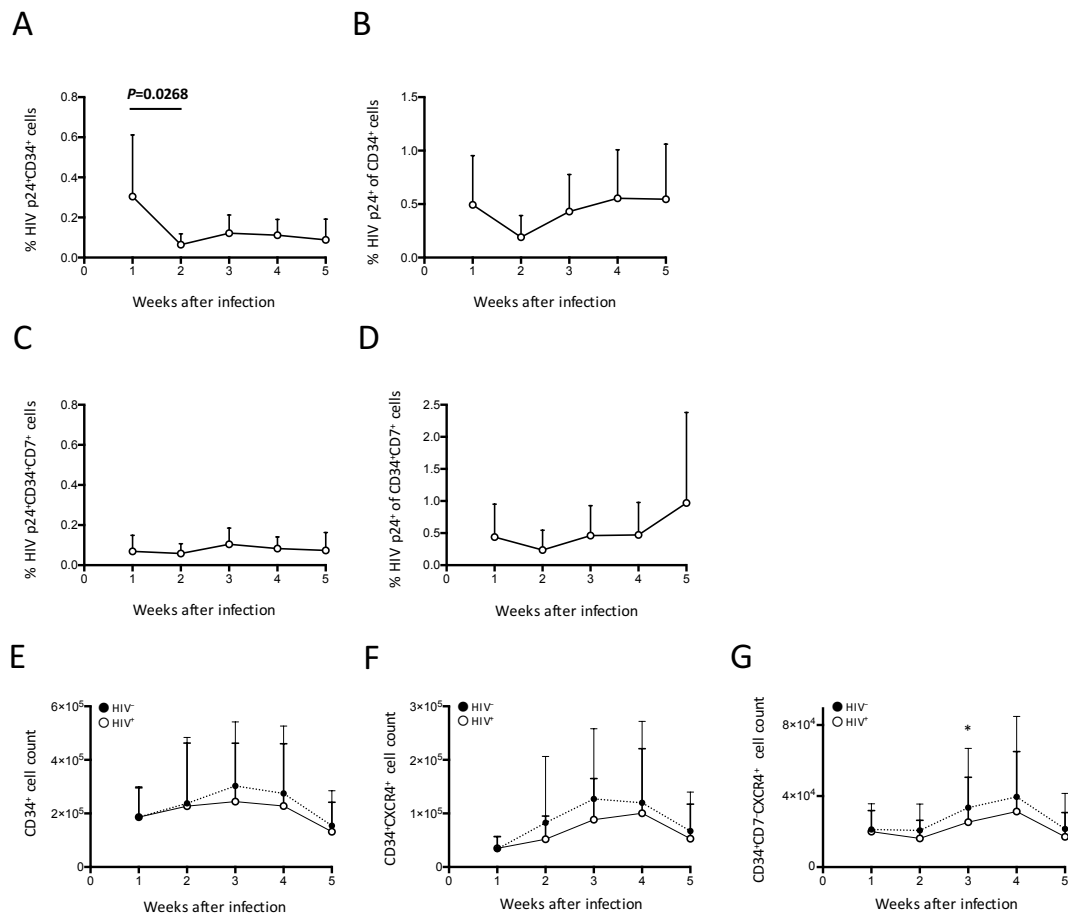
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611 **Extended Data Figure 3.** Gating strategies and analysis of CD34⁺CD4⁺ cells. (A)
612 Flow cytometry plots showing the gates for lymphoid cells (left), live cells (middle),
613 and singlet cells (right). (B) Flow cytometry plots showing the phenotype analysis of
614 CD34⁺CD4⁺ cells in an OP9-DL1 coculture. Representative plots are shown. Cord-
615 derived CD34⁺ cells were cocultured with OP9-DL1 cells for 4 weeks. Cells were
616 collected and analysed by flow cytometry. CD34⁺ cells were gated by CD7 and CD4
617 expression. CD7⁺CD4⁺ and CD7⁻CD4⁺ cells were further gated by CD8 and CD3
618 expression. The analysis revealed that CD7⁺CD4⁺ cells contained different subsets
619 including CD3⁻CD4⁺CD8⁺ and CD3⁺CD4⁺CD8⁻ cells, whereas CD7⁻CD4⁺ cells were
620 mostly CD3⁺CD4⁺CD8⁻ cells. These results indicate that CD4/CD8 double-positive
621 thymocytes, CD7⁺ CD4 T cells and CD7⁻ CD4 T cells were generated via coculture of
622 cord-derived CD34⁺ and OP9-DL1 cells. (C) CD34⁺CD7⁺CD3⁺CD4⁺CD8⁻ cell counts.



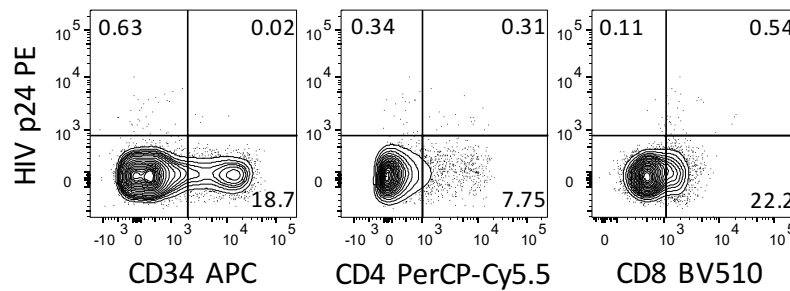
Extended Data Figure 4. Phenotypes of CD34⁺ cells in the OP9-DL1 cocultures were analysed weekly for 5 weeks after HIV-1 infection. Cell counts were compared with those in uninfected samples. (A) CD34⁺CXCR4⁺ cell counts. (B) CD34⁺CD7⁺CXCR4⁺ cell counts. (C) CD34⁺CD7⁻CXCR4⁺ cell counts. Comparisons were performed using the Wilcoxon matched-pairs signed rank test. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

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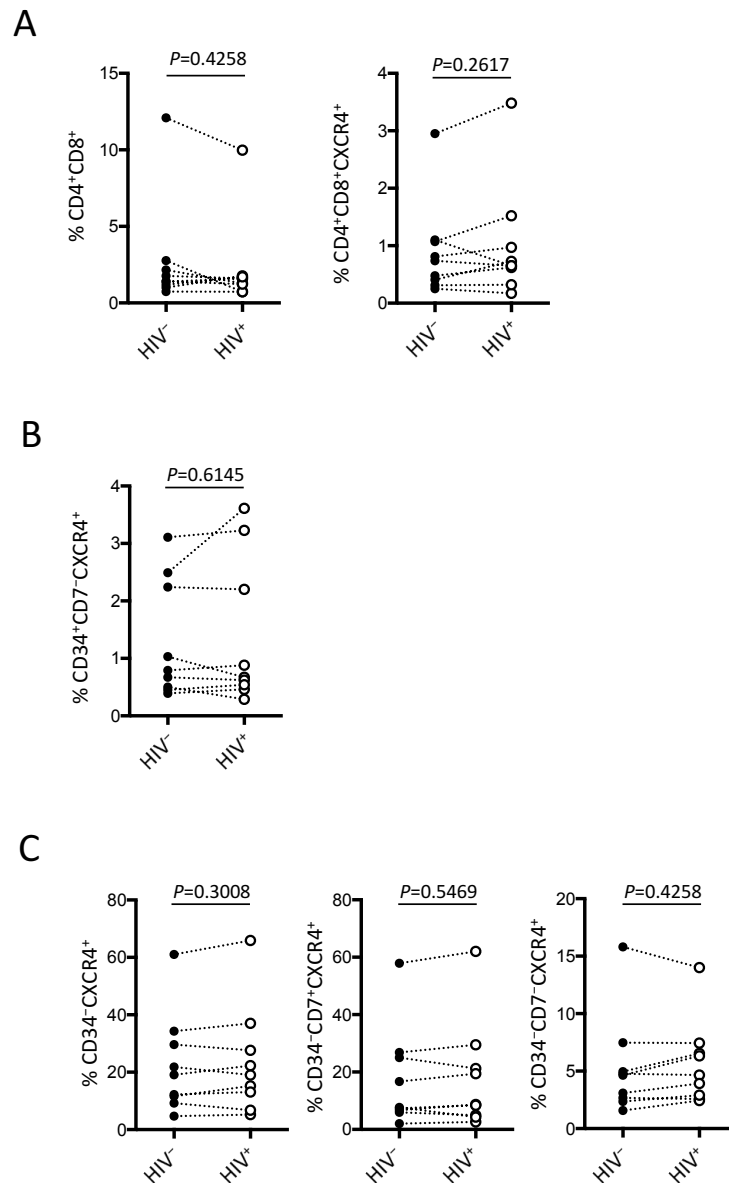
631

632 **Extended Data Figure 5.** (A–D) HIV p24⁺ subset dynamics were analysed. (A)
633 Percent p24⁺CD34⁺ cells. (B) Percent p24⁺ cells among CD34⁺ cells. (C) Percent
634 p24⁺CD34⁺CD7⁺ cells. (D) Percent p24⁺ cells among CD34⁺CD7⁺ cells. (E–G)
635 CD34⁺ subset dynamics were analysed and compared between HIV⁺ and HIV⁻
636 samples. (E) CD34⁺ cell counts. (F) CD34⁺CXCR4⁺ cell counts. (G)
637 CD34⁺CD7⁺CXCR4⁺ cell counts.



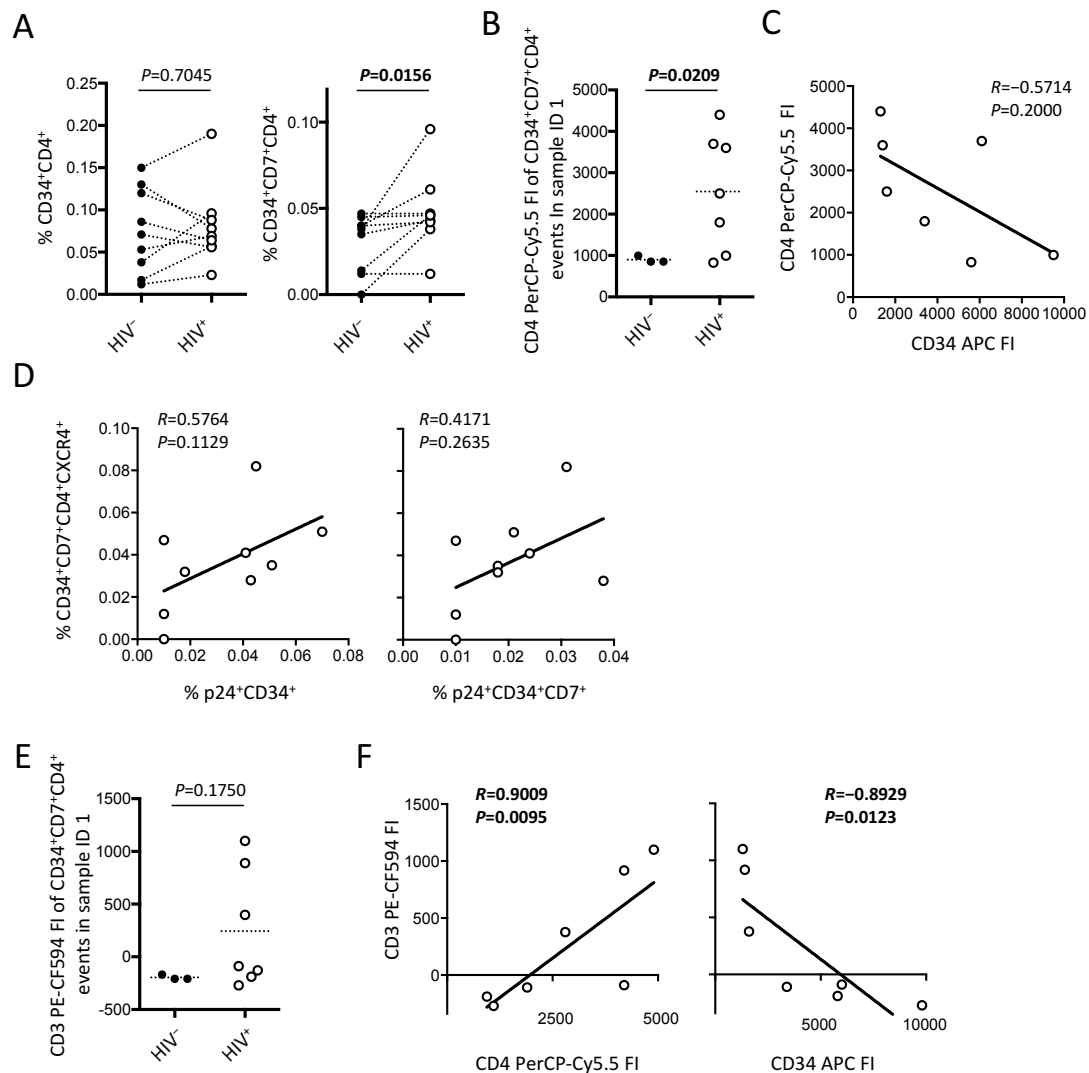
638

639 **Extended Data Figure 6.** Experiment 2 (short coculture, n = 9) was performed to
640 further analyse the dynamics of CD34⁺ cells in the presence of HIV-1. Representative
641 plots showing HIV p24⁺ cells 1 week after infection. The majority of p24⁺ cells were
642 CD34⁻ (left). HIV replication may be causing CD4 downregulation (middle).
643 Frequencies of CD8⁺ cells in p24⁺ cells were variable among the samples (right).

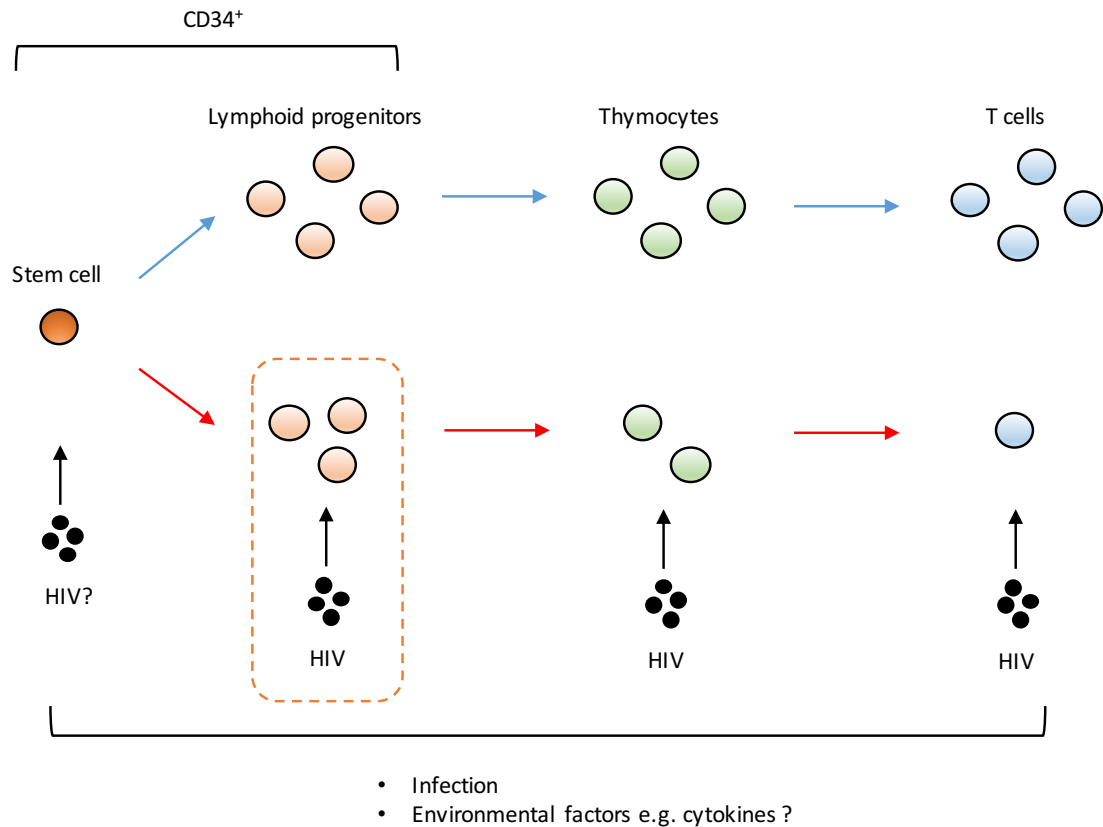


644

645 **Extended Data Figure 7.** The frequencies of subsets were compared between HIV-
646 infected and uninfected samples 1 week after infection. (A) $\text{CD4}^+\text{CD8}^+$ cells. (B)
647 $\text{CD4}^+\text{CD8}^+\text{CXCR4}^+$ cells. (C) $\text{CD34}^-\text{CXCR4}^+$ (left), $\text{CD34}^-\text{CD7}^+\text{CXCR4}^+$ (middle)
648 and $\text{CD34}^-\text{CD7}^-\text{CXCR4}^+$ (right) cells. Comparisons were performed using the
649 Wilcoxon matched-pairs signed rank test.



Extended Data Figure 8. CD34⁺CD7⁺CD4⁺ cells were further analysed. (A) Comparison of CD34⁺CD4⁺ (left) and CD34⁺CD7⁺CD4⁺ (right) frequencies between HIV⁺ and HIV⁻ samples. (B) Fluorescence intensities (FIs) of CD34⁺CD7⁺CD4⁺ cells were compared between HIV⁺ and HIV⁻ samples using the Mann-Whitney test. (C) Correlation between the CD4 and CD34 FI of CD34⁺CD7⁺CD4⁺ cells in the HIV⁺ sample of ID 1 were analysed ($n = 3$ for HIV⁻; $n = 7$ for HIV⁺). (D) CD34⁺CD7⁺CD4⁺CXCR4⁺ cells were tested for correlations with the frequencies of p24⁺CD34⁺ (left) and p24⁺CD34⁺CD7⁺ (right) cells. (E) Comparison of CD3 FIs between HIV-infected and uninfected samples. (F) Correlation analysis between CD3 FIs and CD4 (left) or CD34 (right) FIs. Comparisons were made using the Wilcoxon matched-pairs signed rank test unless otherwise noted. Spearman's correlation coefficients were calculated for correlation analyses.



663

664 **Extended Data Figure 9.** A schematic representation of factors potentially
 665 influencing the decrement of CD4⁺ T cells. Thymocytes and T cells are highly
 666 susceptible to HIV infection. However, the decrease of CD34⁺ lymphoid progenitor
 667 counts occurs early and precedes the decrease of thymocyte and T-cell counts.
 668 Although the mechanisms for the decrease of CD34⁺ lymphoid progenitors are
 669 unclear, this decline may limit the production of thymocytes and T cells.

670

Subset	Cell count in infected samples (mean \pm SD, n = 4)	Percentage in the mean whole-cell count
Whole	390,000 \pm 155,000	100
p24 ⁺	547 \pm 258	0.140
p24 ⁺ CD34 ⁻ CD7 ⁺	326 \pm 242	0.084
p24 ⁺ CD34 ⁻ CD7 ⁻	124 \pm 56	0.032
p24 ⁺ CD34 ⁺	97 \pm 42	0.025
p24 ⁺ CD34 ⁺ CD7 ⁺	80 \pm 31	0.021

671

672 **Extended Data Table 1.** Mean cell counts (n = 4) in different HIV p24⁺ subsets of
673 OP9-DL1 cocultured cells. The whole-cell count data were obtained using four of
674 nine samples from experiment 2 (short coculture), in which cord-derived CD34⁺ cells
675 were first cocultured with OP9-DL1 cells for 4–6 weeks, infected with HIV-1_{NL4-3},
676 cocultured again for 1 week and analyzed by flow cytometry. The percentages in the
677 mean whole-cell count are also shown, and they are comparable to those presented in
678 Fig. 4C. SD, standard deviation.

Subset	Cell count (mean \pm SD, n = 4)		% $\frac{(Infected - Uninfected)}{Uninfected}$
	<i>Uninfected</i>	<i>Infected</i>	
Whole	400,000 \pm 169,000	390,000 \pm 155,000	-2.5
CD4 ⁺ CD8 ⁺	5,790 \pm 3,510	4,920 \pm 2,360	-15.0
CD4 ⁺ CD8 ⁺ CXCR4 ⁺	3,290 \pm 1,760	3,050 \pm 1,840	-7.3
CD34 ⁺	32,800 \pm 18,200	32,100 \pm 21,400	-2.3
CD34 ⁺ CD7 ⁺	16,700 \pm 9,500	16,100 \pm 12,100	-3.6
CD34 ⁺ CXCR4 ⁺	18,300 \pm 11,000	18,300 \pm 14,700	-0.1
CD34 ⁺ CD7 ⁺ CXCR4 ⁺	12,100 \pm 7,800	11,900 \pm 11,100	-2.0
CD34 ⁺ CD7 ⁻ CXCR4 ⁺	6,150 \pm 3,870	6,380 \pm 3,990	+3.7
CD34 ⁺ CD7 ⁺ CD4 ⁺	129 \pm 42	178 \pm 74	+38.0
CD34 ⁻ CXCR4 ⁺	148,000 \pm 164,000	157,000 \pm 167,000	+5.8
CD34 ⁻ CD7 ⁺ CXCR4 ⁺	131,000 \pm 161,000	139,000 \pm 162,000	+5.6
CD34 ⁻ CD7 ⁻ CXCR4 ⁺	16,900 \pm 7,500	18,300 \pm 8,000	+7.8

680

681 **Extended Data Table 2.** Mean cell counts (n = 4) in different subsets of OP9-DL1–
682 cocultured cells. The whole-cell count data were obtained using four of nine samples
683 from experiment 2 (short coculture), in which cord-derived CD34⁺ cells were first
684 cocultured with OP9-DL1 for 4–6 weeks, infected with HIV-1_{NL4-3}, cocultured again
685 for 1 week and analyzed by flow cytometry. Cell counts in HIV-infected samples
686 were compared with those in uninfected control samples. Percent changes in the
687 subset cell counts following HIV infection were calculated, as shown in the right
688 column. Percent changes of >10% are shown in bold. SD, standard deviation.