HIV persistence alters CXCR4 expression patterns in OP9-DL1-mediated differentiation of in vitro infected human cord blood CD34^{+} cells

Tetsuo Tsukamoto [1, 2, 3], Seiji Okada [2]

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

[2] Center for AIDS Research, Kumamoto University, Kumamoto, Japan

[3] Department of Immunology, Kindai University Faculty of Medicine, Osaka, Japan

Author mailing address: Department of Immunology, Kindai University Faculty of Medicine, 377-2 Onohigashi, Osakasayama-shi, 589-8511 Osaka, Japan

Telephone: +81 72 366 0221

Fax: +81 72 367 7660

Email: ttsukamoto@med.kindai.ac.jp

Abstract

CCR5-tropic strains of HIV-1 infect and deplete memory $CD4^+$ T cells, and therefore are considered critical in development of AIDS. In contrast, the impact of CXCR4-tropic HIV-1 strains on the disease is not well understood. CXCR4 is known for its role in the hematopoiesis in the bone marrow and T cell differentiation in the thymus, and therefore its expression on hematopoietic progenitors/precursors is considered essential. Here the authors utilized the OP9-DL1 coculture system that supports in vitro differentiation of human $CD34^+$ hematopoietic stem/progenitor cells to T-lineage cells. Using the system, the present study established an in vitro model to analyze the differentiation process of human $CD34^+$ cells in the presence of HIV-1. HIV-infected cocultures showed sustained HIV replication for five weeks and impaired cell growth. Moreover, HIV-infected samples showed a partial loss of $CD34^+CD7^{hi}CXCR4^{hi}$ cells compared to uninfected samples during the coculture. These results highlight the possible role of CXCR4-tropic HIV-1 strains in the AIDS pathogenesis by disrupting the $CXCR4^+$ cell pools in hematopoiesis and T cell differentiation.

Keywords

human immunodeficiency virus (HIV), acquired immunodeficiency syndrome (AIDS), hematopoietic stem/progenitor cells, C-X-C chemokine receptor type 4 (CXCR4)

Introduction

Hematopoiesis/lymphopoesis environments in the bone marrow and thymus can be mimicked in vitro by using an OP9-DL1 cell line. The OP9-DL1 cell lines were derived from the OP9 mouse stromal cell line by transduction with a notch ligand called delta-like 1 (DL1) (Nakano et al., 1994; Schmitt et al., 2004). The OP9-DL1's functions to support thymopoiesis in vitro were first demonstrated in coculture with mouse cells (Nakano et al., 1994). The cell line is also known to support differentiation of human $CD34^+$ hematopoietic stem/progenitor cells (HSPC) to thymocytes and T cells (De Smedt et al., 2004). There is evidence for production of stromal derived factor-1 (SDF-1, also known as CXCL12), a ligand for CXCR4, in OP9-DL1 (Janas et al., 2010). The OP9-DL1 coculture system is thus a powerful tool to investigate on the events associated with T cell development.

CXCR4 is a co-receptor of HIV-1 (Feng et al., 1996). However, the control of CCR5-tropic strains of HIV-1 is usually considered to be a better correlate of good clinical outcomes (Grivel and Margolis, 1999). This is due to the fact that memory $CD4^+$ T cells express higher levels of CCR5 and are susceptible to CCR5-tropic HIV infection and the following depletion (Schnittman et al., 1990). It might be natural to think that CCR5 is more closely involved in the immunopathogenesis of the HIV disease (Okoye and Picker, 2013).

On the other hand, CXCR4 interacts with SDF-1 and allows CXCR4-expressing cells to home to the loci where SDF-1 is highly expressed (Kucia et al., 2004). For example, the roles of SDF-1 and CXCR4 are essential in human stem cell homing and repopulation of the host with differentiated hematopoietic cells (Kucia et al., 2005; Lapidot and Kollet, 2002). SDF-1 is also produced by thymic epithelial cells and plays

an important role in migration of immature progenitors in the thymus (Plotkin et al., 2003).

It will thus be important to better understand the influence of CXCR4-tropic HIV-1 infection upon T cell development in the thymus and other hematopoiesis events. Bone marrow abnormalities such as dysplasia and abnormal hematopoietic cell development are frequently observed in HIV infected individuals (Tripathi et al., 2005). Thymic dysfunction occurs during HIV disease and is associated with rapid progression in infants with prenatal HIV infection (Ye et al., 2004). There was also an attempt to see the impact of CXCR4-tropic simian-human immunodeficiency virus (SHIV) infection on production of T-lineage cells in the thymus of newborn rhesus macaques (Suzuki et al., 2005a). However, it is not realistic to closely follow the in vivo bone marrow/thymus events in HIV-infected individuals. Instead, humanized mouse models can be beneficial for the purposes (Dudek and Allen, 2013; Marsden et al., 2012). Besides this, to our knowledge there hasn't been an ex-vivo model to assess the differentiation of hematopoietic progenitor/precursor cells to thymocytes in the presence of HIV-1. The OP9-DL1 system was chosen here as a candidate for this.

HIV-1 has recently been shown to infect human $CD34^+$ hematopoietic progenitor cells (Carter et al., 2011; Carter et al., 2010). In addition, there is a report on impaired hematopoiesis by HIV-infected hematopoietic progenitor cells using a humanized BLT mouse model (Nixon et al., 2013). However, it is still unclear what happens to the infected $CD34^+$ progenitor cells and their derivatives in the course of the disease (Akkina, 2013). The present study was aimed at pursuing the in vitro fate of HIV-infected $CD34^+$ cells using the OP9-DL1 coculture system.

Results

Infection of primary human umbilical cord blood (UCB) CD34$^+$ cells with CXCR4-tropic HIV-1$_{NL4-3}$

Two separate in vitro experiments were performed in the study (Table 1). The data for the first experiment (infection-coculture) are shown in Figures 1 to 4. To follow the in vitro fate of HIV-infected CD34$^+$ cells for several weeks, the present study attempted HIV infection of primary human UCB CD34$^+$ cells. Those cells were confirmed to be partially CD4$^+$ and CXCR4$^+$ (Fig 1B). Cells were exposed to a high dose of HIV-1$_{NL4-3}$. Following spinoculation and overnight incubation, the cells were cocultured with OP9-DL1 (Fig 1A). After a week of coculture, the cells were tested for HIV-1 p24 expression (Fig 1C). Intracellular HIV-1 p24 was detected in both CD34$^+$ and CD34$^-$ cells (Fig 1C and 3A). HIV infection was further confirmed by magnetic bead separation of CD34$^+$ and CD34$^-$ cells followed by detection of HIV-1 *gag* DNA using PCR (Fig 1D). In this way, the HIV-infected cells were confirmed to be ready for a longer period of coculture.

The cellular dynamics of the OP9-DL1 cocultures with HIV-infected UCB CD34$^+$ cells

The influence of HIV infection of CD34$^+$ cells upon post-coculture events was evaluated every week until week 5. HIV-infected cultures showed significantly lower whole cell counts at weeks 3, 4, and 5 post-coculture than uninfected cocultures (Fig 2A). This demonstrates that HIV infection of CD34$^+$ cells resulted in impaired cell growth in the cocultures. The counts of cells with different phenotypes in the cocultures were also measured. The CD34$^+$ cell counts were not significantly different between HIV-infected and uninfected cocultures at weeks 3, 4, and 5 (Fig 2B). On the other

hand, $CD4^+CD8^+$ cell counts in HIV-infected cocultures were lower than in uninfected cocultures at weeks 3 and 4 (Fig 2C and D). Some of those HIV-infected samples showed a dramatic decline in the $CD4^+CD8^+$ percentages on flow cytometric analysis compared to the autologous uninfected samples (Fig 2E).

Persistent HIV-1 infection detected in the OP9-DL1 cocultures with UCB $CD34^+$ cells

The above cocultures of HIV-infected $CD34^+$ cells were maintained for five weeks. All the HIV-infected samples showed persistent HIV-1 p24 expression in 0.1-3.5 % of the total cocultured human cells (Fig 3A). Both the HIV-1 $p24^+$ percentages and the cell counts peaked at week 3 (Fig 3A and B). Viral replication levels were further confirmed by measuring HIV-1 p24 amounts in the coculture supernatants using ELISA (Fig 3C). Correlation was found between intracellular HIV-1 $p24^+$ cell counts and supernatant HIV-1 p24 concentrations (Fig 3D).

Impact of HIV-1 infection upon $CD34^+$ cell dynamics in the OP9-DL1 cocultures

Cells were further evaluated for CXCR4 expression levels. $CXCR4^{hi}$ frequencies in the $CD34^+$ fractions of HIV-infected samples were similar to those in uninfected samples at week 1 post coculture but became lower at week 2 post infection. The tendency lasted until week 5 post infection (Fig 4A left). Interestingly, the opposite tendency was observed in $CD34^-$ fractions (Fig 4A right). To better describe the phenotypes of $CD34^+$ cells, downstream gates were set based on expression of CD7, a marker for lymphoid progenitors (Hoebeke et al., 2007). From week 2 to week 5, $CD7^{hi}$ percentages in $CD34^+$ cells were similar between HIV-infected and uninfected samples (Fig 4B left), while the $CD7^{hi}CXCR4^{hi}$ and $CD7^{lo}CXCR4^{hi}$ frequencies in HIV-infected

samples during the week 2-5 period tended to be lower than those in uninfected samples (Fig. 4B middle and right).

HIV-1$_{NL4-3}$ infection of HSPC-derived cells after several weeks' coculture with OP9-DL1

To better describe the effect of HIV infection on the phenotypes of CD34^{+} cells, the second experiment was performed (coculture-infection-coculture, Fig 5A). These results are shown in Figures 5 to 7. Briefly, the OP9-DL1 coculture with UCB CD34^{+} cells, without HIV infection, produced a mixture of cells with different phenotypes, *f.g.* CD4^{+}/CD8^{+} cells, in 4-6 weeks. Cells were then harvested and infected with HIV-1$_{NL4-3}$. The infected cells were cocultured again with a new OP9-DL1 monolayer and incubated for another week. Cells were then collected, intracellularly stained with anti-HIV-1 p24, and analyzed by flow cytometry. HIV replication was detected in all the HIV-infected samples (Fig. 5B, C). Majority of HIV-1 p24^{+} cells were CD8^{+} and CD34^{-} (Fig 5B). The CD8^{+}p24^{+} cells were partly CD4^{lo}, possibly due to CD4 down regulation in HIV infected cells (Fig 5B). The twelve samples tested here showed the average HIV p24^{+} frequency of 0.77 % (Fig 5C). The whole cell counts were not significantly different between HIV-infected and uninfected samples (data not shown).

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

The phenotypes of the cells in the second experiment were analyzed a week after infection. The CD34^{+} frequencies were not significantly different between HIV-infected and uninfected cultures (Fig. 6B). On the other hand, the CXCR4^{hi} frequencies in HIV-infected samples were significantly reduced, when compared to autologous

uninfected samples, in the $CD34^+$ fractions (Fig 6 A and C left). This was not observed in the $CD34^-$ or $CD4^+CD8^+$ fractions (Fig. 6C middle and right).

The $CD34^+$ cells were then gated down to the $CD7^{hi}$ and $CD7^{lo}$ subfractions, and analyzed (Fig 7). In the $CD34^+$ fractions, the HIV-infected samples showed significantly lower $CD7^{hi}$ and $CD7^{hi}CXCR4^{hi}$ percentages than the uninfected samples (Fig 7B left and middle), although the data didn't show a significant difference in $CD7^{lo}CXCR4^{hi}$ percentages (Fig 7B right). These results show that the loss of $CD34^+CXCR4^{hi}$ cells, observed a week after HIV infection of OP9-DL1-cocultured cells (Fig 6C left), reflects the loss of $CD34^+CD7^{hi}CXCR4^{hi}$ cells, despite that the majority of HIV-infected cells were $CD34^-$ (Fig 5B).

The results for the first and second experiments are summarized in Table 1.

Discussion

The impact of HIV infection on the bone marrow/thymus functions has been of great interest. The T-cell restoration by antiretroviral drug treatment appears to be critical in control of disease in HIV-infected patients. However, it has been unclear how HIV impacts the lymphopoietic/thymopoietic process. For this purpose, an in vitro model to mimic the process may be helpful. The present study demonstrates the way to utilize the OP9-DL1 coculture system that enables in vitro follow up of the early lymphopoietic events such as lymphoid progenitor cell generation, that normally occurs in the bone marrow, and $CD4^+$ thymocyte differentiation in the thymus.

Human HSPC were not considered a major virus reservoir (Durand et al., 2012; von Laer et al., 1990). However, HIV-1 was shown to infect those cells (Carter et al., 2010). Although an article reported negative results on detection of HIV-1 DNA in $CD34^+$ hematopoietic progenitor cells (Josefsson et al., 2012), another study showed that $CD133^+$ hematopoietic stem cells could harbor HIV-1 DNA despite long-term viral control (McNamara et al., 2013). It is still debatable whether HSPC are an unignorable viral reservoir (Pace and O'Doherty, 2013).

Another concern is indirect modification of HSPC and hematopoiesis dynamics through HIV infection and depletion of $CD4^+$ cells. HIV disease is known to accompany bone marrow abnormalities (Tripathi et al., 2005). Some patients fail to show $CD4^+$ T-cell recovery even after effective antiretroviral therapy and are called immunological nonresponders. Such immunological nonresponsiveness can be associated with immune activation and/or bone marrow impairment (Hunt et al., 2003; Isgro et al., 2008).

CXCR4 and its ligand SDF-1 have a variety of roles in biological events such as embryogenesis, angiogenesis, hematopoiesis, and lymphopoiesis. CXCR4 is abundantly expressed in mouse embryos and involved in neuronal, vascular, hematopoietic, cardiac, craniofacial, and gastric development (McGrath et al., 1999). The role of a CXCR4 homologue in zebrafish embryosis has been reported (Raz and Mahabaleshwar, 2009). Development of renal vasculature in mice requires the functions of SDF-1 and CXCR4 (Takabatake et al., 2009). CXCR4 acts as a co-stimulator during thymic β-selection (Trampont et al., 2010).

The events observed in the coculture of OP9-DL1 and human $CD34^+$ cells are likely to involve interaction between SDF-1 and CXCR4, since mouse SDF-1 expressed by OP9-DL1 has high identity of amino acid sequence to human SDF-1 (Shirozu et al., 1995). The observation is in accordance with the previous reports showing that SDF-1/CXCR4 pair is crucially involved in homing and repopulation of HSPCs in the specific BM niches (Moll and Ransohoff, 2010) and also in the whole T-cell developmental process in the thymus (Petrie, 2003; Plotkin et al., 2003). Our findings might be worth testing in vivo.

It has been reported that HSPCs and thymocytes express CXCR4 but their CCR5 expression is limited (Nixon et al., 2013; Taylor et al., 2001). Another report says that HIV utilizes CXCR4 when it infects multipotent progenitor cells (Carter et al., 2011). Although CCR5-tropic HIV-1 strains play an essential role in depletion of CCR5-tropic memory $CD4^+$ T cells leading to AIDS (Schnittman et al., 1990), CXCR4-tropic HIV strains might also contribute to the pathogenesis by interfering with hematopoiesis and/or lymphopoiesis (Akkina, 2013; Bordoni et al., 2015; Ho Tsong Fang et al., 2008). Despite the increasing evidence and interests, it is yet to be unveiled whether or how CXCR4-tropic HIV-1 strains impair these processes.

In the present study, the loss of $CD34^{+}CXCR4^{hi}$ cells obverved in the OP9-DL1 cocultures was associated with HIV infection (Table 1). However, only around 1 % of the whole cells were showing active HIV-1 p24 production (Fig 2A and 5B), and the majority of those cells showing HIV replication were $CD4^{+}/CD8^{+}$ cells (Fig 5C). Although the underlying mechanisms are still unknown, possible reasons may be as follows: (1) HIV-infected $CD34^{+}$ cells may be killed by the cytopathic effect of the virus. (2) $CD34^{+}$ cells may have an innate mechanism to sense HIV DNA or RNA, causing a cell death. (3) HIV infection of the part of the cocultured cells may accelerate $CD34^{+}$ cell differentiation, either directly or indirectly. The third hypothesis might be of particular interest, because the HIV-infected samples in the second experiment (coculture-infection-coculture) tended to have larger $CD4^{+}CD8^{+}$ fractions than uninfected samples (Fig 6C), and the possible increase in $CD4^{+}CD8^{+}$ frequencies was accompanied with reduction of $CD34CD7^{hi}CXCR4^{hi}$ fractions (Fig 7B). Nonetheless, regardless of the right answer to the question, our results shed lights on the impact of HIV-1 infection on early events in hematopoiesis, and may even lead to better understanding of the long-term outcomes of HIV-1 persistence in an individual.

The findings in this article will also highlight anti-HIV treatments such as a gene therapy of $CD34^{+}$ HSPC followed by transplantation, because in this way the whole hematopoietic events in the host can be placed under protection against HIV infection even in the absence of effective immune response (Savkovic et al., 2014). The problem is that, while CCR5 is considered a desirable target for knockout or knockdown to prevent infection of CCR5-tropic HIV strains, CXCR4 should be expressed normally in HSPC because of its essential function in our life events (Liu et al., 2014). Therefore, instead of modulation of CXCR4 expression, anti-HIV modalities targeting an HIV gene or component may be promising for protection of hematopoietic cells including

T-lineage cells from CXCR4-tropic HIV infection (Suzuki et al., 2015; Suzuki et al., 2013).

Materials and Methods

Virus stocks. Stocks of HIV-1_{NL4-3} were produced by transfection of the 293T cell line with the molecular clone DNA pNL4-3 (Adachi et al., 1986). After transfection, the culture supernatant was collected and virus titers were determined using an HIV p24 Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (ZeptoMetrix, US).

Cells. Umbilical cord blood (UCB) samples were collected at Fukuda Hospital, Kumamoto following informed consent. Cord blood mononuclear cells were isolated using Pancoll (PAN-Biotech GmbH, Germany) and by centrifugation at 800 rpm for 20 minutes. $CD34^+$ cells were labeled and selected using human CD34 micro beads and LS columns (Miltenyi Biotec, Japan). The purity was constantly more than 92 %. An OP9-DL1 cell line, generated by transduction of the OP9 cell line (ATCC CRL-2749) with human Delta-like 1, was kindly provided by Prof. Seiji Okada (Center for AIDS Research, Kumamoto University, Japan). The cell line was maintained in α-MEM media supplemented with 10 % heat inactivated fetal bovine serum (GE Healthcare, Japan).

Antibodies. The following antibodies were used for the flow cytometric analysis of primary UCB $CD34^+$ cells and human cells cocultured with OP9-DL1. 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 (Tokyo, Japan). 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 (Tokyo, Japan). 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 (Tokyo, Japan). 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 the previously published protocol with modifications (Holmes and Zuniga-Pflucker, 2009). Briefly, 2×10^5 HIV-infected or uninfected UCB $CD34^+$ cells were seeded in a 6-well plate containing a monolayer of OP9-DL1. The coculture was maintained for 5 weeks in the α-MEM media supplemented with 20 % heat inactivated FBS, 5 ng/mL recombinant human FMS-like tyrosine kinase 3 ligand (Flt-3L), and 5ng/mL recombinant human interleukin 7 (IL-7). Cells were passaged every week by vigorous pipetting and filtering through a 70-μm membrane, and cocultured again with a fresh monolayer of OP9-DL1. Surface and intracellular antigen expression was analyzed every week on passage with FACS LSR II (BD Biosciences).

HIV infection. For infection of primary UCB $CD34^+$ cells with HIV-1_{NL4-3}, a 48-well plate was coated with RetroNectin (Takara Bio Inc., Tokyo, Japan) at a concentration of 10 μg/mL (Tsukamoto and Okada, 2017). The plate was incubated overnight and washed with PBS prior to use. $CD34^+$ cells were re-suspended in the OP9-DL1 media, seeded at 2×10^5 per well in the coated plate and infected with 200 ng (p24) of HIV-1_{NL4-3} and using spinoculation at 1,200 g, 34 °C for 30 minutes. Cells were further cultured overnight and cocultured in a 6-well plate with a fresh monolayer of OP9-DL1. For HIV infection of OP9-cocultured human cells, the cell concentration was modified to 5×10^5 per well.

PCR analysis of the HIV DNA. Cellular DNA was extracted using 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 previously (Suzuki et al., 2005b).

Statistical analysis. Statistical analysis was performed using the Graphpad Prism software version 6.0. Statistical significance was defined as $P < 0.05$. Comparison between HIV-infected and uninfected samples was done by paired *t*-tests. Spearman's rank correlation coefficients were calculated for correlation analysis results.

Acknowledgements

The authors cordially thank Drs. Kazuo Matsui and Shoichi Kawakami of Fukuda Hospital, Kumamoto, Japan for their help on cord blood sampling. This work got partial support by grants from the following organizations: Japan Agency for Medical Research and Development (Research program on HIV/AIDS, No. 16fk0410108h0001); Ministry of Education, Culture, Sports, Science and Technology, Japan (Grants-in-Aid for Science Research, No. 25114711); National Health and Medical Research Council, Australia (Project grant). This work was initiated as part of the collaborative project between Prof. Anthony D. Kelleher (Kirby Institute for infection and immunity for society, UNSW Australia) and Prof. Seiji Okada (Center for AIDS Research, Kumamoto University, Japan).

References

Adachi, A., Gendelman, H.E., Koenig, S., Folks, T., Willey, R., Rabson, A., Martin, M.A., 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. Journal of virology 59, 284-291.

Akkina, R., 2013. New insights into HIV impact on hematopoiesis. Blood 122, 2144-2146.

Bordoni, V., Casetti, R., Viola, D., Abbate, I., Rozera, G., Sacchi, A., Cimini, E., Tumino, N., Agrati, C., Orchi, N., Pinnetti, C., Ammassari, A., Martini, F., 2015. Early ART in primary HIV infection may also preserve lymphopoiesis capability in circulating haematopoietic progenitor cells: a case report. The Journal of antimicrobial chemotherapy.

Carter, C.C., McNamara, L.A., Onafuwa-Nuga, A., Shackleton, M., Riddell, J.t., Bixby, D., Savona, M.R., Morrison, S.J., Collins, K.L., 2011. HIV-1 utilizes the CXCR4 chemokine receptor to infect multipotent hematopoietic stem and progenitor cells. Cell host & microbe 9, 223-234.

Carter, C.C., Onafuwa-Nuga, A., McNamara, L.A., Riddell, J.t., Bixby, D., Savona, M.R., Collins, K.L., 2010. HIV-1 infects multipotent progenitor cells causing cell death and establishing latent cellular reservoirs. Nature medicine 16, 446-451.

De Smedt, M., Hoebeke, I., Plum, J., 2004. Human bone marrow CD34+ progenitor cells mature to T cells on OP9-DL1 stromal cell line without thymus microenvironment. Blood cells, molecules & diseases 33, 227-232.

Dudek, T.E., Allen, T.M., 2013. HIV-specific CD8(+) T-cell immunity in humanized bone marrow-liver-thymus mice. The Journal of infectious diseases 208 Suppl 2, S150-154.

Durand, C.M., Ghiaur, G., Siliciano, J.D., Rabi, S.A., Eisele, E.E., Salgado, M., Shan, L., Lai, J.F., Zhang, H., Margolick, J., Jones, R.J., Gallant, J.E., Ambinder, R.F., Siliciano, R.F., 2012. HIV-1 DNA is detected in bone marrow populations containing CD4+ T cells but is not found in purified CD34+ hematopoietic progenitor cells in most patients on antiretroviral therapy. The Journal of infectious diseases 205, 1014-1018.

Feng, Y., Broder, C.C., Kennedy, P.E., Berger, E.A., 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. Science (New York, N.Y.) 272, 872-877.

Grivel, J.C., Margolis, L.B., 1999. CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nature medicine 5, 344-346.

Ho Tsong Fang, R., Colantonio, A.D., Uittenbogaart, C.H., 2008. The role of the thymus in HIV infection: a 10 year perspective. AIDS (London, England) 22, 171-184.

Hoebeke, I., De Smedt, M., Stolz, F., Pike-Overzet, K., Staal, F.J., Plum, J., Leclercq, G., 2007. T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34(+)CD38(-)CD7(+) common lymphoid progenitors expressing lymphoid-specific genes. Leukemia 21, 311-319.

Holmes, R., Zuniga-Pflucker, J.C., 2009. The OP9-DL1 system: generation of T-lymphocytes from embryonic or hematopoietic stem cells in vitro. Cold Spring Harbor protocols 2009, pdb.prot5156.

Hunt, P.W., Martin, J.N., Sinclair, E., Bredt, B., Hagos, E., Lampiris, H., Deeks, S.G., 2003. T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. The Journal of infectious diseases 187, 1534-1543.

Isgro, A., Leti, W., De Santis, W., Marziali, M., Esposito, A., Fimiani, C., Luzi, G., Pinti, M., Cossarizza, A., Aiuti, F., Mezzaroma, I., 2008. Altered clonogenic capability and stromal cell function characterize bone marrow of HIV-infected subjects with low CD4+ T cell counts despite viral suppression during HAART. Clin Infect Dis 46, 1902-1910.

Janas, M.L., Varano, G., Gudmundsson, K., Noda, M., Nagasawa, T., Turner, M., 2010. Thymic development beyond beta-selection requires phosphatidylinositol 3-kinase activation by CXCR4. The Journal of experimental medicine 207, 247-261.

Josefsson, L., Eriksson, S., Sinclair, E., Ho, T., Killian, M., Epling, L., Shao, W., Lewis, B., Bacchetti, P., Loeb, L., Custer, J., Poole, L., Hecht, F.M., Palmer, S., 2012. Hematopoietic precursor cells isolated from patients on long-term suppressive HIV therapy did not contain HIV-1 DNA. The Journal of infectious diseases 206, 28-34.

Kucia, M., Jankowski, K., Reca, R., Wysoczynski, M., Bandura, L., Allendorf, D.J., Zhang, J., Ratajczak, J., Ratajczak, M.Z., 2004. CXCR4-SDF-1 signalling, locomotion, chemotaxis and adhesion. Journal of molecular histology 35, 233-245.

Kucia, M., Reca, R., Miekus, K., Wanzeck, J., Wojakowski, W., Janowska-Wieczorek, A., Ratajczak, J., Ratajczak, M.Z., 2005. Trafficking of normal stem cells and metastasis of cancer stem cells involve similar mechanisms: pivotal role of the SDF-1-CXCR4 axis. Stem cells (Dayton, Ohio) 23, 879-894.

Lapidot, T., Kollet, O., 2002. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. Leukemia 16, 1992-2003.

Liu, Y., Zhou, J., Pan, J.A., Mabiala, P., Guo, D., 2014. A novel approach to block HIV-1 coreceptor CXCR4 in non-toxic manner. Molecular biotechnology 56, 890-902.

Marsden, M.D., Kovochich, M., Suree, N., Shimizu, S., Mehta, R., Cortado, R., Bristol, G., An, D.S., Zack, J.A., 2012. HIV latency in the humanized BLT mouse. Journal of virology 86, 339-347.

McGrath, K.E., Koniski, A.D., Maltby, K.M., McGann, J.K., Palis, J., 1999. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Developmental biology 213, 442-456.

McNamara, L.A., Onafuwa-Nuga, A., Sebastian, N.T., Riddell, J.t., Bixby, D., Collins, K.L., 2013. CD133+ hematopoietic progenitor cells harbor HIV genomes in a subset of optimally treated people with long-term viral suppression. The Journal of infectious diseases 207, 1807-1816.

Moll, N.M., Ransohoff, R.M., 2010. CXCL12 and CXCR4 in bone marrow physiology. Expert review of hematology 3, 315-322.

Nakano, T., Kodama, H., Honjo, T., 1994. Generation of lymphohematopoietic cells from embryonic stem cells in culture. Science (New York, N.Y.) 265, 1098-1101.

Nixon, C.C., Vatakis, D.N., Reichelderfer, S.N., Dixit, D., Kim, S.G., Uittenbogaart, C.H., Zack, J.A., 2013. HIV-1 infection of hematopoietic progenitor cells in vivo in humanized mice. Blood 122, 2195-2204.

Okoye, A.A., Picker, L.J., 2013. CD4(+) T-cell depletion in HIV infection: mechanisms of immunological failure. Immunological reviews 254, 54-64.

Pace, M., O'Doherty, U., 2013. Hematopoietic stem cells and HIV infection. The Journal of infectious diseases 207, 1790-1792.

Petrie, H.T., 2003. Cell migration and the control of post-natal T-cell lymphopoiesis in the thymus. Nature reviews. Immunology 3, 859-866.

Plotkin, J., Prockop, S.E., Lepique, A., Petrie, H.T., 2003. Critical role for CXCR4 signaling in progenitor localization and T cell differentiation in the postnatal thymus. Journal of immunology (Baltimore, Md. : 1950) 171, 4521-4527.

Raz, E., Mahabaleshwar, H., 2009. Chemokine signaling in embryonic cell migration: a fisheye view. Development (Cambridge, England) 136, 1223-1229.

Savkovic, B., Nichols, J., Birkett, D., Applegate, T., Ledger, S., Symonds, G., Murray, J.M., 2014. A quantitative comparison of anti-HIV gene therapy delivered to hematopoietic stem cells versus CD4+ T cells. PLoS computational biology 10, e1003681.

Schmitt, T.M., de Pooter, R.F., Gronski, M.A., Cho, S.K., Ohashi, P.S., Zuniga-Pflucker, J.C., 2004. Induction of T cell development and establishment of T cell competence from embryonic stem cells differentiated in vitro. Nature immunology 5, 410-417.

Schnittman, S.M., Lane, H.C., Greenhouse, J., Justement, J.S., Baseler, M., Fauci, A.S., 1990. Preferential infection of CD4+ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. Proceedings of the National Academy of Sciences of the United States of America 87, 6058-6062.

Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., Honjo, T., 1995. Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 28, 495-500.

Suzuki, H., Motohara, M., Miyake, A., Ibuki, K., Fukazawa, Y., Inaba, K., Masuda, K., Minato, N., Kawamoto, H., Hayami, M., Miura, T., 2005a. Intrathymic effect of acute pathogenic SHIV infection on T-lineage cells in newborn macaques. Microbiology and immunology 49, 667-679.

Suzuki, K., Ahlenstiel, C., Marks, K., Kelleher, A.D., 2015. Promoter Targeting RNAs: Unexpected Contributors to the Control of HIV-1 Transcription. Molecular therapy. Nucleic acids 4, e222.

Suzuki, K., Hattori, S., Marks, K., Ahlenstiel, C., Maeda, Y., Ishida, T., Millington, M., Boyd, M., Symonds, G., Cooper, D.A., Okada, S., Kelleher, A.D., 2013. Promoter Targeting shRNA Suppresses HIV-1 Infection In vivo Through Transcriptional Gene Silencing. Molecular therapy. Nucleic acids 2, e137.

Suzuki, K., Shijuuku, T., Fukamachi, T., Zaunders, J., Guillemin, G., Cooper, D., Kelleher, A., 2005b. Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. J RNAi Gene Silencing 1, 66-78.

Takabatake, Y., Sugiyama, T., Kohara, H., Matsusaka, T., Kurihara, H., Koni, P.A., Nagasawa, Y., Hamano, T., Matsui, I., Kawada, N., Imai, E., Nagasawa, T., Rakugi, H., Isaka, Y., 2009. The CXCL12 (SDF-1)/CXCR4 axis is essential for the development of renal vasculature. Journal of the American Society of Nephrology : JASN 20, 1714-1723.

Taylor, J.R., Jr., Kimbrell, K.C., Scoggins, R., Delaney, M., Wu, L., Camerini, D., 2001. Expression and function of chemokine receptors on human thymocytes:

implications for infection by human immunodeficiency virus type 1. Journal of virology 75, 8752-8760.

Trampont, P.C., Tosello-Trampont, A.C., Shen, Y., Duley, A.K., Sutherland, A.E., Bender, T.P., Littman, D.R., Ravichandran, K.S., 2010. CXCR4 acts as a costimulator during thymic beta-selection. Nature immunology 11, 162-170.

Tripathi, A.K., Misra, R., Kalra, P., Gupta, N., Ahmad, R., 2005. Bone marrow abnormalities in HIV disease. The Journal of the Association of Physicians of India 53, 705-710.

Tsukamoto, T., Okada, S., 2017. Use Of RetroNectin In Studies Requiring In Vitro HIV Infection Of Human Hematopoietic Stem/Progenitor Cells. bioRxiv, 142075; doi: https://doi.org/142010.141101/142075.

von Laer, D., Hufert, F.T., Fenner, T.E., Schwander, S., Dietrich, M., Schmitz, H., Kern, P., 1990. CD34+ hematopoietic progenitor cells are not a major reservoir of the human immunodeficiency virus. Blood 76, 1281-1286.

Ye, P., Kirschner, D.E., Kourtis, A.P., 2004. The thymus during HIV disease: role in pathogenesis and in immune recovery. Current HIV research 2, 177-183.

Figure legends

Figure 1. Primary umbilical cord blood (UCB) $CD34^+$ cells are susceptible to HIV infection. (A) Schematic representation of the first experiment (infection-coculture). Primary UCB $CD34^+$ cells were infected with HIV-1_{NL4-3}, and cocultured with OP9-DL1 for 5 weeks. (B) $CD34^+$ cells were gated and tested for expression of CD4 and CXCR4 by flow cytometry. Representative plots are shown. The CD4 signal levels were higher than those of the cells stained with an isotype control antibody. (C) Intracellular HIV p24 expression was tested a week after infection and coculture with OP9-DL1. HIV $p24^+$ cells were found in both $CD34^+$ and $CD34^-$ fractions of HIV-infected samples. Representative plots are shown. (D) $CD34^+$ and $CD34^-$ fractions of an HIV-infected sample were separated by the CD34 microbead method. Total DNA was isolated and analyzed by PCR. HIV *gag* DNA was detected in both $CD34^+$ and $CD34^-$ fractions. A PCR sample using an HIV molecular plasmid pNL4-3 was placed as a control.

Figure 2. HIV infection of primary UCB $CD34^+$ cells affected dynamics of OP9-DL1 cocultured cells (n = 12). Whole cell counts (A), $CD34^+$ cell counts (B), and $CD4^+CD8^+$ cell counts (C) were compared between autologous HIV-infected and uninfected samples using a paired *t*-test. (D) $CD4^+CD8^+$ cell counts obtained at weeks 3 and 4 are compared between HIV-infected and uninfected samples. (E) Representative plots for those samples that showed reduced $CD4^+CD8^+$ frequencies 3-5 weeks after HIV infection of primary $CD34^+$ cells and coculture. The plots were taken from week 4 samples. **: $p < 0.01$, ***: $p < 0.001$.

Figure 3. Coculture of HIV-infected primary UCB $CD34^+$ cells with OP9-DL1 resulted in persistent viral replication for five weeks (n = 12). (A) HIV $p24^+$ frequencies of HIV-infected OP9-DL1 coculture samples tested every week by intracellular staining and flow cytometry. (B) HIV $p24^+$ cell counts were calculated from HIV $p24^+$ percentages and whole cell counts. (C) Coculture supernatants of HIV-infected samples were tested every week for HIV p24 concentrations. (D) Correlation analysis between HIV $p24^+$ frequencies and HIV p24 concentrations. All the data in B and C are included (four time points including weeks 2, 3, 4, and 5 post infection from 12 coculture samples, n = 48 in total).

Figure 4. HIV infection of primary UCB $CD34^+$ cells affected dynamics of $CD34^+$ cells in OP9-DL1 cocultures (n = 14). (A) $CXCR4^{hi}$ frequencies in $CD34^+$ (left) and $CD34^-$ (right) fractions were compared between autologous HIV-infected and mock-infected samples. (B) The frequencies of $CD7^{hi}$ (left), $CD7^{hi}CXCR4^{hi}$ (middle), and $CD7^{lo}CXCR4^{hi}$ (right) sub fractions of $CD34^+$ cells were further analyzed. (C) Representative plots showing the reduced $CD7^{hi}CXCR4^{hi}$ frequency in $CD34^+$ cells at week 3 post HIV infection. A paired *t*-test was used for comparison. *: $p < 0.05$, **: $p < 0.01$.

Figure 5. (A) Schematic representation of the second experiment (coculture-infection-coculture). Primary UCB $CD34^+$ cells were cocultured with OP9-DL1 for 4-6 weeks and then collected and infected with HIV-1_{NL4-3}. The infected cells were cocultured again with OP9-DL1 for another week, collected and analyzed. (B) Representative plots showing HIV $p24^+$ cells after the post-infection coculture and their phenotypes. A

majority of $p24^+$ cells were $CD8^+$ and $CD34^-$. (C) HIV $p24^+$ frequencies measured 1 week after the post-infection coculture (n = 12).

Figure 6. $CD34^+$ cells in the second experiment (coculture-infection-coculture, as described in Fig 5A) were analyzed 1 week after the post-infection coculture (n = 12). (A) Representative plots showing reduction of $CXCR4^+$ frequencies in HIV-infected cocultures compared to autologous uninfected counterparts. (B) $CD34^+$ frequencies were not significantly affected by HIV infection. (C) Loss of $CXCR4^{hi}$ cells was observed in $CD34^+$ cells (left) but not in $CD34^-$ (middle) or $CD4^+CD8^+$ (right) cells. A paired *t*-test was used for comparison.

Figure 7. In the second experiment (coculture-infection-coculture, as described in Fig 5A), $CD34^+$ cells 1 week after the post-infection coculture were further analyzed in detail (n = 9). (A) Representative plots showing the changes in CD7/CXCR4 expression levels in HIV-infected samples compared to their autologous uninfected counterparts. (B) $CD7^{hi}$ (left), $CD7^{hi}CXCR4^{hi}$ (middle), and $CD7^{lo}CXCR4^{hi}$ (right) frequencies in the $CD34^+$ fractions were compared between autologous HIV-infected and mock-infected samples. A paired *t*-test was used for comparison.

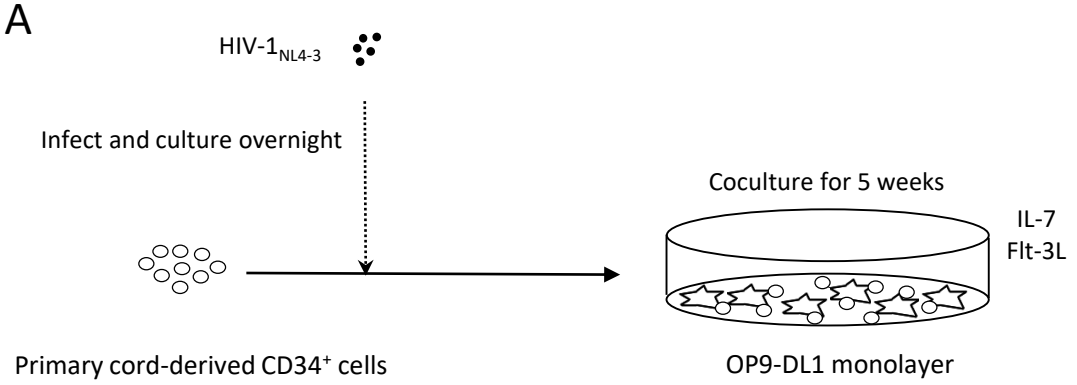
Tables

	Experiment 1 [Fig1-4] (infection-coculture)	Experiment 2 [Fig5-7] (coculture-infection-coculture)
Whole cell count	↓ [Fig2A]	→ [Text]
$CD4^+CD8^+$ cell count	↓ [Fig2C]	→ [Fig6C/Text]
$CD34^+$ cell count	→ [Fig2B]	→ [Fig6B/Text]
% $CD7^{hi}$ in CD34	→ [Fig4B]	↓ [Fig7B]
% $CD7^{hi}CXCR4^{hi}$ in CD34	→ ～ ↓ [Fig4B]	↓ [Fig7B]
% $CD7^{lo}CXCR4^{hi}$ in CD34	→ ～ ↓ [Fig4B]	→ [Fig7B]

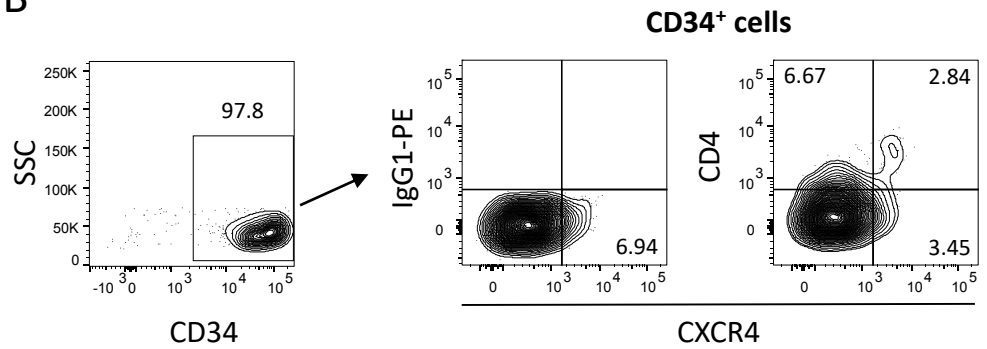
Table 1. Summary for the two separate coculture experiments. Comparison was done between HIV-infected and uninfected samples. →: not different, ↓: lower in HIV-infected samples, → ～ ↓: lower in HIV-infected samples at a time point. Highlight means the same tendencies between Experiments 1 and 2. Superscripts indicate the related figures (or text).

Figure 1

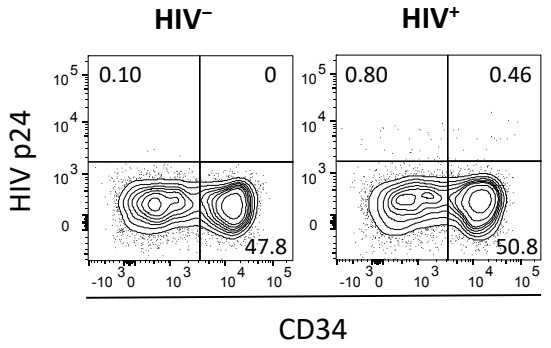
A



B



C



D

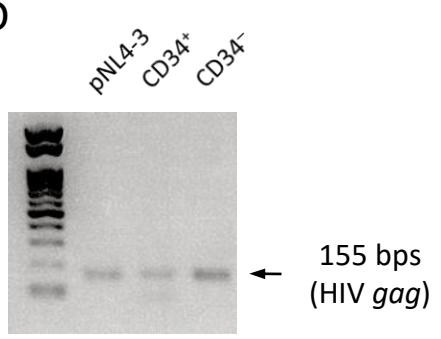
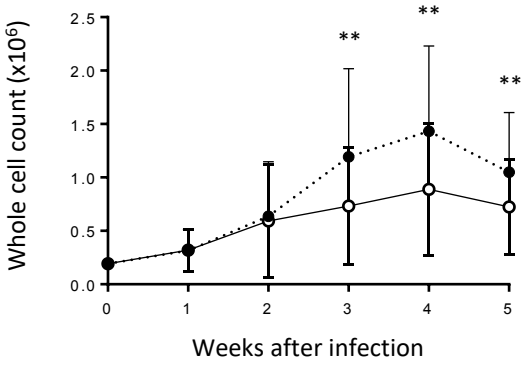
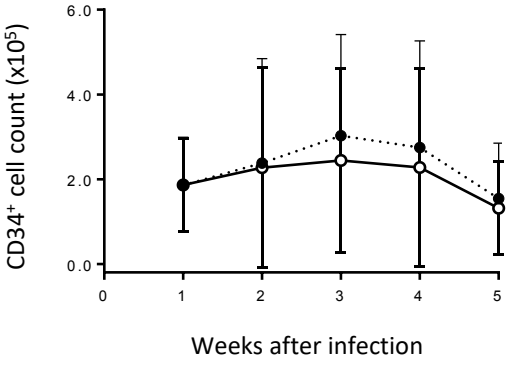


Figure 2

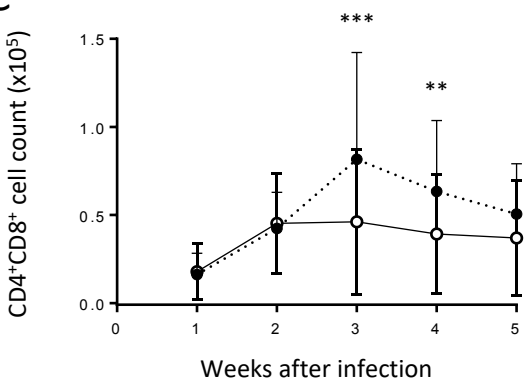
A



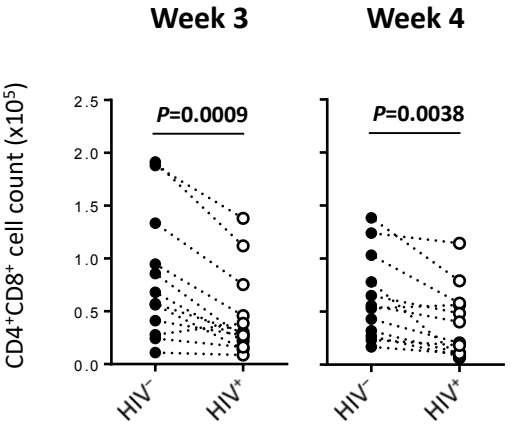
B



C



D



E

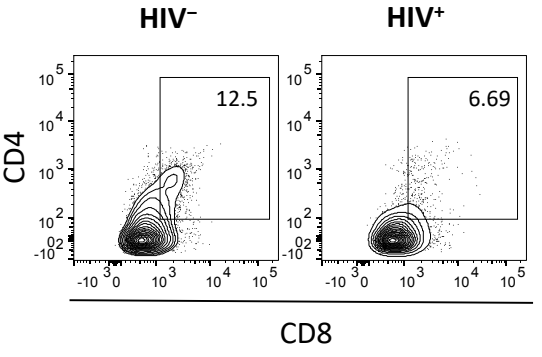


Figure 3

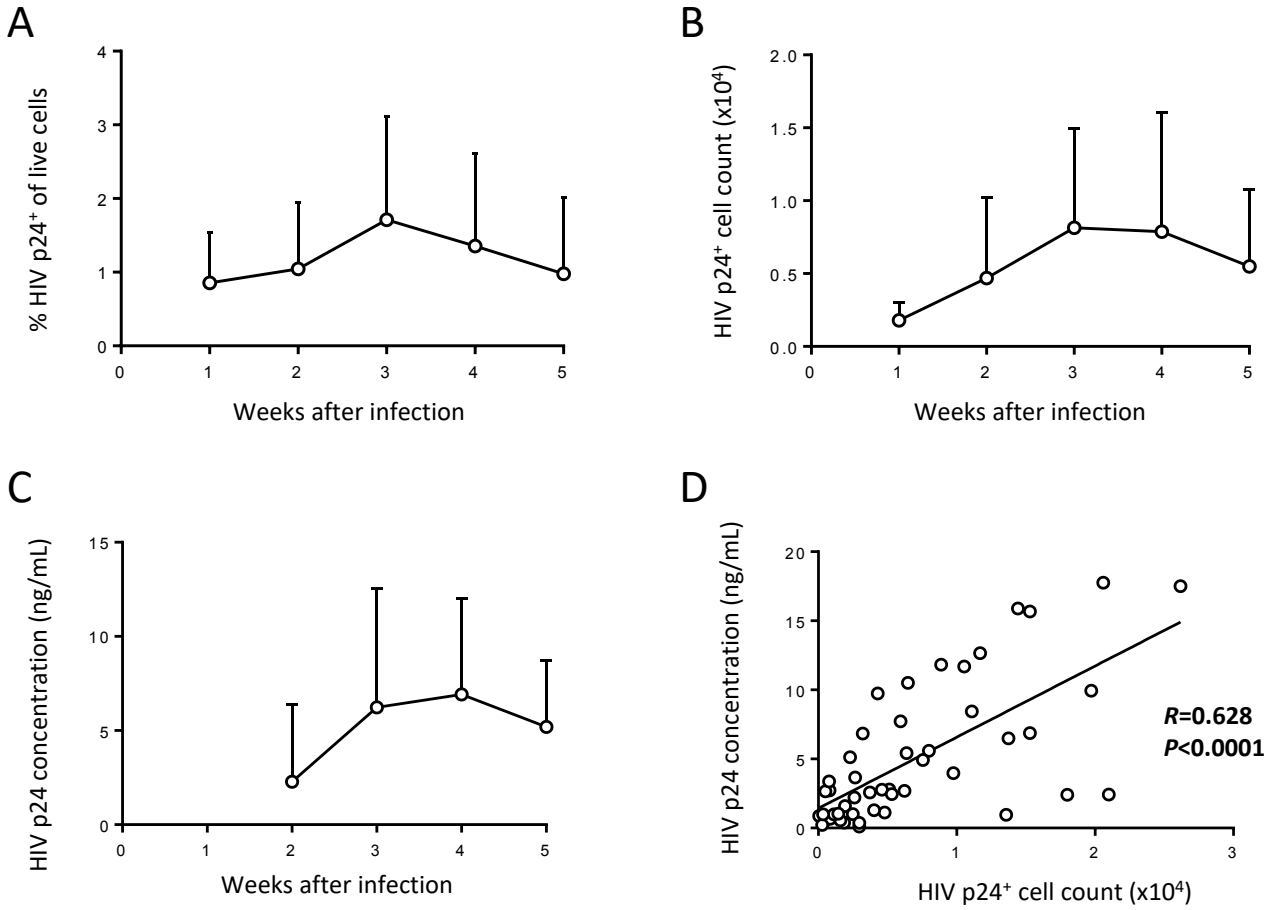
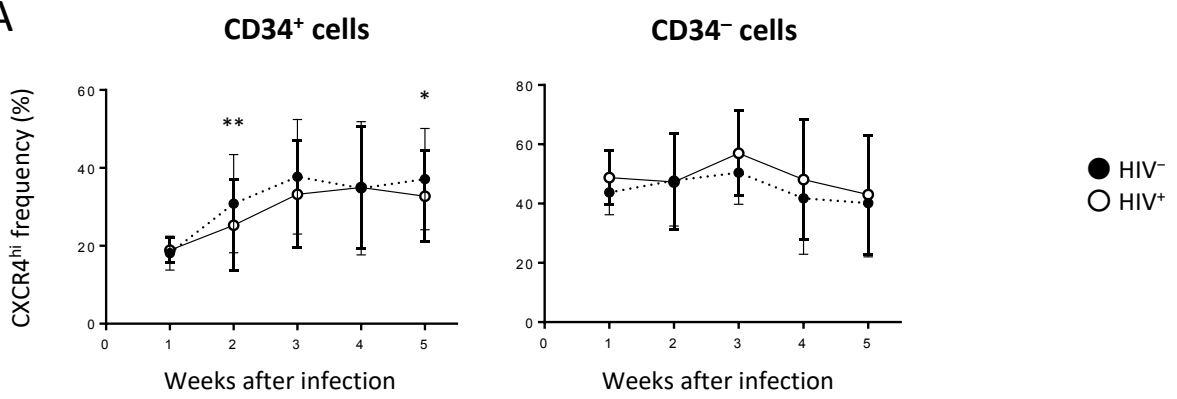
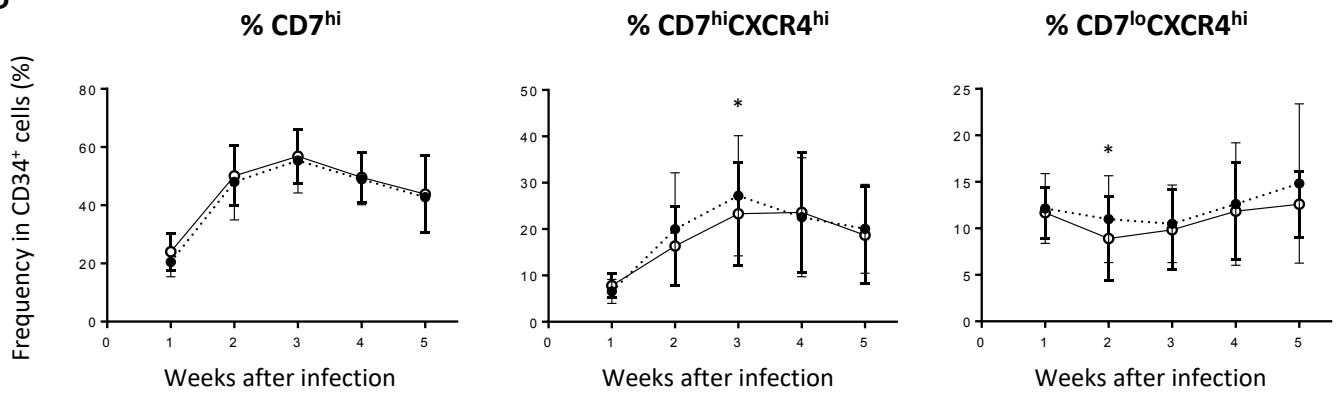

A
% HIV p24+ of live cells
Weeks after infection
B
HIV p24+ cell count (x10^4)
Weeks after infection
C
HIV p24 concentration (ng/mL)
Weeks after infection
D
HIV p24 concentration (ng/mL)
HIV p24+ cell count (x10^4)
R=0.628
P<0.0001

Figure 4

A


CD34+ cells
CD34− cells
CXCR4hi frequency (%)
Weeks after infection
Weeks after infection
**
*
HIV−
HIV+

B


% CD7hi
% CD7hiCXCR4hi
% CD7loCXCR4hi
Frequency in CD34+ cells (%)
Weeks after infection
Weeks after infection
Weeks after infection
*
*

C

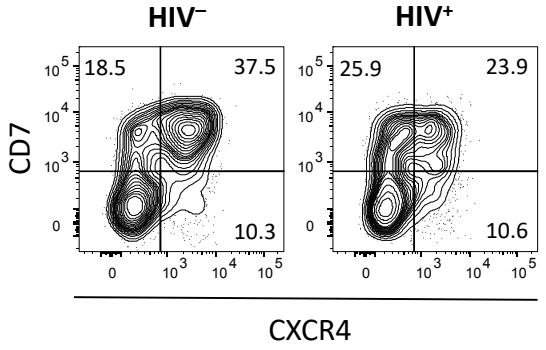
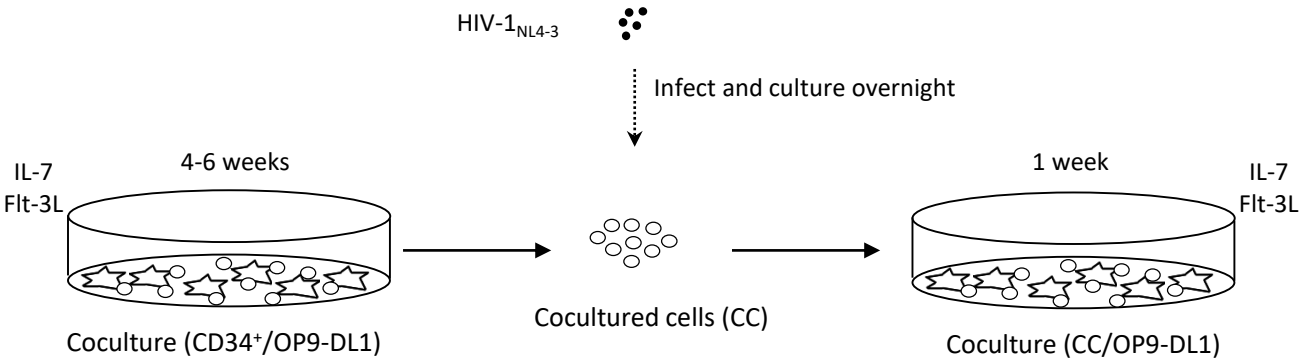
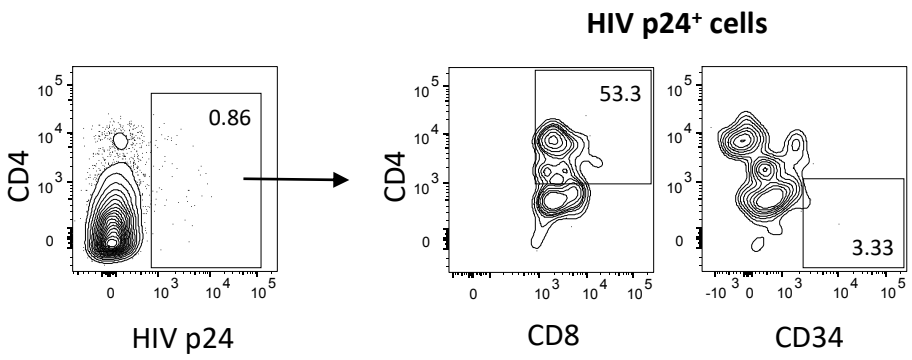

HIV−
HIV+
18.5
37.5
10.3
25.9
23.9
10.6
CD7
CXCR4

Figure 5

A


HIV-1NL4-3
Infect and culture overnight
IL-7
Flt-3L
4-6 weeks
Coculture (CD34+/OP9-DL1)
Cocultured cells (CC)
1 week
IL-7
Flt-3L
Coculture (CC/OP9-DL1)

B


HIV p24+ cells
CD4
0.86
HIV p24
53.3
CD8
3.33
CD34

C

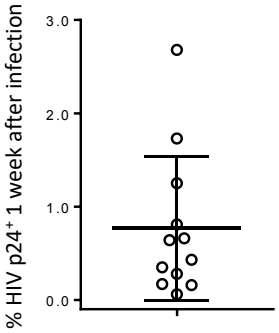

% HIV p24+ 1 week after infection
3.0
2.0
1.0
0.0

Figure 6

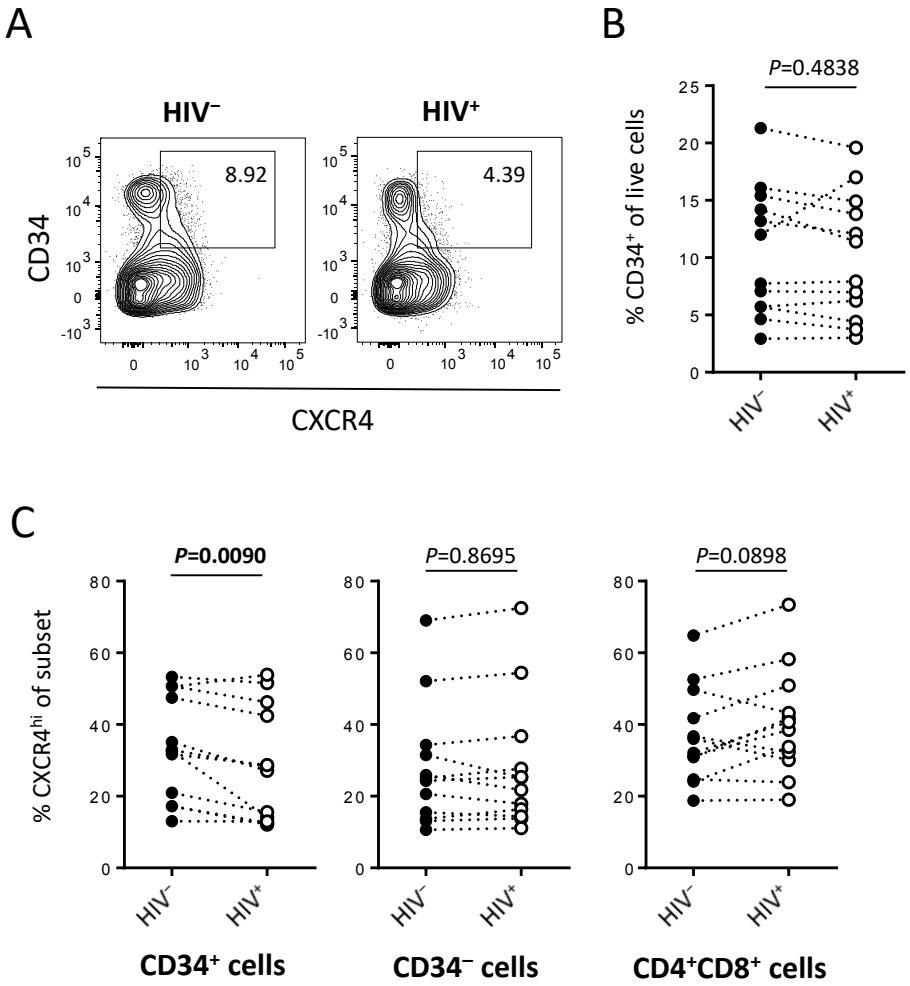
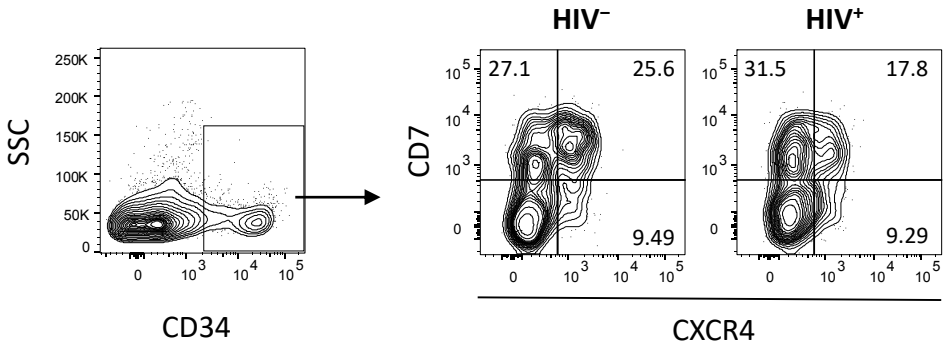
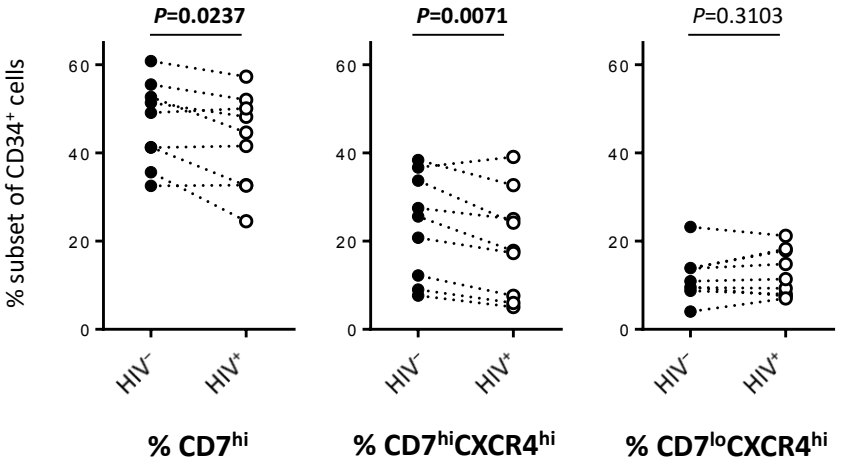

A
HIV⁻
HIV⁺
8.92
4.39
CD34
CXCR4
B
P=0.4838
% CD34⁺ of live cells
HIV⁻
HIV⁺
C
P=0.0090
P=0.8695
P=0.0898
% CXCR4hi of subset
CD34⁺ cells
CD34⁻ cells
CD4⁺CD8⁺ cells

Figure 7

A


HIV⁻
HIV⁺
SSC
CD34
CD7
CXCR4
27.1
25.6
9.49
31.5
17.8
9.29

B


P=0.0237
P=0.0071
P=0.3103
% subset of CD34⁺ cells
HIV⁻
HIV⁺
% CD7hi
% CD7hiCXCR4hi
% CD7loCXCR4hi