Loss of bone marrow cells including CD34^{+} cells following CCR5-tropic HIV-1 infection of humanized NOD/SCID/JAK3^{null} mice and partial protection of those cells by a promotor-targeting shRNA

Tetsuo Tsukamoto [1, 2, 3]

[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

Corresponding 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

Hematological abnormalities are common among HIV-infected patients. Of those, bone marrow changes such as anemia and pancytopenia can be observed in advanced HIV disease. However, experimental evidence for those manifestations in animal immunodeficiency virus infection models has been limited. In the present study, NOD/SCID/JAK3^{null} (NOJ) mice transplanted with human cord-derived CD34^{+} cells were infected with CCR5-tropic HIV-1$_{JRFL}$ 16 weeks after transplantation. At week 2 post infection, massive HIV replication was observed in the peripheral blood and the spleen. This was accompanied with a loss of peripheral/spleen CD4^{+}CCR5^{+} T cells and bone marrow cells including CD34^{+} stem/progenitor cells. On the other hand, the lentiviral transfer of a promoter-targeting shRNA called PromA in CD34^{+} cells prior to transplantation rendered the humanized NOJ mice resistant to HIV replication in the peripheral blood, spleen, and bone marrow, resulting in the preservation of CD4^{+}CCR5^{+} T cells and bone marrow CD34^{+} cells. These results implicate the importance of evaluating hematopoietic stem/progenitor cell pools in addition to peripheral CD4^{+} T-cell counts for the assessment of HIV disease. Moreover, it was suggested that the stable gene transfer of PromA to hematopoietic stem cells not only limit HIV replication, but lead to preservation of different subsets of hematopoietic cells including the bone marrow stem/progenitor cells.

Keywords

Human immunodeficiency virus (HIV), acquired immunodeficiency syndrome (AIDS), hematopoietic stem/progenitor cells (HSPC), transcriptional gene silencing (TGS)

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection is associated with hematological changes [1]. Antiretroviral therapy (ART) is effective on the control of viremia and the treatment of AIDS. However, some patients fail to show sufficient T-cell immune restoration despite being aviremic during treatment [2]. Such immunological nonresponsiveness can be associated with immune activation and/or bone marrow impairment [3,4]. Bone marrow abnormalities, such as dysplasia and abnormal hematopoietic cell development, have frequently been observed in HIV-infected individuals [5]. Therefore, a method of protecting the whole hematopoietic cell populations from HIV would be desirable for preventing immunological/hematological disorders associated with HIV infection.

Transcriptional gene silencing (TGS) occurs through small non-coding RNAs that direct epigenetic changes such as DNA methylation and heterochromatin formation. TGS was first described in plants [6]. Since then, the application of TGS in humans has been investigated for the development of new therapeutic agents [7]. For example, previous reports showed sustained, profound, highly specific suppression of viral replication by siRNA– and short hairpin RNA (shRNA)–induced TGS of HIV-1 and simian immunodeficiency virus in various *in vitro* models, through a mechanism that results in chromatin compaction [8-14]. Because HIV-1 has identical long terminal repeats (LTRs) at the 5′ and 3′ ends of the integrated virus, any promoter-targeted siRNA can potentially act through post-transcriptional gene silencing (PTGS). With the lead candidate called PromA, it was found that the contribution of PTGS is limited [8].

In the previous collaborative study including *in vivo* animal experiments, a lentiviral delivery system was used to express the previously described shRNA PromA

targeting the HIV-1 promoter region to transduce human PBMCs, whereby an antiviral effect of the TGS produced by this construct on HIV-1 infection *in vivo* was demonstrated using NOD/SCID/Jak3^{null} (NOJ) mice [15] transplanted with the lentivirally transduced PBMCs [13]. In the present study, we tested whether the TGS through the shRNA (shPromA) may be applied to more profound gene therapy such as gene-modified hematopoietic stem cell transplantation to replace patients' immune system to an HIV-resistant one. Humanized mouse models have been characterized for tissue distribution of human hematopoietic cells and HIV infection of those cells [16]. To take better advantage of the NOJ-based humanized mouse model, we transplanted irradiated newborn NOJ mice with human cord-derived CD34^{+} cells as described previously [17]. We demonstrate that the generated hematopoietic/immune cells, especially CD4^{+} T cells, are resistant to the replication of the challenged HIV-1$_{JRFL}$ *in vivo*. We also show that bone marrow CD34^{+} cells expressing shPromA may be better protected and preserved after HIV infection.

Materials and Methods

Virus stocks. Stocks of HIV-1_{JRFL} were produced by transfection of 293T cell line with the molecular clone DNA pJRFL. After the transfection, the culture supernatant was collected and virus titers were determined using an HIV p24 Enzyme-Linked ImmunoSorbent Assay (ELISA) kit (ZeptoMetrix, NY, USA).

Cells. Umbilical cord blood (UCB) samples from healthy newborn infants were collected at Fukuda Hospital, Kumamoto, Japan following informed consent. Cord blood mononuclear cells were isolated using Pancoll (PAN-Biotech GmbH, Aidenbach, Germany) and by density gradient centrifugation. $CD34^+$ cells were labelled and selected using human CD34 micro beads and LS columns (Miltenyi Biotec, NSW, Australia). The purity was constantly above 92 % on flow cytometric analysis.

Production of lentivirus. The construction of self-inactivating lentiviral vectors expressing shRNA PromA (shPromA) or PromA M2 (shPromA-M2) (Fig. 1) were previously described [13]. Vesicular stomatitis virus-G (VSV-G) pseudotyped lentiviral vectors were prepared by transduction of plasmid DNA into 293T cells using Lipofectamin 2000 (Thermo Fisher Scientific, VIC, Australia). The virus was concentrated from supernatant as previously described [18,19], and stocks were titrated on 293T cells based on GFP expression.

Transduction of $CD34^+$ cells with lentivirus. Fresh cord-derived $CD34^+$ cells were cultured in X-VIVO 10 (Lonza, NSW, Australia). Cells were transduced with a lentiviral vector at a multiplicity of infection (MOI) of 10 following pre-stimulation

with 50 ng/mL each of stem cell factor (SCF), FMS-related tyrosine kinase 3 Ligand (Flt-3L), and thrombopoietin (TPO) (R&D Systems, MN, USA) for 24 hours. Cells were collected and cryopreserved 24 hours after transduction until the day of transplantation. A small portion of cell culture was further incubated for 48 hours to check the GFP expression level by flow cytometry.

Colony formation assay. In vitro differentiation of shPromA-transduced $CD34^+$ cells to erythrocytes, granolocytes, or monocytes were assessed in vitro by a colony formation assay. Cells were diluted to 500 /mL in MethoCult Optimum methylcellulose media (STEMCELL Technologies, VIC, Australia) containing recombinant human cytokines including SCF, granulocyte macrophage-colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), interleukin 3 (IL-3), and erythropoietin. Cells were seeded in 6-well plate (Corning, VIC, Australia) using a syringe and BD blunt plastic cannula (BD Biosciences, NSW, Australia). Colony-forming units per 1,000 $CD34^+$ cells were counted after 14 days of culture.

Transplantation of cord-derived human $CD34^+$ cells into NOJ mice and HIV-1 infection of mice. Human cell-transplanted NOJ mice were generated as described previously [17]. Briefly, newborn NOJ mice were irradiated (1.0 Gy) and shPromA–transduced or unmanipulated $CD34^+$ cells (2×10^5) were resuspended in phosphate-buffered saline (PBS) (0.1 mL) and infused intrahepatically into each mouse. At week 14 after transplantation, peripheral blood was collected from the retro-orbital sinus for flow cytometric analysis of PBMCs to check the engraftment of human hematopoietic cells (Fig. 3). At week 16 after transplantation, a dose (200 ng p24) of HIV-1_{JRFL}, which was determined by HIV-1 p24 antigen ELISA, was inoculated intraperitoneally into

each mouse with successful engraftment (Fig. 3). Blood was collected at weeks 1 and 2 after infection to obtain plasma and PBMCs. Mice were sacrificed at week 2, followed by collection of splenocytes as well as bone marrow cells in the left femur. All animal experiments were performed according to the guidelines of the Kumamoto University Graduate School of Medical Science.

PCR/RT-PCR analysis. Cellular DNA was extracted using Kaneka Easy DNA Extraction Kit (Kaneka, Osaka, Japan). Cellular RNA was extracted using High Pure RNA Tissue Kit (Roche Diagnostics, Tokyo, Japan). DNA/RNA extraction was followed by the PCR/RT-PCR analysis using an HIV *gag* primer set (sense: 5′-AGTGGGGGGACATCAAGCAGCCATGCAAAT-3′, antisense: 5′-TACTAGTAGTTCCTGCTATGTCACTTCC-3′) as described previously [9]. Both HIV DNA and RNA levels were normalized against GAPDH. All the quantitative PCR/RT-PCR analyses were performed using a syber green method on an ABI Prism machine (Thermo Fisher Scientific).

Flow cytometric analysis of $CD4^+/CD8^+$ T cells and intracellular p24-expressing cells. PBMCs and splenocytes of HIV-infected NOJ mice were analyzed by flow cytometry. Briefly, blood samples were treated with red cell lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1mM EDTA) for 15 minutes, and resuspended in the staining media containing 2 % fetal bovine serum (FBS) and 0.05 % sodium azide. Cells were surface stained with the Live/Dead Fixable Near-IR Dead Cell Stain dye (Thermo Fisher Scientific), and the following monoclonal antibodies for 15 minutes: anti-mouse CD45 PerCP-Cy5.5, anti-human CD45 Brilliant Violet (BV) 510, anti-human CD3 PE-Cy7, anti-human CD4 PerCP-Cy5.5, anti-human CD8 Alexa Fluor

(AF) 647, and anti-human CCR5 PE-CF594. Cells were washed and stained with anti-HIV-1 p24 PE using the CytoFix/CytoPerm kit (BD Biosciences). The stained cells were acquired on an FACS LSR II (BD Biosciences). The flow cytometry data were analyzed with FlowJo v10.8 (FlowJo, OR, USA).

Sequence analysis. The 5'LTR region of viral RNA was amplified by PCR using a viral RNA-specific primer set (sense: 5′-GACCATCAAGCGGCTATGCAGATT-3′, antisense: 5′-GTAAATGTTGCCTACTGGTATGGG-3′), followed by a sequence analysis of the region around the two NF-κB binding sites using the BigDye Terminator version 3.1 and a sequence analyzer (Thermo Fisher Scientific).

In vitro generation and HIV-1$_{JRFL}$ infection of macrophages. After transduction of CD34^{+} cells either with shPromA or shPromA-M2, cells were stimulated and expanded for 2 or 3 weeks in 12-well plate using X-VIVO 10 media supplemented with 4 % FBS, 50 ng/mL SCF, 15 ng/mL TPO, 30 ng/mL Flt-3L, and 30 ng/mL IL-3 [20]. Cells were then washed and further cultured for 4-7 days using the RPMI 1640 media supplemented with 20 % FBS, 25 ng/mL SCF, 30 ng/mL Flt-3L, 30 ng/mL IL-3, and 30 ng/mL macrophage colony-stimulating factor (M-SCF). Development of shPromA–expressing macrophages were detected on a fluorescence microscope as GFP^{+} cells with typical fried-egg shapes (Fig. 6A). Cells were then seeded at 1×10^5 per well in a 96-well plate and infected with 50 ng (p24) of HIV-1$_{JRFL}$. Cells were collected 5 days after infection, stained for surface markers and intracellular HIV p24, and analyzed by flow cytometry.

Antibodies and reagents for flow cytometry. Anti-human CD34 APC and anti-human CD38 PE (BD Biosciences) were used for analysis of magnetically separated $CD34^+$ cells. The following antibodies were used for the flow cytometric analysis of NOJ mouse peripheral blood mononuclear cells (PBMCs), splenocytes, and bone marrow cells after transplantation of human $CD34^+$ cells. Anti-mouse CD45 PerCP-Cy5.5, anti-mouse CD45 PE, anti-human CD45 Brilliant Violet (BV) 510, anti-human CCR5 PE-CF594, anti-human CD56 PerCP-Cy5.5, anti-human CD11c BV510, anti-human CD19 PE-CF594, anti-human CD33 PE-Cy7, anti-human CD66b Alexa Fluor 647 (AF647) (BD Biosciences), anti-human CD3 PE-Cy7, anti-human CD4 BV421, anti-human CD8 AF647, anti-human CD14 PerCP-Cy5.5 (BioLegend, CA, USA), anti-human CD123 PE (Thermo Fisher Scientific), and anti-HIV-1 p24 PE (Beckman Coulter, Tokyo, Japan). In addition, the following antibodies were used for the staining of in vitro generated macrophages. Anti-human CD4 APC, anti-human CD11b BV421, anti-HLA-DR BV510 (BD Biosciences), anti-human CD14 PE-Cy7, anti-human CD16 AF700, and anti-human CD163 PerCP-Cy5.5 (BioLegend). Dead cells were stained and excluded from analysis using the Live/Dead Fixable Near-IR Dead Cell Stain Kit (Thermo Fisher Scientific).

Statistical analysis. Statistical analysis was performed using the GraphPad Prism software version 7 (GraphPad Software, CA, USA). Statistical significance was defined as $P < 0.05$. Comparison between 2 samples was done by the unpaired Mann-Whitney test or Wilcoxon matched-pairs signed rank test. Nonparametric multiple comparison analysis was performed with the Dunn's method.

Results

Cord-derived CD34$^+$ cells were efficiently transduced with lentivirus expressing the shPromA customized for HIV-1$_{JRFL}$

The siRNAs PromA and PromA M2 were described previously [13]. Because of the one-base mismatch in the PromA region between HIV-1$_{NL4-3}$ and HIV-1$_{JRFL}$, in the present study we used the PromA that is customized for HIV-1$_{JRFL}$, which we call PromA in the present article. The sequences of HIV-1$_{JRFL}$, PromA, and PromA-M2 are described in Fig. 1. The lentiviral vectors expressing PromA and PromA M2 in their shRNA forms (shPromA and shPromA-M2, respectively) were prepared for transduction of CD34$^+$ cells.

The volumes of the cord blood samples were about 20–130 mL per batch. Those samples contained about 5×10^6–2×10^8 mononuclear cells per batch, of which about 0.1–6 % were CD34$^+$ cells on flow cytometric analysis. After mononuclear cell isolation and CD34$^+$ micro bead selection, about 5×10^4–2×10^6 CD34$^+$ cells were obtained. The average purity of the CD34$^+$ cells after selection was 96.7 %, while the average CD38^{lo} frequency was 25.6 % (Fig. S1A and C). The CD34$^+$ cell batches with more than 4×10^5 cells were used for the following lentiviral transduction of shPromA. Fresh CD34$^+$ cells were prestimulated in X-VIVO 10 media containing SCF, Flt-3L, and TPO for 24 hours and then transduced with lentivirus expressing shPromA at an MOI of 10. Cells were cryopreserved 24 hours after transduction except for a small portion of cells cultured for another 48 hours to evaluate the transduction efficiency, expressed as percent of GFP$^+$ cells in the culture. The average GFP$^+$ frequency was 65.1 % (Fig. S1B and C).

$CD34^+$ cells transduced with lentivirus expressing shPromA showed colony forming capability in vitro relevant to untransduced $CD34^+$ cells

In order to confirm the quality of $CD34^+$ cells in terms of their potential for differentiation, we performed colony formation assays using methylcellulose culture containing cytokines (Fig. S2). Colonies expressing shPromA were detected by checking the GFP fluorescence emission on fluorescence microscopy (Fig S2A). No significant difference was found in the colony forming units between untransduced samples and shPromA-treated samples (Fig. S2B). After counting colonies, the methylcellulose culture cells were collected, and analyzed by flow cytometry. The untransduced $CD34^+$ cells and shPromA-treated $CD34^+$ cells showed similar CD33/CD14 differentiation patterns (Fig. S2C). Also, there was no obvious skew in the expression of CD33 or CD14 between GFP^+ and GFP^- cells in the shPromA-treated sample. (Fig. S2C).

Transplantation of irradiated newborn NOJ mice with shPromA-expressing $CD34^+$ cells resulted in efficient engraftment and differentiation of shPromA-expressing human lymphocytes and myeloid cells in vivo

Newborn NOJ mice were irradiated and intrahepatically transplanted with $CD34^+$ cells. Around 12–14 weeks after transplantation, peripheral blood was obtained from the retroorbital sinus. PBMCs were isolated and analyzed by flow cytometry. Twenty mice transplanted with shPromA-transduced cells (group PromA) and 12 mice transplanted with unmanipulated $CD34^+$ cells (group CD34) were alive at the time of the engraftment assessment. After excluding poorly engrafted mice showing low human $CD45^+$ frequencies (< 3.0 %) or poorly differentiated $CD3^+$ cells, human $CD45^+$ frequencies were compared between groups PromA and CD34, and there was no

significant difference (Fig. 2B). These mice were included in the following HIV challenge experiment (Fig. 3). The average GFP^+ frequency of human $CD45^+$ cells was 40.8 % in group PromA (Fig. S3A). This was lower than the average transduction efficiency (65.1 %, Fig. S1C). Three of the mice transplanted with shPromA-transduced cells were sacrificed 15 weeks after transplantation without HIV challenge. Splenocytes and bone marrow cells were collected and tested for the expression of GFP, the marker for shPromA. Cells were also checked for the expression of CD45 (hematopoietic cells), CD3 (T cells), CD4, CD8, CD19 (B cells), CD56 (subset of NK cells), CD123 (subset of dendritic cells), CD33 (myeloid cells), CD11c, CD66b (granulocytes), and CD14 (monocytes). There was no obvious skew in the expression of GFP among all the phenotypes of cells tested (Fig. 2A and C).

Suppression of viral replication after HIV-1_{JRFL} challenge observed in NOJ mice transplanted with shRNA PromA-expressing $CD34^+$ cells

The 2 groups of mice (PromA and CD34) were challenged with HIV-1_{JRFL} 14-16 weeks after transplantation (Fig. 3). Peripheral blood was collected at weeks 1 and 2 post-challenge. Mice were sacrificed at week 2 and splenocytes and bone marrow cells (from the left femur) were collected for further analysis (Fig. 3). Intracellular HIV Gag p24 expression levels were measured in PBMC (weeks 1 and 2), splenocytes (week 2), and bone marrow cells (week 2). There were significant differences in HIV $p24^+$ frequencies in $CD3^+CD8^-$ T cells at weeks 1 and 2, indicating the impact of PromA on suppression of HIV replication (Fig. 4A and B, Fig. S4). Cell-associated HIV DNA/RNA copy numbers in splenocytes (week 2) relative to human GAPDH were measured by quantitative PCR/RT-PCR. In group PromA, the average relative RNA copy number was lower than in group CD34, while relative DNA copy numbers were

significantly higher (Fig. 4C left and middle). The ratios of the HIV RNA expression levels to the HIV DNA levels were calculated. The average RNA/DNA ratio in group PromA was more than 10 times lower than that in group CD34 (Fig. 4C right), showing the suppressed numbers of HIV RNA, including transcripts, per DNA. The HIV RNA didn't show a mutation in the PromA region (data not shown).

NOJ mice transplanted with shPromA$^+$ CD34$^+$ cells showed notable differences in the dynamics in human CD4$^+$CCR5$^+$ T cells and CD34$^+$ cells after HIV-1$_{JRFL}$ challenge

The frequencies of human CD45$^+$ cells and their subsets were analyzed using PBMC (weeks 1 and 2), splenocytes (week 2), and bone marrow cells (week 2) (Fig. S5, Table 1). In PBMC, human CD45$^+$ percentages were significantly lower in group PromA than in group CD34 (Fig. S5A). The peripheral blood human CD45$^+$ cell counts also showed significant difference at week 1 (Fig. S5B). However, the blood CD45+ cell counts at week 2 were not significantly different between the 2 groups (Fig. S5B). Moreover, the CD45+ cell counts in spleens and bone marrows were much higher in group PromA than in group CD34 (Table 1). The reasons for these discrepancies are not clear.

There was a marked decline in the splenocyte CD4$^+$CCR5$^+$ frequencies in group CD34 at week 2 compared to the uninfected control group, presumably due to their depletion following HIV replication (Fig. 5A and B). On the other hand, the mice in group PromA showed preserved splenocyte CD4$^+$CCR5$^+$ T-cell frequencies, supporting the protective effect of PromA against the CCR5-tropic challenge strain of HIV-1 (Fig. 5A and B). Similar results were found by the analysis of PBMCs sampled at week 2 post challenge showed the similar results (Fig. S6). To better clarify the protective effect of PromA on human hematopoietic cells in HIV infection, we analyzed bone

marrow $CD34^+$ cells (Fig. 5C and D). At week 2 post challenge, $CD34^+$ cells were severely depleted in group CD34 compared to the uninfected control group (Fig. 5D). The $CD34^+$ frequencies in group PromA were significantly higher than those in CD34 group (Fig. 5D), indicating that shPromA was associated with protection of bone marrow cells including hematopoietic stem/progenitor cells (HSPCs). The calculation of bone marrow $CD34^+$ cell counts showed similar results. (Fig. S7A). We further analyzed shRNA (GFP) expression levels in the bone marrow cells of group PromA mice. Interestingly, $CD34^+$ cells had greater GFP^+ frequencies than $CD45^+$ cells (Fig. S7B), implicating the possible role for shPromA in protection of $CD34^+$ cells from HIV infection.

In vitro generated PromA-expressing macrophages showed resistance to HIV-1_{JRFL} replication in vitro

In parallel with the HIV challenge experiment with the humanized mice, the effect of shPromA on suppression of HIV replication was tested with macrophages generated in vitro from cord-derived $CD34^+$ cells. PromA-expressing (GFP^+) macrophages with the typical fried-egg shapes were successfully generated (Fig. 6A). The average GFP^+ level in $CD11b^+$ cells were 42.6 %. The most $CD11b^+$ cells were $CD14^+$, $CD16^+$, $CD4^+$ and $CCR5^+$ (Fig. 6B). The in vitro generated macrophages were infected with HIV-1_{JRFL}. Five days after infection, HIV replication levels, measured as intracellular HIV $p24^+$ percentages, were compared between autologous untransduced, shPromA-M2-transduced, and shPromA-transduced macrophages. As shown in Fig 6C and D, macrophages transduced with shPromA showed suppression of HIV replication.

Discussion

One of the challenges with the current standard HIV therapies is that there is no effective way so far to repair the immune system that is supposed to be already partly broken at the time of treatment initiation even without manifestation of AIDS [21]. Gene therapy of HSPCs has been of great interest, because this could possibly recover the lost naïve/memory T-cell repertoire, which then would enable re-immunization of the individual against pathogens including HIV [22]. The most successful cases so far are those using the knockout or knockdown of CCR5, a coreceptor used by those strains of HIV-1 infecting memory $CD4^+$ T cells [23]. In addition, researchers have been finding other host and non-host targets that would make the host cells more resistant to HIV replication [24]. The PromA region in the HIV genome has proved to be among safe and powerful non-host targets [25].

In the present study, we performed transplantation of newborn NOJ mice with lentivirally transduced $CD34^+$ cells expressing shPromA. This resulted in engraftment of shPromA-expressing $CD34^+$ cells and their differentiation to most major subtypes of myeloid and lymphoid cells in vivo, including $CD4^+/CD8^+$ T cells, $CD19^+$ B cells, $CD56^+$ NK cells, and $CD11c^+/CD14^+/CD66b^+/CD123^+$ myeloid cells (Fig. 2A-C, Table 1). This suggests that the off-target effects associated with the expression of PromA do not interfere with differentiation of hematopoietic cells in vivo [13]. The method we tested here is thus promising and also supporting the previous reports showing that retroviral transduction does not alter the immune functions [26].

In the following HIV-1_{JRFL} challenge experiment, the mice engrafted with shPromA-transduced cells showed suppression of HIV replication (Fig. 4) and preservation of $CD4^+CCR5^+$ T cells (Fig. 5A and B), which was relevant to the previous

in vivo results using adult NOJ mice transplanted with shPromA-transduced PBMCs [13]. It was also confirmed here that the mice in group PromA also showed preserved human $CD34^+$ cells in the bone marrow (Fig. 5C and D, Fig. S7). This implicates the advantages of the anti-HIV gene therapy of $CD34^+$ HSPCs over the identical gene therapy of PBMC, because in the former, shPromA can even confer protection of HSPCs from HIV-1 as well as their differentiation processes [21,27]. As multiple types of hematopoietic progenitor cells can harbor CCR5- or CXCR4-tropic virus for a long time [28], the expression of an anti-HIV modality in all of those cell types may be highly effective on protection of the hematopoietic system in HIV-infected patients.

The limited duration of the collaboration, the limited number of mice available, the limited technical expertise in the field of humanized mouse models, and the lack of an empty lentivirus vector for the patented shPromA lentivirus vector, prevented the inclusion of most appropriate control animals transduced with a control lentivirus vector. Briefly, the mice transplanted with unmanipulated $CD34^+$ cells were placed as the control group. However, unmanipulated $CD34^+$ cells are different from PromA-transduced cells at multiple facets: the lack of the shRNA, the lack of the lentivirus used for transduction, and the lack of stimulation with the transduction media containing SCF-1, TPO, and Flt-3L. These differences may have caused substantial discrepancies in the physiological conditions of the cells at the time of transplantation, and thus the engraftment rates in the following 4 months. To partly compensate for the control problems with the in vivo experiment, the in vitro viral suppression assays with macrophages, as shown in Fig. 6, were designed to include both untransduced cells and cells transduced with shPromA-M2 (having a 3-base mismatch to shPromA) as controls. In addition to the control problems, the use of the PC3 facility in Japan was allowed for a strictly limited duration of time. Therefore, the HIV challenge study was planned as

a 2-week acute infection experiment with injection of a high dose HIV-1_{JRFL} per animal. However, a chronic infection study starting from injection of a low dose HIV-1_{JRFL} should be more desirable to address the long-term effect of the shPromA-expressing hematopoietic/immune system upon control of persistent HIV infection that is most commonly observed with HIV-infected patients. This will further help clarify whether PromA is able to support preservation of bone marrow $CD34^+$ cells and their functions for a long time [29]. These future plans will better address whether the gene therapy approach with TGS may restore the hematopoietic system functions of HIV-infected patients and extend the periods of symptom-free lives.

In summary, even with those limitations, the present study demonstrates that the gene therapy of HSPCs with shPromA targeting a non-host, non-coding gene sequence may be beneficial for preservation of the hematopoiesis potential in the bone marrow [16]. This is a remarkable step toward a clinical application of TGS, including combination of PromA and other modalities *e.g.* targeting of CCR5 and antiretroviral therapies.

References

1 Parinitha, S. & Kulkarni, M. Haematological changes in HIV infection with correlation to CD4 cell count. *Australas Med J* **5**, 157-162, doi:10.4066/AMJ.20121008 (2012).

2 Corbeau, P. & Reynes, J. Immune reconstitution under antiretroviral therapy: the new challenge in HIV-1 infection. *Blood* **117**, 5582-5590, doi:10.1182/blood-2010-12-322453 (2011).

3 Hunt, P. W. *et al.* T cell activation is associated with lower CD4+ T cell gains in human immunodeficiency virus-infected patients with sustained viral suppression during antiretroviral therapy. *J Infect Dis* **187**, 1534-1543, doi:10.1086/374786 (2003).

4 Isgro, A. *et al.* 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, doi:10.1086/588480 (2008).

5 Tripathi, A. K., Misra, R., Kalra, P., Gupta, N. & Ahmad, R. Bone marrow abnormalities in HIV disease. *J Assoc Physicians India* **53**, 705-710 (2005).

6 Matzke, M. A., Primig, M., Trnovsky, J. & Matzke, A. J. M. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. *The EMBO Journal* **8**, 643-649 (1989).

7 Weinberg, M. S. & Morris, K. V. Transcriptional gene silencing in humans. *Nucleic Acids Research* **44**, 6505-6517, doi:10.1093/nar/gkw139 (2016).

8 Suzuki, K. *et al.* Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter region. *J Biol Chem* **283**, 23353-23363, doi:10.1074/jbc.M709651200 (2008).

9 Suzuki, K. *et al.* Prolonged transcriptional silencing and CpG methylation induced by siRNAs targeted to the HIV-1 promoter region. *J RNAi Gene Silencing* **1**, 66-78 (2005).

10 Lim, H. G., Suzuki, K., Cooper, D. A. & Kelleher, A. D. Promoter-targeted siRNAs induce gene silencing of simian immunodeficiency virus (SIV) infection in vitro. *Mol Ther* **16**, 565-570, doi:10.1038/sj.mt.6300380 (2008).

11 Yamagishi, M. *et al.* Retroviral delivery of promoter-targeted shRNA induces long-term silencing of HIV-1 transcription. *Microbes Infect* **11**, 500-508, doi:10.1016/j.micinf.2009.02.003 (2009).

12 Suzuki, K. *et al.* Transcriptional gene silencing of HIV-1 through promoter targeted RNA is highly specific. *RNA Biol* **8**, 1035-1046, doi:10.4161/rna.8.6.16264 (2011).

13 Suzuki, K. *et al.* Promoter Targeting shRNA Suppresses HIV-1 Infection In vivo Through Transcriptional Gene Silencing. *Mol Ther Nucleic Acids* **2**, e137, doi:10.1038/mtna.2013.64 (2013).

14 Ahlenstiel, C. *et al.* Novel RNA Duplex Locks HIV-1 in a Latent State via Chromatin-mediated Transcriptional Silencing. *Mol Ther Nucleic Acids* **4**, e261, doi:10.1038/mtna.2015.31 (2015).

15 Hattori, S. *et al.* Potent Activity of a Nucleoside Reverse Transcriptase Inhibitor, 4′ -Ethynyl-2-Fluoro-2′ -Deoxyadenosine, against Human Immunodeficiency Virus Type 1 Infection in a Model Using Human Peripheral Blood Mononuclear Cell-Transplanted NOD/SCID Janus Kinase 3

Knockout Mice. *Antimicrobial Agents and Chemotherapy* **53**, 3887-3893, doi:10.1128/aac.00270-09 (2009).
16 Arainga, M., Su, H., Poluektova, L. Y., Gorantla, S. & Gendelman, H. E. HIV-1 cellular and tissue replication patterns in infected humanized mice. *Sci Rep* **6**, 23513, doi:10.1038/srep23513 (2016).
17 Okada, S., Harada, H., Ito, T., Saito, T. & Suzu, S. Early development of human hematopoietic and acquired immune systems in new born NOD/Scid/Jak3null mice intrahepatic engrafted with cord blood-derived CD34 + cells. *Int J Hematol* **88**, 476-482, doi:10.1007/s12185-008-0215-z (2008).
18 Miyoshi, H., Takahashi, M., Gage, F. H. & Verma, I. M. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proceedings of the National Academy of Sciences* **94**, 10319-10323 (1997).
19 Zhang, B. *et al.* A highly efficient and consistent method for harvesting large volumes of high-titre lentiviral vectors. *Gene Ther* **8**, 1745-1751, doi:10.1038/sj.gt.3301587 (2001).
20 Stec, M. *et al.* Expansion and differentiation of CD14+CD16(-) and CD14+ +CD16+ human monocyte subsets from cord blood CD34+ hematopoietic progenitors. *J Leukoc Biol* **82**, 594-602, doi:10.1189/jlb.0207117 (2007).
21 Tsukamoto, T., Yamamoto, H., Okada, S. & Matano, T. Recursion-based depletion of human immunodeficiency virus-specific naive CD4(+) T cells may facilitate persistent viral replication and chronic viraemia leading to acquired immunodeficiency syndrome. *Med Hypotheses* **94**, 81-85, doi:10.1016/j.mehy.2016.06.024 (2016).
22 Rosenzweig, M. *et al.* Intracellular immunization of rhesus CD34+ hematopoietic progenitor cells with a hairpin ribozyme protects T cells and macrophages from simian immunodeficiency virus infection. *Blood* **90**, 4822-4831 (1997).
23 Myburgh, R. *et al.* Lentivector Knockdown of CCR5 in Hematopoietic Stem and Progenitor Cells Confers Functional and Persistent HIV-1 Resistance in Humanized Mice. *J Virol* **89**, 6761-6772, doi:10.1128/jvi.00277-15 (2015).
24 Kitchen, S. G., Shimizu, S. & An, D. S. Stem cell-based anti-HIV gene therapy. *Virology* **411**, 260-272, doi:10.1016/j.virol.2010.12.039 (2011).
25 Ahlenstiel, C. L., Suzuki, K., Marks, K., Symonds, G. P. & Kelleher, A. D. Controlling HIV-1: Non-Coding RNA Gene Therapy Approaches to a Functional Cure. *Front Immunol* **6**, 474, doi:10.3389/fimmu.2015.00474 (2015).
26 Duarte, R. F. *et al.* Functional impairment of human T-lymphocytes following PHA-induced expansion and retroviral transduction: implications for gene therapy. *Gene Ther* **9**, 1359-1368, doi:10.1038/sj.gt.3301807 (2002).
27 Tsukamoto, T. HIV disrupts CD34+ progenitors involved in T-cell differentiation. *bioRxiv*, 142539, doi:10.1101/142539 (2017).
28 Sebastian, N. T. *et al.* CD4 is expressed on a heterogeneous subset of hematopoietic progenitors, which persistently harbor CXCR4 and CCR5-tropic HIV proviral genomes in vivo. *PLoS Pathog* **13**, e1006509, doi:10.1371/journal.ppat.1006509 (2017).
29 Terahara, K. *et al.* Expansion of activated memory CD4+ T cells affects infectivity of CCR5-tropic HIV-1 in humanized NOD/SCID/JAK3null mice. *PLoS One* **8**, e53495, doi:10.1371/journal.pone.0053495 (2013).

Acknowledgements

We thank Drs. Kazuo Matsui and Shoichi Kawakami of Fukuda Hospital, Kumamoto, Japan for their assistance with cord blood sampling. We thank Prof. Seiji Okada of Center for AIDS Research, Kumamoto University and his staff for their assistance with animal experiments. We thank Ms. Michelle Millington, Ms. Maureen Boyd, and Dr. Geoff Symonds for providing lentiviral vector constructs and the transduction protocol. We thank Prof. Anthony Kelleher, Dr. Kazuo Suzuki and Ms. Katherine Marks of the Kirby Institute, UNSW Australia for their support and providing the shRNA-expressing lentivirus vectors. This work was supported by the grants from National Health and Medical Research Council, Australia (Project grant). The work was also partly supported 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).

Competing interests

The authors have no competing interests to declare.

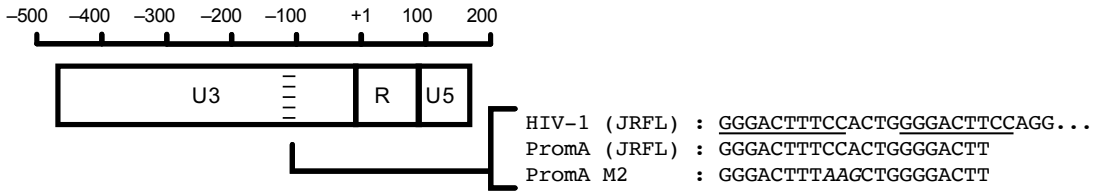

Fig 1. A schematic representation of the siRNAs PromA (JRFL) and PromA-M2. The two NF-κB binding sites in the HIV-1_{JRFL} proviral DNA sequence are underlined. The 3 mismatched bases in PromA-M2 compared to $PromA_{JRFL}$ are indicated with italic letters.

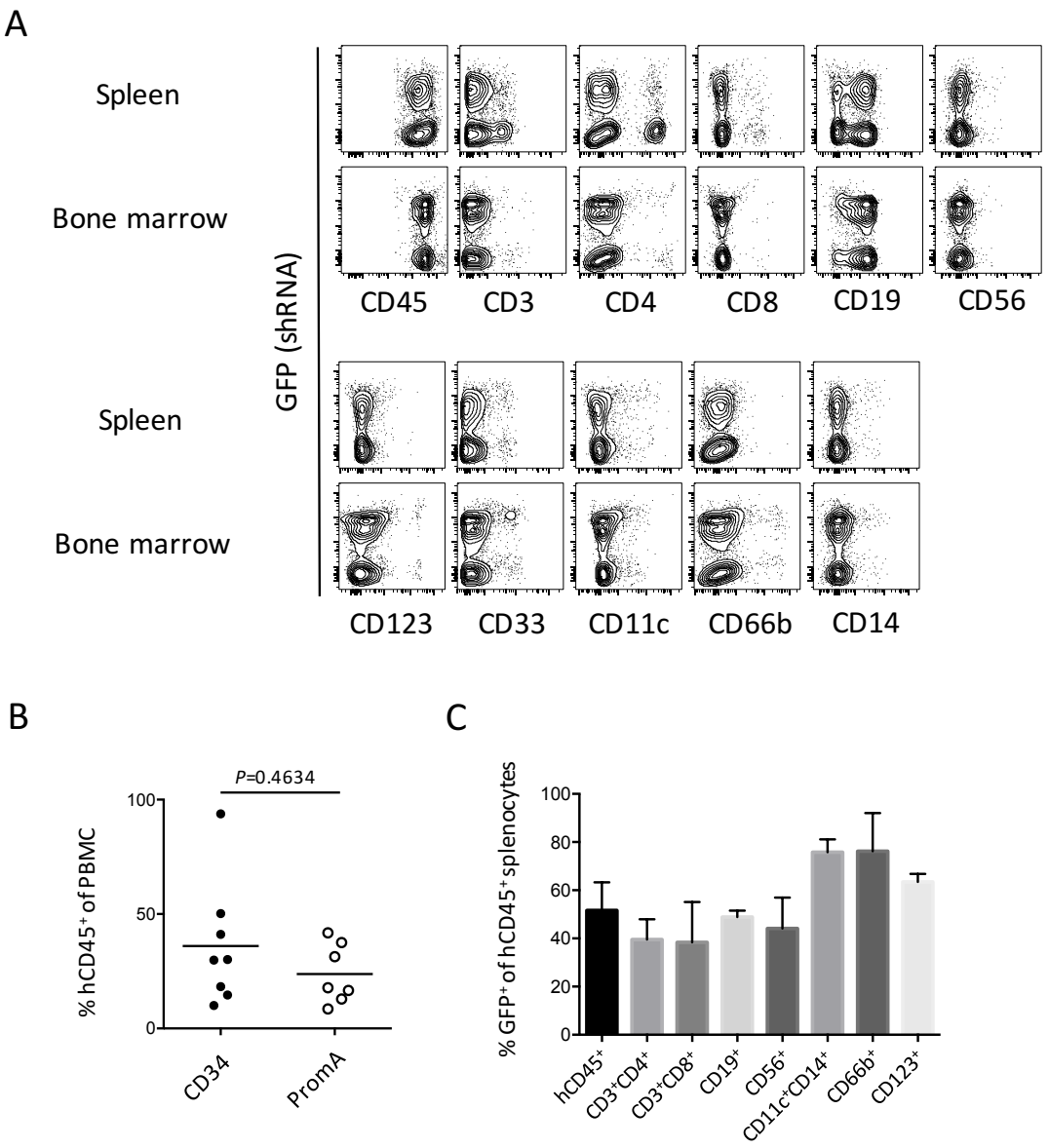

Fig 2. Engraftment of human cells in transplanted NOJ mice was tested 15 weeks after transplantation. (A) Three mice were sacrificed without HIV challenge 15 weeks after transplantation with shPromA-transduced cells . Splenocytes and bone marrow cells were collected from the 3 mice and tested for GFP expression levels in different major subsets of human $CD45^+$ hematopoietic cells. Representative plots showing both GFP^+ and GFP^- cells in all the different subsets of human hematopoietic cells tested. (B) Comparison of human $CD45^+$ frequencies between group CD34 (transplanted with unmanipulated $CD34^+$ cells) and group PromA (transplanted with shPromA-transduced cells) mice that were included in the following HIV challenge experiment. (C) The mean shRNA-expressing (GFP^+) percentages in the different subsets of cells in spleen. Statistical analysis was performed with the Mann-Whitney test.

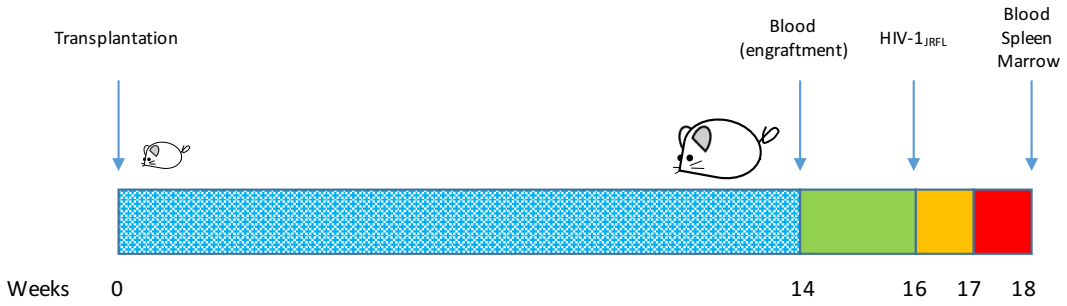

Fig 3. A schematic representation of the in vivo study plan. The new-born NOJ mice were irradiated and transplanted with PromA-transduced or unmanipulated $CD34^+$ cells. 14 weeks later, blood was sampled at the retro-orbital sinus and the PBMCs were tested for the expression of human CD45. Two weeks later, mice were intraperitoneally challenged with HIV-1_{JRFL}. Blood was collected at weeks 1 and 2 post challenge. Mice were sacrificed at week 2 for collection of splenocytes and bone marrow cells.

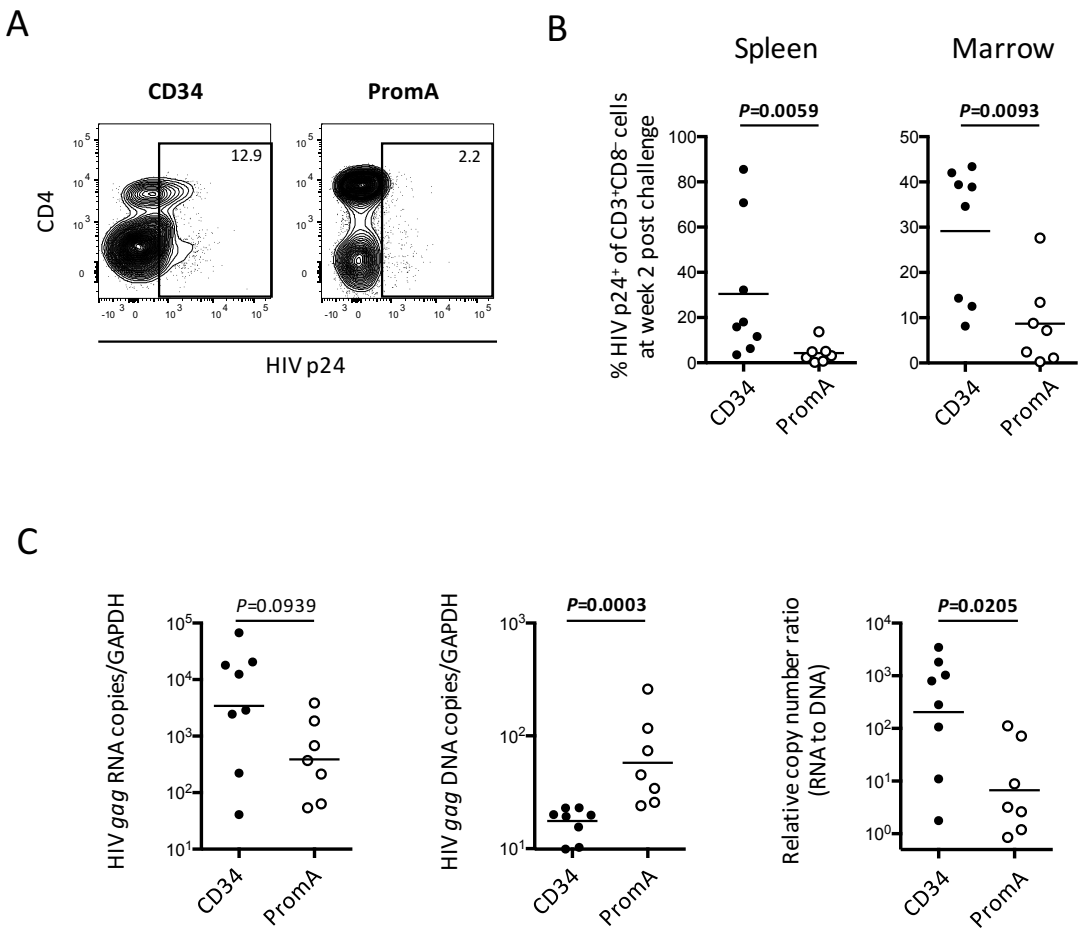

Fig 4. PromA suppressed viral replication in NOJ mice. (A) Representative plots showing suppression of intracellular HIV p24 expression in PromA-transduced mice. (B) Intracellular HIV p24^{+} frequencies in CD3^{+}CD8^{-} T cells were compared between groups CD34 and PromA. The analysis was done using splenocytes (left) and bone marrow cells (right) obtained at week 2 post challenge. (C) Cell-associated RNA (left) and DNA (middle) copies normalized to GAPDH. Relative RNA copies per DNA were calculated from these data (right). Statistical analysis was performed by the Mann-Whitney test.

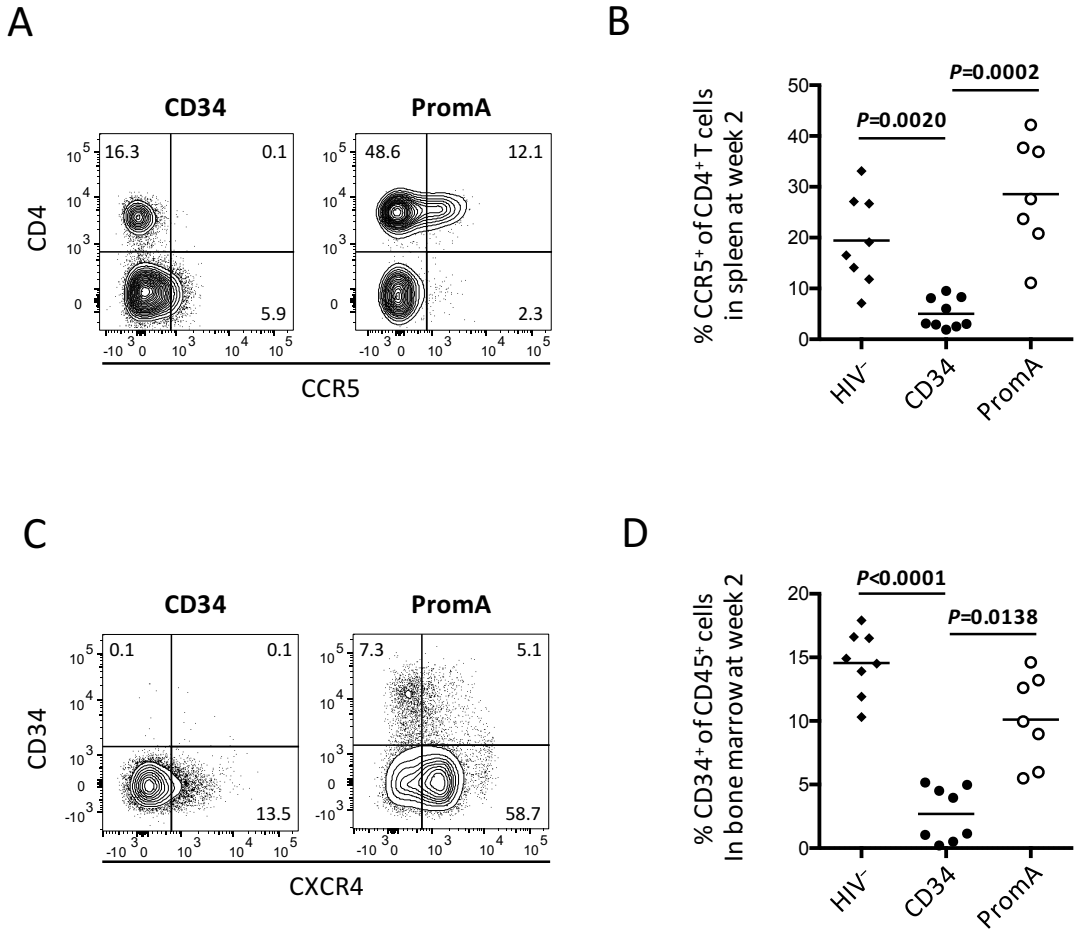

Fig 5. (A) Representative plots showing preserved $CD4^{+}CCR5^{+}$ cells in the PromA-transduced mice compared to the control mice. (B) $CCR5^{+}$ frequencies of $CD4^{+}$ T cells in spleen at week 2 post challenge. (C) Representative plots showing preserved bone marrow human $CD34^{+}$ cells in the $PromA_{JRFL}$-transduced cells compared to the control mice. (D) $CD34^{+}$ frequencies in the bone marrow $CD45^{+}$ cells. Nonparametric multiple comparison analysis was performed with the Dunn's method.

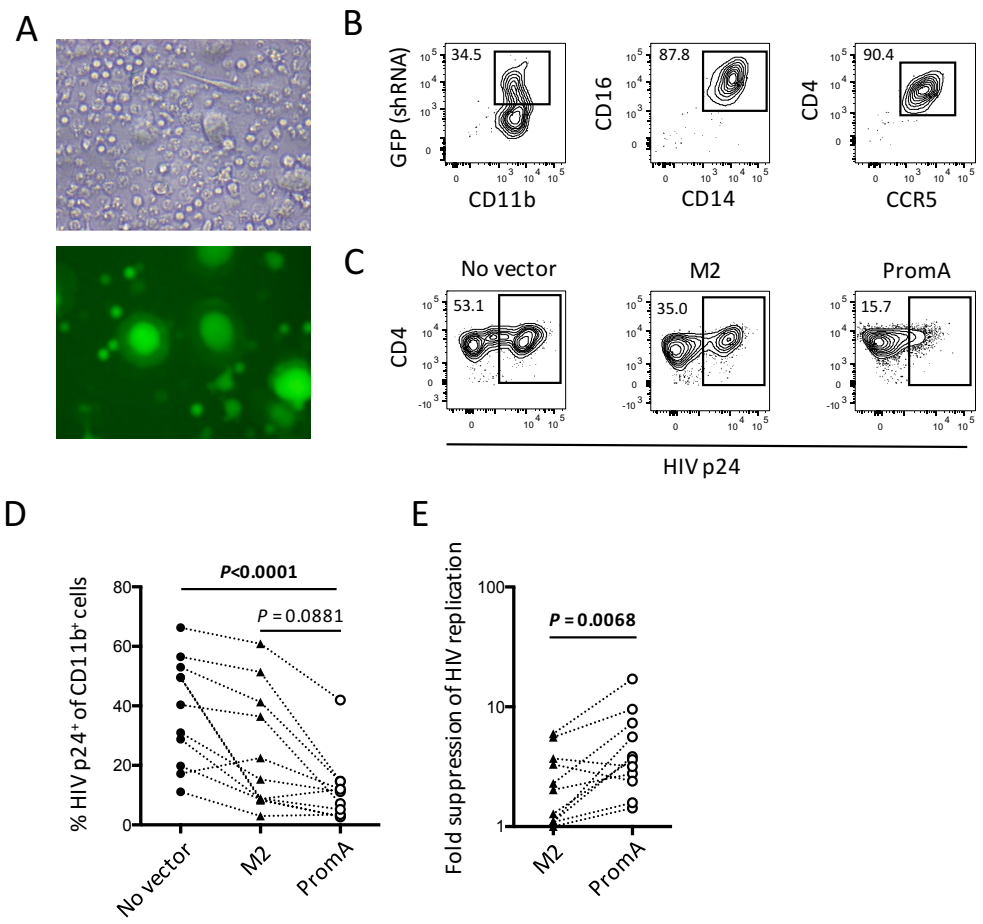

Fig 6. Freshly isolated CD34$^+$ cells were transduced with shPromA and differentiated to macrophages in vitro. Cells were then infected with HIV-1$_{JRFL}$. HIV replication levels were compared to autologous mock-transduced or shPromA-M2-transduced macrophages on day 5 post infection. (A) Fluorescence microscopy analysis showed PromA-expressing (GFP$^+$) macrophages with the typical fried-egg shapes. (B) Representative plots showing expression patterns of GFP (PromA)/CD11b (left) in live cells, as well as expression patterns of CD14/CD16 (middle), and CD4/CCR5 (right) in CD11b$^+$ cells. (C) Representative plots showing intracellular HIV p24$^+$ frequencies in mock-transduced, shPromA M2-transduced, and shPromA-transduced macrophages on day 5 post infection. (D) HIV replication was significantly lower in the shPromA-transduced samples than in mock-transduced samples. Nonparametric multiple comparison analysis was performed with the Dunn's method. (E) Fold suppression of HIV replication relative to mock-transduced samples was calculated for PromA M2-transduced and shPromA$_{JRFL}$-transduced samples. The shPromA$_{JRFL}$-transduced macrophages showed significantly higher suppressive capacity. Comparison was done by the Wilcoxon matched-pairs signed rank test or Dunn's method of multiple comparison analysis.

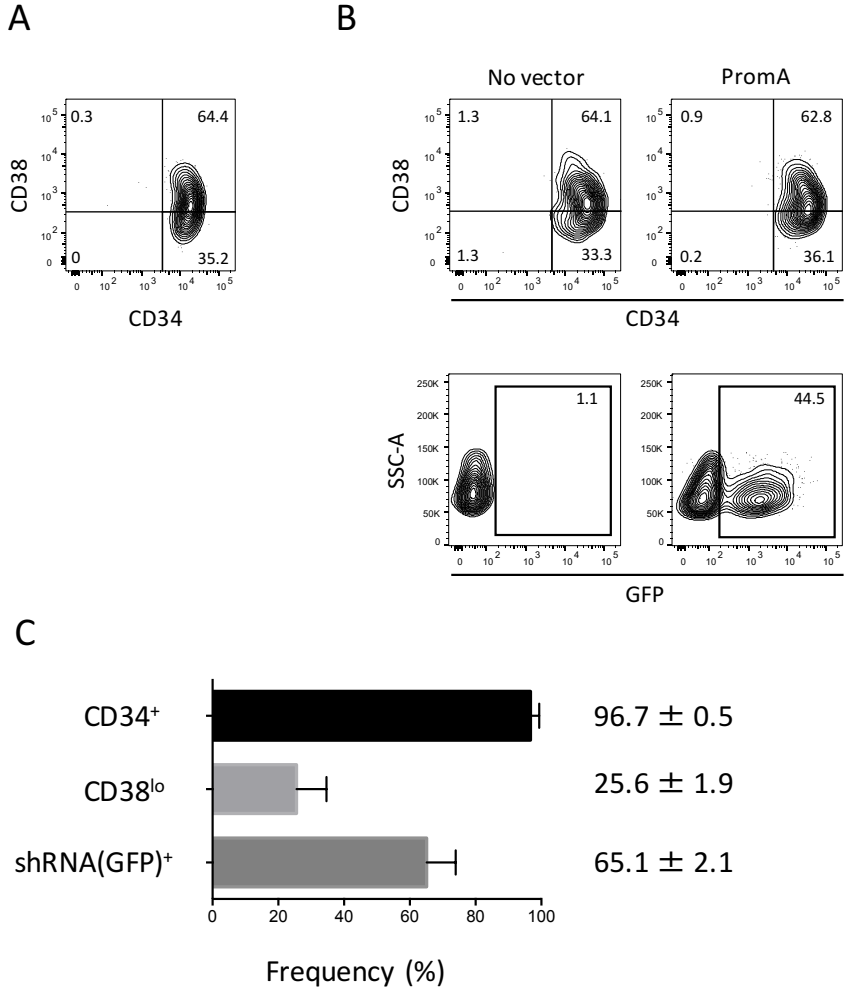

Fig S1. Fresh cord-derived CD34$^+$ cells were isolated and transduced with a lentivirus vector expressing the shRNA PromA adapted to HIV-1$_{JRFL}$ (shPromA$_{JRFL}$ or simply shPromA). (A) A representative plot showing the CD34/CD38 expression pattern in freshly isolated CD34$^+$ cells. (B) Representative plots showing the lentiviral transduction efficiency measured by GFP expression. (D) Summary of the CD34$^+$ cell isolation results (upper and middle bars) and the efficiency of the following lentiviral transduction (lower bar).

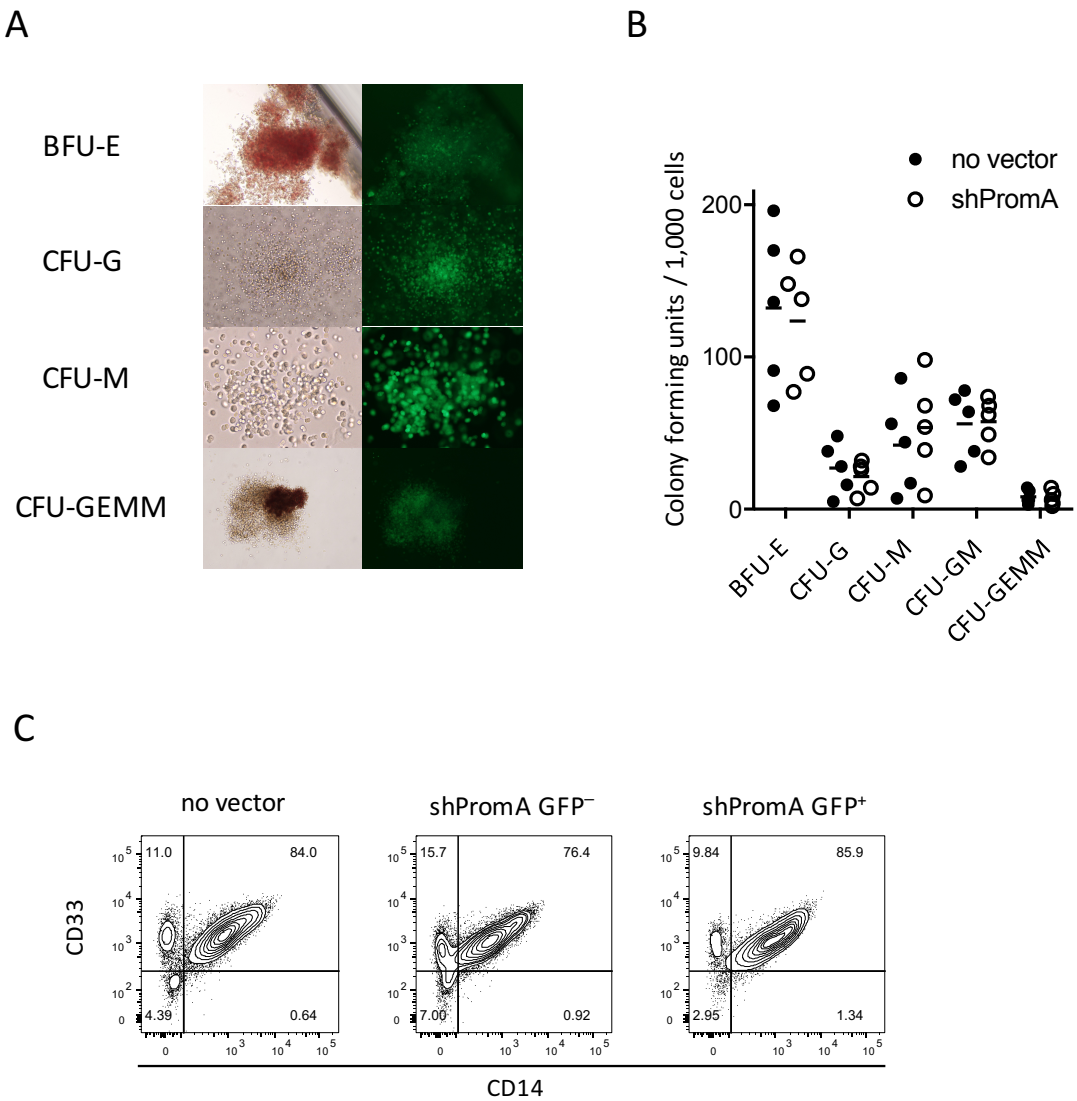

Fig S2. PromA-transduced CD34$^+$ cells showed the same colony forming capability to mock-transduced CD34$^+$ cells. (A) Different types of GFP$^+$ colonies were found. (B) PromA-transduced CD34$^+$ cells showed no significant difference to mock-transduced cells in colony forming units of all the different colony types tested. (C) Colony forming cells were collected together for flow cytometric analysis. CD14/CD33 expression levels were compared between mock-transduced, shPromA-transduced GFP$^-$, and PromA-transduced GFP$^+$ cells.

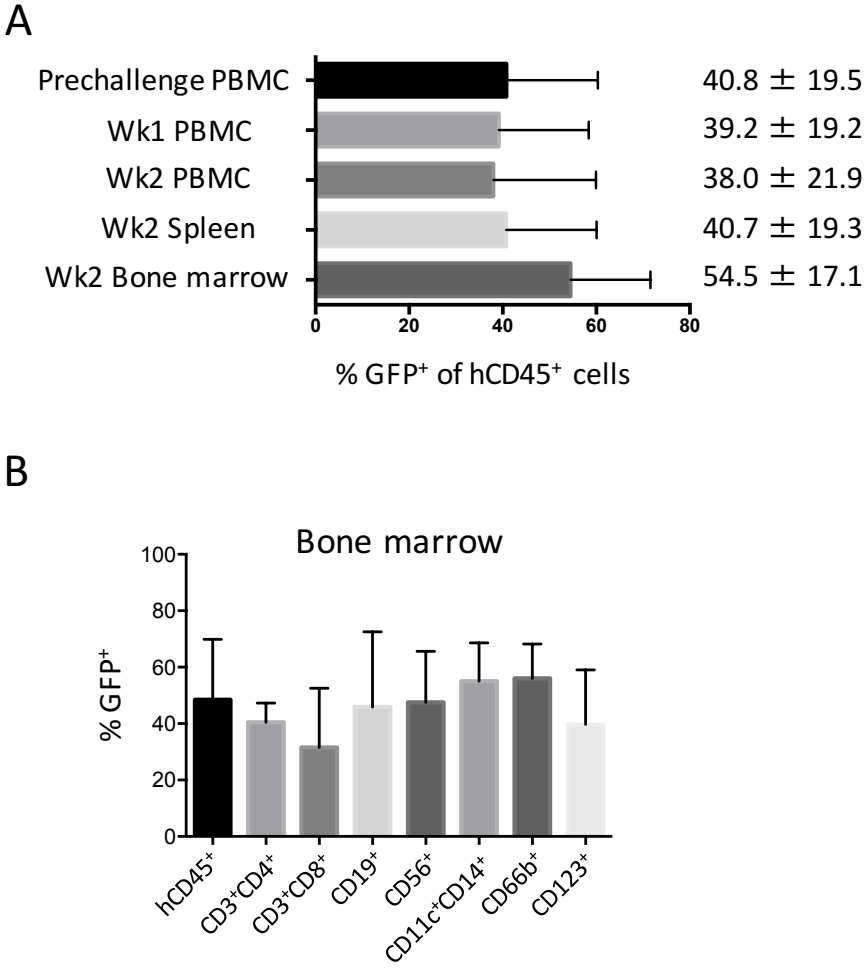

Fig S3. Engraftment of human cells in transplanted NOJ mice was tested 15 weeks after transplantation. Data associated with Fig 2. (A) PromA-expressing frequencies in human $CD45^+$ cells, expressed as GFP^+ frequencies, in group PromA are shown. These were tested in mouse PBMC (prechallenge, week 1, week 2), splenocytes (week 2) and bone marrow cells (week 2) (B) The mean GFP^+ percentages in the different subsets of cells in bone marrow.

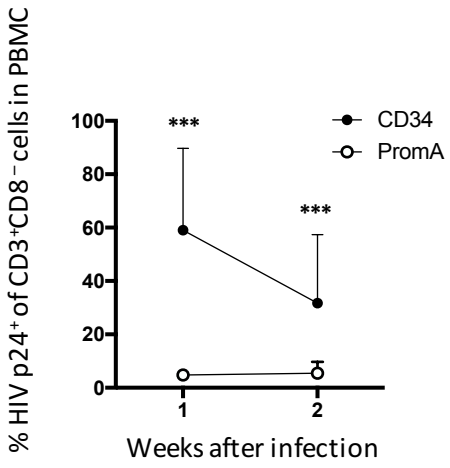

Fig S4. Data associated with Fig 4. Intracellular HIV p24^{+} frequencies in CD3^{+}CD8^{-} T cells were compared between group CD34 and PromA. The analysis was done using PBMC obtained at weeks 1 and 2 post challenge. Comparison was done by the Mann-Whitney test. ***: $P<0.001$.

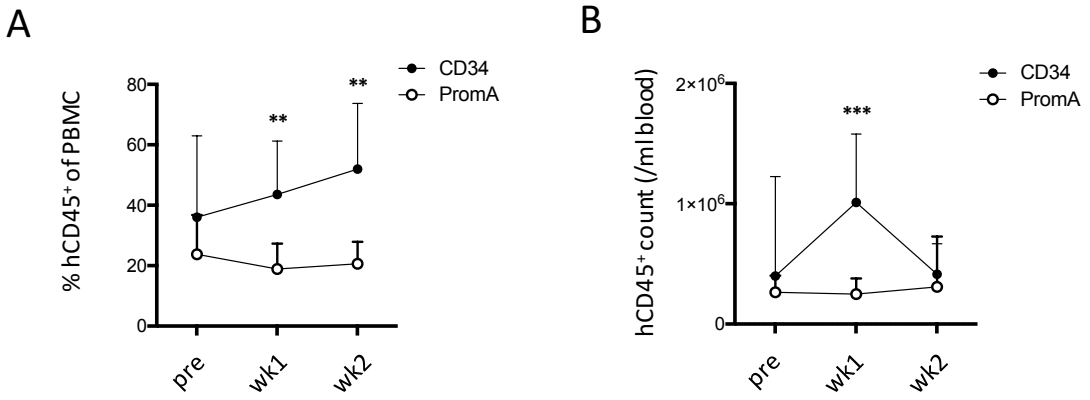

Fig S5. Data associated with Table 1. Blood CD45$^+$ frequencies were analyzed. (A) Human CD45$^+$ percentages in PBMC at pre-challenge, week 1, and week 2. (B) Human CD45$^+$ cell counts in blood (/ml) at pre-challenge, week 1, and week 2. Comparison was done by the Mann-Whitney test. **: P<0.01, ***: P<0.001.

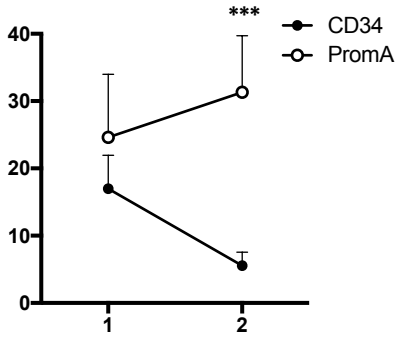

Fig S6. Data associated with Fig 5. $CD4^{+}CCR5^{+}$ T-cell frequencies in PBMCs were analyzed at weeks 1 and 2 post challenge. Comparison was done by the Mann-Whitney test. ***: $P<0.001$.

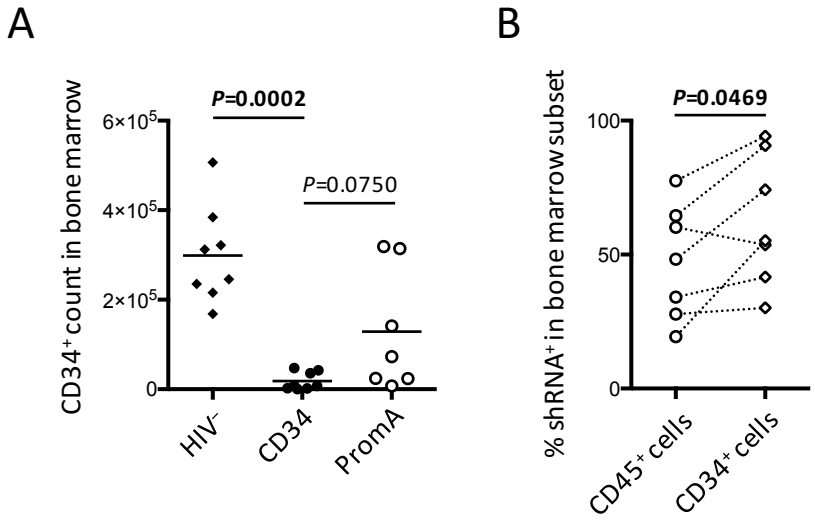

Fig S7. Data associated with Fig 5. Bone marrow cells were collected from the left femur of each animal 2 weeks after infection and analyzed. (A) $CD34^+$ cell counts. Nonparametric multiple comparison analysis was performed with the Dunn's method. (B) The shPromA-expressing (GFP^+) frequencies in $CD45^+$ and $CD34^+$ cells. Comparison was done by the Wilcoxon matched-pairs signed rank test (B).

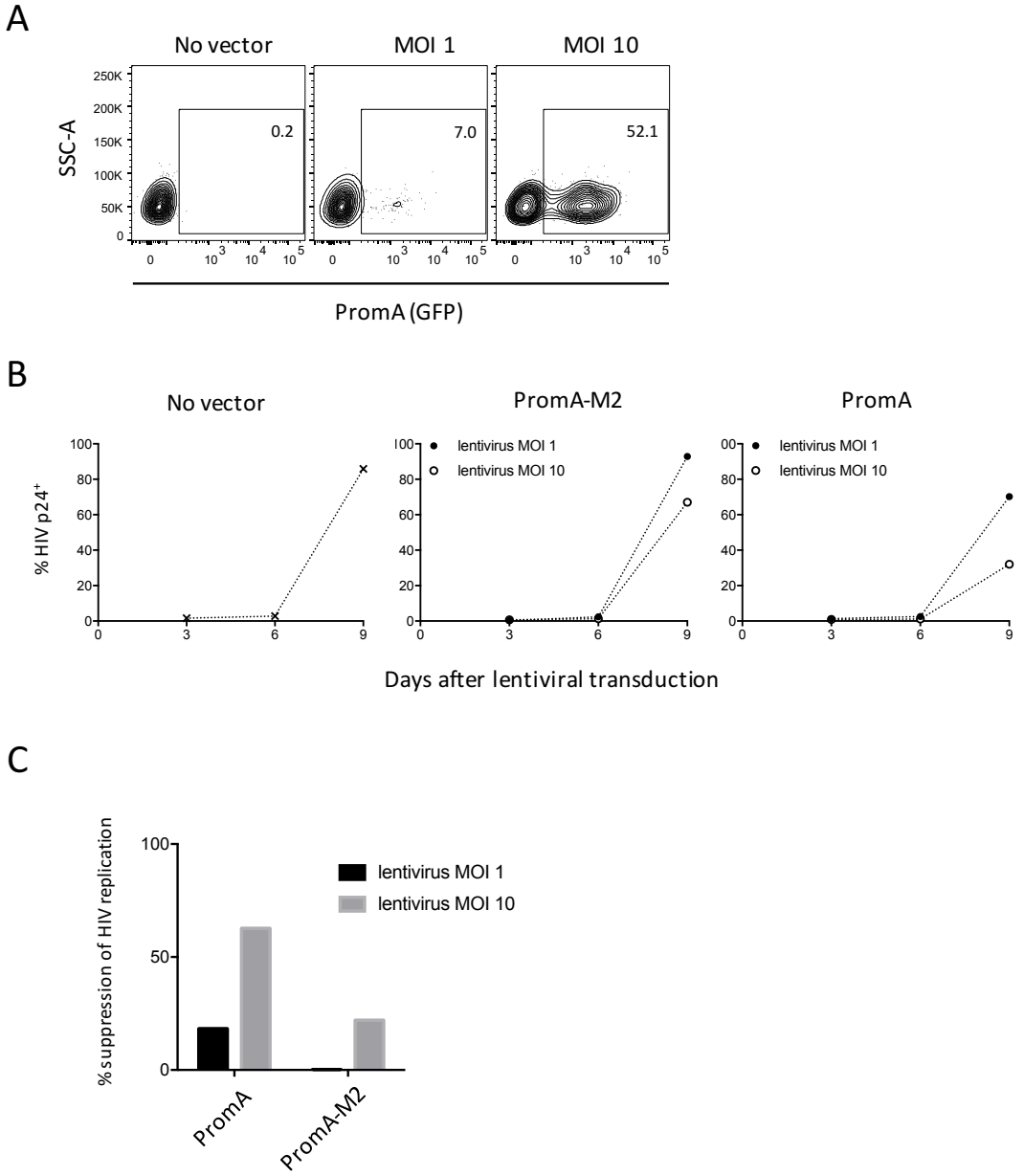

Fig S8. Data associated with Fig 6. The efficacy of shPromA to suppress HIV infection was tested in vitro using the PM1-CCR5 cell line. Cells were infected with HIV-1_{JRFL} at an MOI of 0.01. Twenty-four hours later, cells were seeded in RetroNectin-coated 48-well plate at the concentration of 5,000 cells/well and transduced with the lentivirus expressing shPromA at an MOI of 1 or 10. Cells were analyzed at days 3, 6, and 9 post-transduction for the intracellular expression of HIV $p24^+$. (A) Transduction rates as determined by the expression of GFP analyzed by flow cytometry. The lentiviral MOIs were indicated. (B) Intracellular HIV $p24^+$ percentages monitored at days 3, 6, and 9 post-transduction. (C) Percentages of suppression of HIV replication relative to the untransduced control at day 9 post-transduction were calculated by the data shown in B.

Tables

	CD34 uninfected	CD34 infected	PromA uninfected	PromA infected
No. of samples	8	8	3	7
Spleen: $CD45^+$ count	$1.7\times10^7 \pm 1.0\times10^7$	$3.4\times10^6 \pm 3.3\times10^6$	$4.7\times10^6 \pm 1.2\times10^6$	$8.7\times10^6 \pm 6.2\times10^6$
Spleen: % $CD45^+$	94.5 ± 3.3	53.8 ± 22.0	94.6 ± 5.6	55.2 ± 19.1
Spleen $CD45^+$: % GFP^+	0	0	51.6 ± 11.7	40.7 ± 19.3
Spleen $CD3^+$: % GFP^+	0	0	36.0 ± 4.5	32.5 ± 25.5
Marrow: $CD45^+$ count	$2.1\times10^6 \pm 9.9\times10^5$	$5.4\times10^5 \pm 3.9\times10^5$	$7.1\times10^5 \pm 6.2\times10^5$	$1.1\times10^6 \pm 9.9\times10^5$
Marrow: % $CD45^+$	90.1 ± 5.4	77.3 ± 21.7	62.0 ± 28.3	37.9 ± 17.9
Marrow $CD45^+$: % GFP^+	0	0	48.4 ± 21.5	47.4 ± 21.3
Marrow $CD34^+$: % GFP^+	0	0	N.D.	62.8 ± 24.3

Table 1. Comparison of spleen/bone marrow cells among the animal groups untransduced (CD34) uninfected, untransduced (CD34) infected, PromA-transduced uninfected, and PromA-transduced infected. Values are presented as mean ± SD (standard deviation). Bone marrow cells were collected from the left femur of each animal. Samples were collected at week 15–18 post transplantation, or at week 2 post challenge for HIV-infected animals (Fig. 3). N.D.: not determined.