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In vivo selection of anti-HIV-1 gene-modified human hematopoietic stem/progenitor cells to enhance engraftment and HIV-1 inhibition

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Hematopoietic stem/progenitor cell (HSPC)–based anti-HIV-1 gene therapy holds great promise to eradicate HIV-1 or to provide long-term remission through a continuous supply of anti-HIV-1 gene-modified cells without ongoing antiretroviral therapy. However, achieving sufficient engraftment levels of anti-HIV gene-modified HSPC to provide therapeutic efficacy has been a major limitation. Here, we report an *in vivo* selection strategy for anti-HIV-1 gene-modified HSPC by introducing 6-thioguanine (6TG) chemoresistance through knocking down hypoxanthine-guanine phosphoribosyl transferase (HPRT) expression using RNA interference (RNAi). We developed a lentiviral vector capable of co-expressing short hairpin RNA (shRNA) against HPRT alongside two anti-HIV-1 genes: shRNA targeting HIV-1 co-receptor CCR5 and a membrane-anchored HIV-1 fusion inhibitor, C46, for efficient *in vivo* selection of anti-HIV-1 gene-modified human HSPC. 6TG-mediated preconditioning and *in vivo* selection significantly enhanced engraftment of HPRT-knockdown anti-HIV-1 gene-modified cells (>2-fold, $p < 0.0001$) in humanized bone marrow/liver/thymus (huBLT) mice. Viral load was significantly reduced (>1 log fold, $p < 0.001$) in 6TG-treated HIV-1-infected huBLT mice compared to 6TG-untreated mice. We demonstrated that 6TG-mediated preconditioning and *in vivo* selection considerably improved engraftment of HPRT-knockdown anti-HIV-1 gene-modified HSPC and repopulation of anti-HIV-1 gene-modified hematopoietic cells in huBLT mice, allowing for efficient HIV-1 inhibition.

INTRODUCTION

HIV-1 infection has been a significant health problem in humans for more than 40 years.[1–3] Highly active antiretroviral therapy (HAART) has significantly improved patient life expectancy and quality of life.[4,5] However, HAART is limited by the presence of latent HIV-1 viral reservoirs, which force the need for lifelong treatment.[6–8] As patients live longer, they can face major health challenges such as drug side effects, difficulty in adherence, accumulated costs, and long-term complications of HIV-1 infection.[9–13] These challenges highlight the importance of developing novel therapeutic approaches capable of HIV-1 cure.[7,14]

The recent cases of HIV-1 cure by using naturally HIV-1 resistant CCR5Δ32/Δ32 bone marrow for hematopoietic stem cell transplantation (HSCT) offer great promise for the development of an hematopoietic stem/progenitor cell (HSPC)–based anti-HIV-1 gene therapy for long-term HIV-1 remission or cure.[15–20] However, due to the limitations in the efficiency of vector transduction and engraftment of anti-HIV-1 gene-modified HSPC, previous clinical trials had low levels of gene-modified HSPC reconstitution and thus were unable to achieve therapeutic benefit in patients.[21–24]

To enhance gene-modified HSPC engraftment, we previously developed a combined preconditioning and *in vivo* selection strategy using a clinically available guanosine analog antimetabolite, 6-thioguanine (6TG), for the selection of hypoxanthine-guanine phosphoribosyl transferase (HPRT)-knockout mouse bone marrow in C57BL/6J mice.[25,26] 6TG is metabolized by HPRT, and the resulting active toxic metabolite is incorporated into DNA and RNA, causing cytotoxicity. HPRT-deficient cells can prevent the formation of the active toxic metabolite and enable the enrichment of HPRT-deficient HSPC. This combined 6TG-mediated preconditioning and *in vivo* selection strategy consistently achieved >95% engraftment of HPRT-deficient donor bone marrow in C57BL/6J mice without significant toxicity and in the absence of irradiation or any other preconditioning regimen.[26] Additional studies achieving HPRT knockdown by lentiviral-mediated expression of short hairpin RNA (shRNA) against

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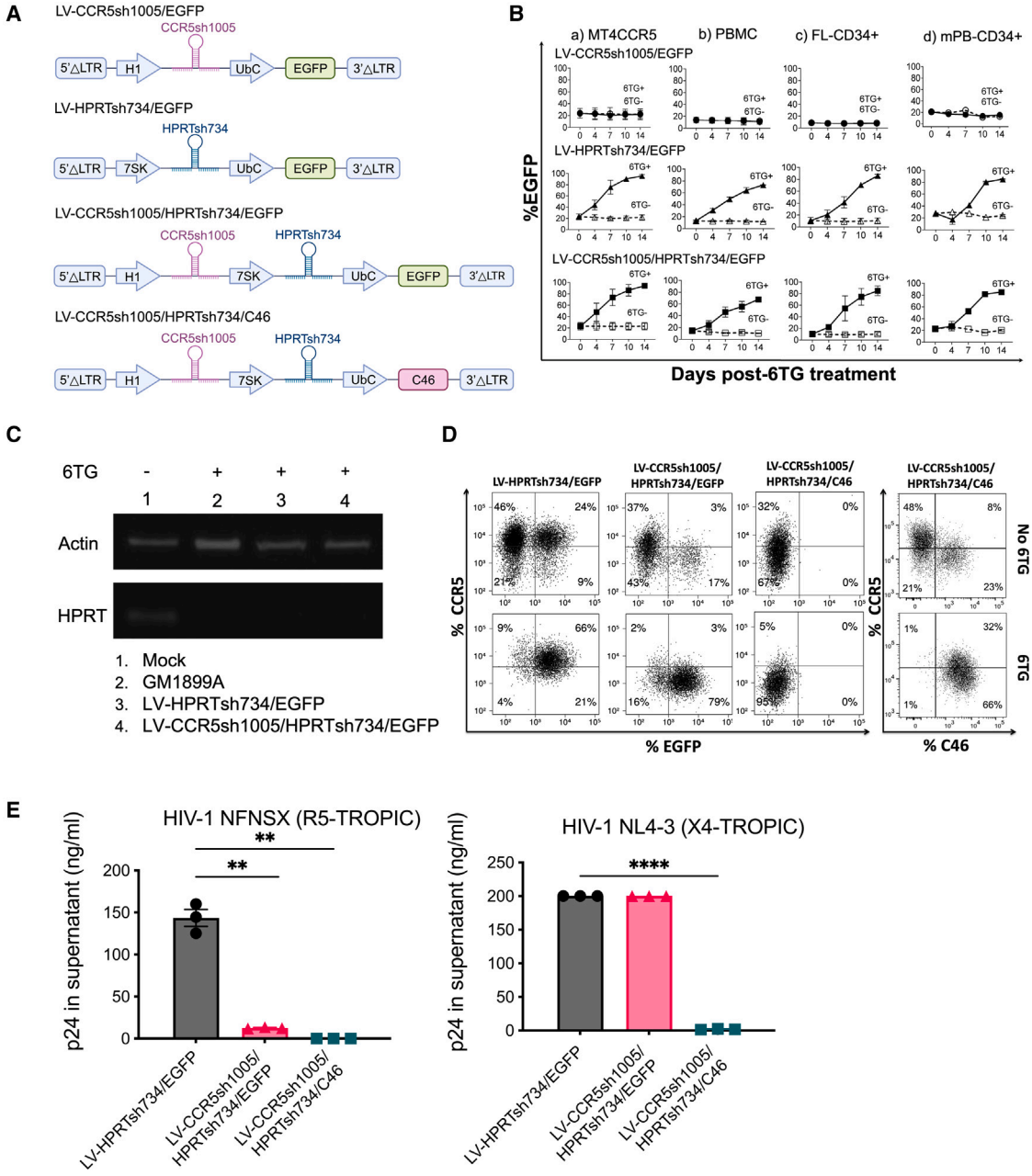

Figure 1. *In vitro* characterization of 6TG selectable anti-HIV lentiviral vectors with HPRTsh734

(A) Lentiviral vectors for expressing HPRTshRNA734, a fluorescent marker EGFP gene, and/or anti-HIV-1 genes (CCR5shRNA1005, C46). 7SK, 7SK RNA polymerase III promoter; H1, H1 RNA polymerase III promoter; LTR, long terminal repeat; UbC, ubiquitin C RNA polymerase II promoter. (B) 6TG-mediated selection of HPRTsh734 vector-transduced cells. Various human cells (CCR5 MT4 cell line, PBMC, FL-CD34$^+$ cells, mPB CD34$^+$ cells) were transduced at 10%–20% EGFP$^+$, with lentiviral vectors expressing HPRTsh734 (LV-HPRTsh734/EGFP or LV-CCR5sh1005/HPRTsh734/EGFP). LV-CCR5sh1005/EGFP vector was used as a negative control. Vector-transduced cells were cultured for 14 days with 6TG (0.3 μM) or without 6TG. %EGFP expressions were monitored by flow cytometry during the 14-day 6TG treatment. Solid lines and closed circle symbols indicate 6TG treatment (6TG$^+$). Dotted lines and open circle symbols indicate without 6TG treatment (6TG$^-$). Bars and error bars show means ± standard deviations (SDs). (C) HPRT downregulation in 6TG selected HPRTsh734 vector-transduced CCR5 MT4 cell line. Whole-cell lysates were probed for HPRT by anti-HPRT antibodies and analyzed HPRT protein expression by western blot. Mock transduced cells without 6TG (lane 1) were used as a control to detect HPRT expression. GM1899A, an HPRT-deficient lymphoblast cell line (lane 2), was used as a control for absence of HPRT expression. Actin was included as the loading control. (D) CCR5 downregulation by the vectors with CCR5sh1005 in vector-transduced MT4-CCR5 cell line. Representative flow cytometry plots of EGFP or C46 (x axis) versus CCR5 (y axis) are shown without 6TG (top, 6TG$^-$) or with 6TG (bottom, 6TG$^+$) treatment. LV-HPRTsh734/EGFP vector-transduced cells were used as a control for CCR5 expression

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HPRT have shown to confer 6TG resistance *in vitro* and to enable highly efficient selection of HPRT-shRNA transduced murine and human HSPC *in vivo*.[26–30]

In the present study, we applied 6TG-mediated preconditioning and *in vivo* selection for anti-HIV-1 HSPC-based gene therapy. We developed a lentiviral vector capable of co-expressing shRNA against HPRT alongside shRNA against HIV-1 co-receptor CCR5 and a membrane-anchored HIV-1 fusion inhibitor, C46, allowing for 6TG-mediated selection of anti-HIV-1 gene-modified HSPC. We further investigated whether combined 6TG-mediated preconditioning and *in vivo* selection can be used to significantly improve the engraftment and repopulation of anti-HIV-1 gene-modified HSPC in the humanized bone marrow/liver/thymus (huBLT) mouse model, thereby allowing for efficient HIV-1 inhibition.

RESULTS

Development of a 6TG-selectable anti-HIV-1 lentiviral vector

We cloned 26 shRNA candidates targeting different HPRT mRNA sequences into a lentiviral vector expressing EGFP. These 26 shRNAs were screened in the vector-transduced K562 cell lines by 6TG-mediated selection *in vitro* (Figure S1). The most efficient shRNA (designated HPRTsh734) was selected for further development of a 6TG-selectable anti-HIV-1 lentiviral vector (Figure 1A). We used 7SK and H1 RNA polymerase III promoters to co-express HPRTsh734 and our previously identified shRNA against CCR5 (CCR5sh1005),[31] respectively, to avoid promoter interference and vector recombination within a lentiviral vector. Average titers of 100-fold concentrated vectors were consistently high at 1–2 × 10^8 IU/mL based on EGFP expression. Human MT4-CCR5 cell line, human peripheral blood mononuclear cells (PBMCs), human fetal liver (FL)–derived FL-CD34$^+$, and human mobilized peripheral blood (mPB)-CD34$^+$ were transduced with relatively low transduction efficiencies of 10%–20% EGFP expression to examine 6TG-mediated selection. EGFP expression remained stable at 10%–20% in the control non-HPRTsh734 vector (LV-CCR5sh1005/EGFP) transduced cells with and without 6TG treatment (Figure 1B). In contrast, 6TG treatment increased EGFP expression from 10%–20% to 90% in the HPRTsh734 vector-transduced cells (LV-CCR5sh1005/HPRTsh734/EGFP and LV-HPRTsh734/EGFP) (Figure 1B). HPRT protein expression was downregulated in 6TG-treated HPRTsh734 vector-transduced MT4-CCR5 cells (Figure 1C). CCR5 expression was downregulated in 6TG-untreated and 6TG-treated EGFP$^+$ vector-transduced MT4-CCR5 cells (Figure 1D). Finally, we substituted EGFP with a membrane anchored HIV-1 fusion inhibitor C46 to inhibit both R5 and X4-tropic HIV-1 (Figure 1A). CCR5 expression was downregulated in 6TG-untreated and 6TG-treated C46$^+$ vector-transduced MT4-CCR5 cells (Figure 1D). R5 tropic HIV-1_{NFNSX} and X4-tropic HIV-1_{NL4-3} infections were significantly inhibited in 6TG-selected vector-transduced MT4-CCR5 cells (>7-fold, $p < 0.01$, and >40-fold, $p < 0.0001$, respectively) (Figure 1E). These results demonstrated that our lentiviral vector expressing HPRTsh734 and two anti-HIV-1 genes (CCR5sh1005 and C46) allowed for highly efficient 6TG-mediated selection of vector-transduced cells and HIV-1 inhibition *in vitro*. We next investigated whether 6TG-mediated preconditioning and *in vivo* selection could enhance engraftment of HPRT-knockdown anti-HIV-1 gene-modified HSPC in huBLT mice.

6TG preconditioning and *in vivo* selection improved engraftment of anti-HIV gene-modified HSPC in huBLT mouse model

We initially examined whether the previously optimized 6TG regimen in C57BL/6J mice[26] could be applied for preconditioning and *in vivo* selection of HPRT-downregulated anti-HIV-1 gene-modified human HSPC in an huBLT mouse model. NSG mice were preconditioned with 6TG (10 mg/kg by intraperitoneal injection) and transplanted with human FL-CD34$^+$ HSPC transduced with a lentiviral vector expressing HPRTshRNA734, CCR5shRNA1005, and C46 and a piece of human fetal thymus (Figures 2A and 2B). huBLT mice were injected with 5 mg/kg 6TG every 3 days for *in vivo* selection of HPRT-knockdown vector-transduced cells. However, the huBLT mice presented significant weight loss (Figure S2) after 3 weeks of 6TG injections, suggesting that tolerability to 6TG may be lower in NSG mice than in C57/BL6 mice. Consequently, we reduced the frequency of 6TG treatment to once per week, and the mice remained healthy without significant weight loss for 8 weeks (Figure S2). No significant difference was observed in mouse health status and survival between 6TG-treated and -untreated groups when the adjusted 6TG dose was used for *in vivo* selection (Figure S3). Reconstitution of human multi-lineage hematopoietic cells was similar in 6TG-treated and 6TG-untreated huBLT mice in peripheral blood (Figure 2C) and various tissues including the human thymus transplant, spleen, bone marrow, and lung for 17 weeks postvector-transduced HSPC transplantation (Figure 2D). To assess whether the 6TG treatment improved the engraftment of HPRT-knockdown anti-HIV-1 gene vector-transduced HSPC, we measured vector copy number (VCN) per human cell (β-globin DNA copy) by digital PCR (dPCR). Average VCN per human cell in peripheral blood (Figure 2E) increased approximately 2-fold ($p < 0.0001$) in 6TG-treated huBLT mice (1.34 ± 0.48 vector copies/cell) compared to 6TG-untreated huBLT mice (0.68 ± 0.33 copies/cell) over the course of the experiment for 16 weeks postvector-transduced HSPC transplantation, and achieved similar levels in spleen and bone marrow tissue (Figure 2F). The reproducibility of successful selection was 83% determined by a >2-fold increase in vector marking in 5 of 6 independent experiments. These results demonstrated that 6TG-mediated preconditioning and *in vivo* selection could effectively enhance the engraftment of HPRT-downregulated anti-HIV-1 gene-modified cells in huBLT mice.

in EGFP$^+$ population (left). (E) HIV-1 inhibition *in vitro*. After 6TG-mediated selection, LV-CCR5sh1005/HPRTsh734/EGFP, LV-CCR5sh1005/HPRTsh734/C46 vector, or LV-HPRTsh734/EGFP vector-transduced MT4-CCR5 cell lines were challenged with R5 tropic HIV-1_{NFNSX} (MOI 1) or X4-tropic HIV-1_{NL4-3} (MOI 5), respectively. Levels of HIV-1 p24 production (ng/mL) were measured in culture supernatant by ELISA at 7 days post-HIV-1_{NFNSX} challenge and 4 days post-HIV-1_{NL4-3} challenge. Data are shown from a single experiment performed in triplicate for each different tropic virus. The range of p24 detection was determined from a standard curve to be 0.031–200 ng/mL. The Mann-Whitney *U* test was performed to calculate significance. Bars and error bars show means ± SDs. **$p < 0.01$ and ***$p < 0.001$.

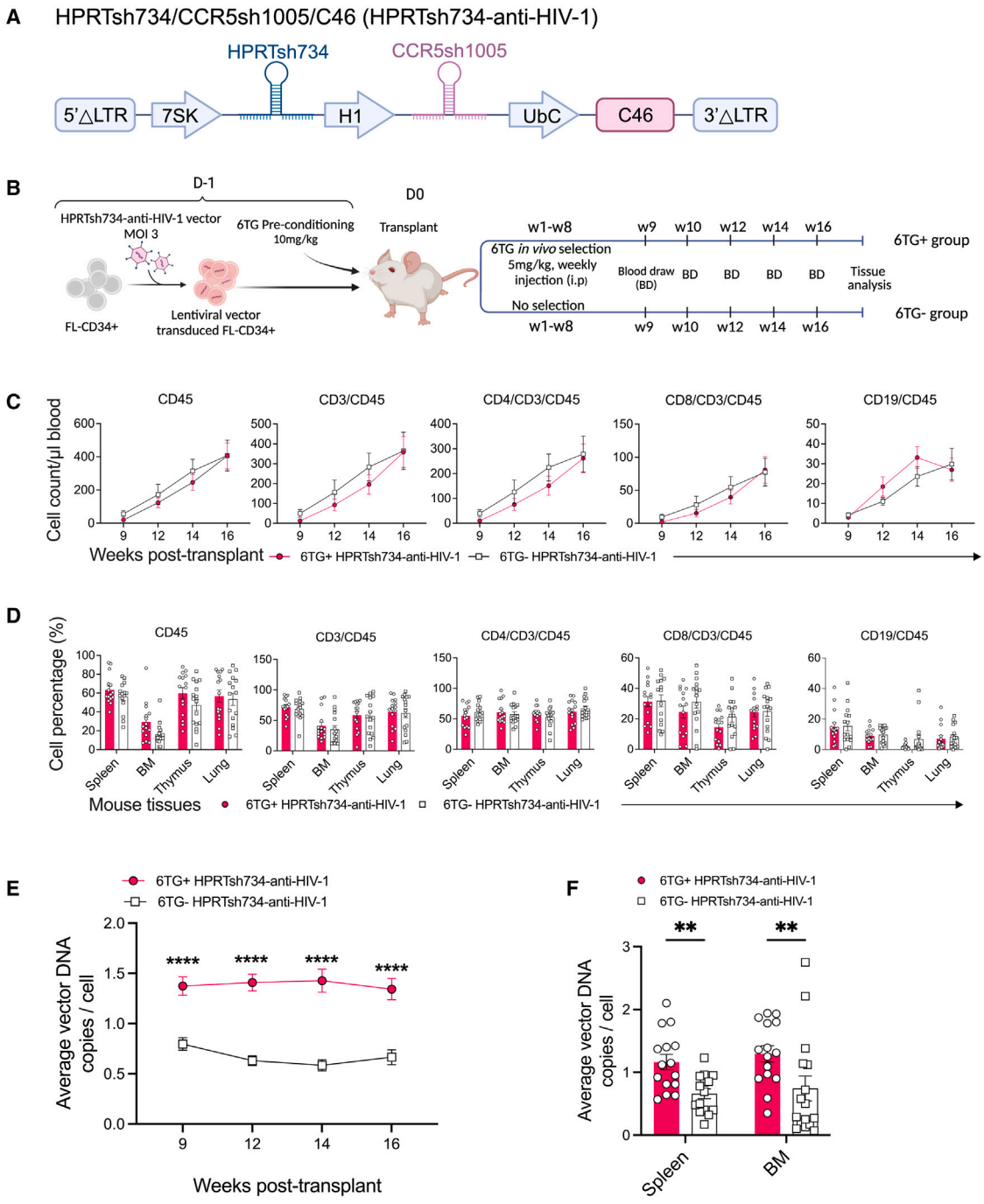

Figure 2. 6TG-mediated preconditioning and *in vivo* selection resulted in enhancement of HPRTsh734-anti-HIV-1 vector-modified human hematopoietic cells in huBLT mice

(A) Schematic review of a selectable HPRTsh734-anti-HIV-1 lentiviral vector. (B) The experimental design for the 6TG-mediated preconditioning and *in vivo* selection of vector-modified $CD34^+$ HSPC. HPRTsh734-anti-HIV-1 vector expresses HPRTsh734, CCR5sh1005, and C46. Human FL-$CD34^+$ cells were transduced with HPRTsh734-anti-HIV-1 lentiviral vector at MOI 3 on day −1. NSG mice were preconditioned with 6TG (10 mg/kg) at day −1. Mice were transplanted with the vector-transduced $CD34^+$ HSPC and human thymus pieces on day 0. huBLT mice were either treated ($6TG^+$) or untreated ($6TG^-$) with 6TG (5 mg/kg) weekly for 8 weeks (w). (C) Multi-lineage human hematopoietic cell reconstitution in peripheral blood. Absolute human cell (CD45, CD3, CD4, CD8, CD19) count per μL of human peripheral blood were monitored from 9 to 16 weeks postvector-transduced HSPC transplantation in $6TG^+$ and $6TG^-$ huBLT mice. Bars and error bars show means ± SEMs. (D) Multi-lineage human hematopoietic cell reconstitution in tissues (spleen, bone marrow, human thymus implant, lung) in $6TG^+$ and $6TG^-$ huBLT mice. Bars and error bars show means ± SEMs. (E) Vector marked

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6TG preconditioning and *in vivo* selection enabled efficient HIV inhibition in HPRT-knockdown anti-HIV gene-modified HSPC transplanted huBLT mice

To examine HIV-1 inhibition, we transduced human FL-CD34$^+$ HSPC with an HPRTsh734-anti-HIV-1 vector (HPRTshRNA734/CCR5sh 1005/C46) or a control HPRTsh734 vector (HPRTshRNA734/CCR5sh 1005/EGFP) (Figure 3A). Vector-transduced HSPC were transplanted in 6TG-preconditioned NSG mice to create huBLT mice (Figure 3B). 6TG-mediated *in vivo* selection significantly enhanced the engraftment of HPRTsh734-anti-HIV-1 vector-transduced HSPC approximately 3-fold in 6TG-treated huBLT mice (1.10 ± 0.19 VCNs/cell) compared to 6TG-untreated huBLT mice (0.33 ± 0.18 VCNs/cell) at 9 weeks post-HSPC transplantation (Figure 3C). 6TG-mediated selection also significantly enhanced the engraftment of HPRTsh734 vector-transduced HSPC in the control group (1.71 ± 0.84 VCN/cell). We previously demonstrated R5-tropic HIV-1 inhibition with anti-HIV-1 vector-transduced HSPC transplanted huBLT mice.[32] In the present study, we extended our investigation for X4-tropic HIV-1 inhibition. Consequently, these huBLT mice were challenged with X4-tropic HIV-1_{NL4-3} (200 ng/p24 via intravenous injection) (Figure 3B). Viral loads were significantly reduced (>1-log fold, $p < 0.05$) in 6TG-treated HPRTsh 734-anti-HIV-1 vector-transduced HSPC-transplanted huBLT mice compared to 6TG-untreated HPRTsh734-anti-HIV-1 vector-transduced HSPC-transplanted huBLT mice at 2 and 4 weeks post-HIV-1 challenge (Figure 3D). Viral loads were high in the control HPRTsh 734 vector huBLT mice because X4-tropic HIV-1 should not be inhibited by the control vector due to the lack of C46 expression. Successful HIV-1 inhibition by HSPC-based gene therapy is dependent in part on achieving sufficient levels of anti-HIV-1 gene modification to protect cells from HIV-1 infection. We examined whether a certain threshold level of anti-HIV-1 gene-modified cells would be required to effectively reduce viral load in huBLT mice (Figure 3E). Viral load decreased from above 10^5 viral RNA copies/mL plasma to under 10^4 viral RNA copies/mL plasma as average vector DNA copies increased to 0.5 copies per cell. Beyond 0.5 copies per cell, viral load plateaued at $\sim 10^3$ copies/mL at week 4 postinfection in 6TG-treated huBLT mice. A moderate inverse correlation was seen between vector copies and viral load ($r = -0.549$, $p = 0.006$). We therefore estimated that 0.5 copies/cell of our HPRTsh734-anti-HIV-1 vector is the minimum vector marking the level required to inhibit HIV-1_{NL4-3} infection >1-log in huBLT mice. These results demonstrated that 6TG-mediated *in vivo* selection enriched anti-HIV-1 gene-modified HSPC in huBLT, thereby allowing efficient HIV-1 inhibition.

DISCUSSION

In this study, we investigated whether combined 6TG-mediated preconditioning and *in vivo* selection improves the engraftment of HPRT-knockdown anti-HIV-1 gene-modified HSPC in the huBLT mouse model. We developed a lentiviral vector that successfully co-expresses HPRTshRNA734 to downregulate HPRT expression for 6TG-mediated *in vivo* selection alongside two anti-HIV-1 genes: shRNA1005 to downregulate HIV-1 co-receptor CCR5 and HIV-1 fusion inhibitor C46. Our results demonstrated that combined 6TG-mediated preconditioning and *in vivo* selection significantly improved the engraftment of HPRT-knockdown anti-HIV-1 gene-modified HSPC, allowing for efficient HIV-1 inhibition in the huBLT mouse model.

Successful HIV-1 cures have been achieved for a handful of HIV-1 patients who had developed leukemia and were treated by HSCT using naturally HIV-1-resistant homozygous *CCR5* Δ32/Δ32 allogeneic donors.[15–20] Although these HIV-1 cures have provided proof of concept that HIV-1 eradication is possible, they required intensive myeloablative conditioning by chemotherapeutic drugs and irradiation for the complete engraftment of HIV-1-resistant homozygous *CCR5* Δ32/Δ32 allogeneic donor cells, which could be an unfavorable risk–benefit calculus for the majority of HIV-1 patients. Furthermore, naturally HIV-1-resistant homozygous *CCR5* Δ32/Δ32 allogeneic donors are very rare, and the human leukocyte antigen matching required for allogeneic HSCT reduces further the likelihood of finding a suitable donor.[33]

A successful HSPC-based anti-HIV-1 gene therapy holds great hope for HIV-1 eradication. However, the successful development of an HSPC-based anti-HIV-1 gene therapy has been facing major limitations due to low engraftment levels of gene-modified HSPC, which failed to achieve significant levels of reconstitution to inhibit HIV-1.[34–37] Thus, an effective strategy to enhance the engraftment of anti-HIV-1 gene-modified HSPC is highly desirable for the efficient inhibition and functional cure of HIV-1 infection. One successful *in vivo* selection strategy for anti-HIV-1 HSPC gene therapy to date employed $MGMT_{P140K}$, which confers resistance to DNA damaging agents such as 1,3-bis (2chloroethyl)-1-nitrosurea and O^6-benzylguanine. $MGMT_{P140K}$ expressed from retroviral/lentiviral vectors enables the selection of transduced HSPC in mice[38,39] and pigtail macaques,[39–41] and has been tested in clinical trials for myeloprotection in glioblastoma patients.[42] $MGMT_{P140K}$ *in vivo* selection was applied for a C46 anti-HIV-1-expressing vector-transduced HSPC transplantation study in pigtail macaques.[43]

Our *in vivo* selection strategy using RNAi-based technology to downregulate HPRT expression to confer resistance to 6TG has advantages over existing *in vivo* selection strategies. For example, chemoresistance is conferred by small and non-immunogenic shRNA. Because our lentiviral vector co-expresses anti-HIV-1 genes (CCR5shRNA1005 and C46), decreasing the size of the vector by using an shRNA is advantageous over a large protein molecule such as $MGMT_{P140K}$. It would also facilitate vector design and manufacturing. Naturally occurring HPRT deficiency has a negligible impact on stem cell pluripotency and steady-state hematopoiesis,[44–47] although generalized HPRT deficiency in humans

cell level in peripheral blood of 6TG$^+$ or 6TG$^-$ huBLT mice. (F) Vector marked cell level in tissues of 6TG$^+$ or 6TG$^-$ huBLT mice. The levels of vector DNA marking were determined by a vector-specific (WPRE) and human β-globin gene-specific dPCR assay from 9 to 16 weeks in peripheral blood cells (E) and tissues (spleen and bone marrow) (F) of 6TG$^+$ and 6TG$^-$ huBLT mice. The average vector DNA copies per human cell were calculated by (VCN) = WPRE copies/(β-globin copies/2). Bars and error bars show means ± SEMs. Symbols represent individual mice. The Mann-Whitney *U* test was performed to calculate significance. **p < 0.01; ***p < 0.001; and ****p < 0.0001.

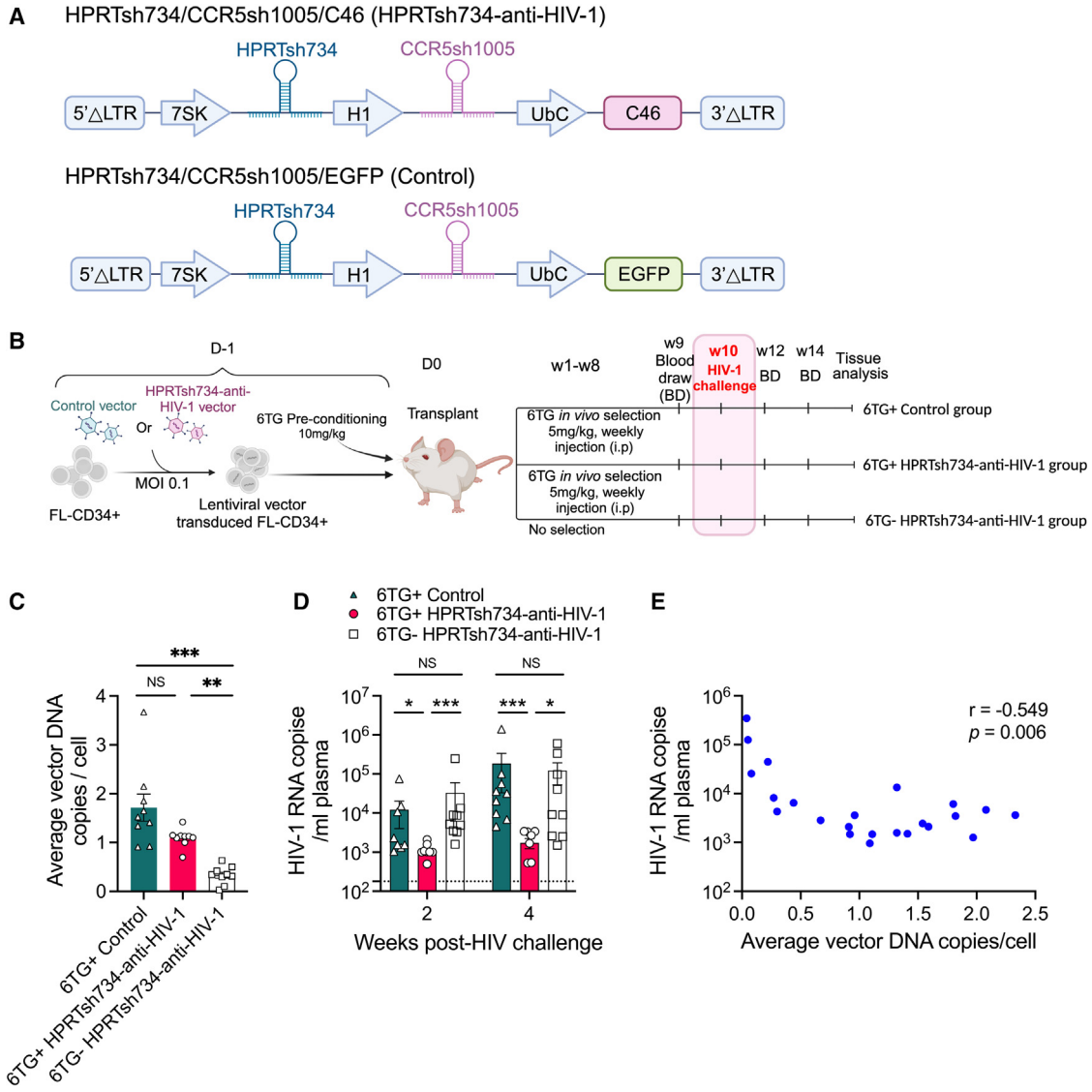

Figure 3. HIV-1 inhibition in 6TG selected HPRTsh734-anti-HIV-1 vector-modified HSPC transplanted huBLT mice

(A) Lentiviral vectors for HIV-1 inhibition in the huBLT mouse experiments. HPRTsh734-anti-HIV-1 lentiviral vector expresses HPRTsh734, CCR5sh1005, and C46. HPRTsh734/CCR5sh1005/EGFP lentiviral vector, in which C46 was replaced with EGFP, was used as a negative control for X4-tropic HIV-1_{NL4-3} infection. (B) Experimental design to investigate HIV-1 inhibition in huBLT mice. Human FL-CD34$^+$ cells were transduced either with a control vector or HPRTsh734-anti-HIV-1 lentiviral vector at MOI 0.1 on day −1. NSG mice were preconditioned with 6TG (10 mg/kg) at day −1. Mice were transplanted with the vector-transduced CD34$^+$ HSPC and human thymus pieces on day 0. huBLT mice were either treated (6TG$^+$) or untreated (6TG$^-$) with 6TG (5 mg/kg) weekly for 8 weeks. Mice were challenged with HIV-1_{NL4-3} (200 ng of p24/mouse) at 10 weeks posttransplant. (C) Vector marked cell level in peripheral blood of 3 different groups huBLT mice at week 9 posttransplant. Bars and error bars show means ± SEMs. Symbols represent individual mice. The Mann-Whitney *U* test was performed to calculate significance. NS, not significant; **$p < 0.01$; and ***$p < 0.001$. (D) Efficient HIV-1 inhibition in 6TG$^+$ HPRTsh734-anti-HIV-1 vector-modified HSPC transplanted huBLT mice. Control HPRTsh734 vector-transplanted huBLT mice with 6TG treatment (6TG + Control) were used as a negative control. HIV-1 RNA copies per mL of mouse plasma collected every 2 weeks postchallenge was calculated by detecting for HIV-1_{NL4-3} gag region by dPCR. Data are shown as means ± SEMs. The Mann-Whitney *U* test was performed to calculate significance. NS, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p < 0.0001$. (E) Moderate inverse correlation between vector copies and viral load. Plasma HIV-1 RNA copies/mL and anti-HIV vector average marking levels in mice PBMCs were detected by dPCR at week 4 after HIV-1 challenge. Inverse correlations were analyzed using the Spearman nonparametric correlation test.

is associated with Lesch-Nyhan syndrome, an inborn error of purine metabolism associated with hyperuricemia and a spectrum of neurological manifestations depending on the degree of the HPRT enzymatic deficiency.[47] HPRT-null mice exhibit largely normal hematopoiesis and pluripotency upon reaching steady state.[44] The lack of a divergent hematopoietic and immune system phenotype is also reflected in HPRT

knockout mice.[26,47] 6TG has been used for decades for leukemia and autologous HSCT in adult and pediatric patients.[48,49] Hence, there is considerable data on the pharmacokinetic distribution, metabolism, safety profile, and toxicity in humans. Our current results in the huBLT mouse model and further investigation of the 6TG mediated selection for a longer period in a non-human primate model should provide us with a more concrete basis and rationale for a Phase I clinical trial in the future. Altogether, these results may provide a strong premise for the clinical application of 6TG for *in vivo* selection of HPRT-knockdown anti-HIV-1 gene-modified HSPC.

Thus far, the most successful HSPC-based gene therapy strategies have been developed for X-linked severe combined immunodeficiency disorder, adenosine deaminase–deficient severe combined immunodeficiency, and Wiskott-Aldrich syndrome, in which the gene-transduced HSPC and their progenies have a strong selective advantage for achieving highly efficient engraftment in patients.[50–52] However, many other diseases have not been effectively treated by HSPC-based gene therapy due to the lack of a selective advantage of gene-modified cells, resulting in low engraftment. We developed a safe and effective 6TG-mediated preconditioning and *in vivo* selection strategy for our HSPC-based anti-HIV-1 gene therapy strategy. Our strategy to down-regulate HPRT expression to provide resistance to 6TG to gene-modified cells for efficient *in vivo* positive selection could be applied for HSPC-based gene therapy for other hematopoietic diseases in which the low level of gene-modified cells is the primary limitation for providing therapeutic effects, such as sickle cell disease and thalassemia, among others.[53,54] Altogether, improving the engraftment of gene-modified cells constitutes a strong catalyst in the development of sustainable therapies against HIV-1 infection as well as many other diseases that have been difficult to provide therapeutic benefit for by HSPC-based gene therapy strategies.

MATERIALS AND METHODS

Lentiviral vector plasmid DNA construction

To effectively knock down HPRT expression, 26 shRNA candidates targeting different HPRT mRNA sequences were cloned into a lentiviral vector plasmid DNA expressing EGFP as a reporter gene (Figure S1). Candidate vectors were screened to identify the most efficient HPRT-targeting shRNA. The chosen shRNA, HPRTshRNA734, was synthesized by annealing the following oligo DNA primers: forward primer: 5′-CTC-AGGATATGCCCTTGACTAT-TTGTCCGAC-ATAGTCA AGGGCATATCCT-TTTTTGAGC-3′; reverse primer: 5’-GCTCAA AAA- AGGATATGCCCTTGACTAT-GTCGGACAA-ATAGTCAA GGGCATATCCT-GAGGTAC-3′. The annealed oligo DNA was cloned into a 7SK promoter containing plasmid DNA (pVAX-7SK2; shuttle vector) to generate an HPRTsh734 expression cassette. The DNA sequence was confirmed by DNA Sanger sequencing. The 7SK promoter-HPRTsh734 expression cassette was inserted between the EcoRI and AfeI restriction enzyme sites in FG12 EGFP,[55] FG12H1CCR5sh1005,[28,56] and LVsh5/C46 lentiviral vector plasmid DNAs.[32,57] Resulting lentiviral vector plasmid DNAs were confirmed for insertion of the 7SK-HPRTsh734 expression cassette by restriction enzyme digest and DNA Sanger sequencing.

Cell culture

MT4-CCR5 cells are a human T lymphotropic virus type 1–transformed human $CD4^+$ T cell line that stably expresses CCR5. MT4-CCR5 cells were kindly provided by Dr. Koki Morizono (UCLA, Los Angeles, CA). MT4-CCR5 cells were generated by transducing MT4 cells with a lentiviral vector expressing human CCR5 under the control of the internal SFFV promoter. These cells were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Cytiva, Marlborough, MA), 2 mM L-glutamine, 100 U penicillin, and 100 μg/mL streptomycin (GPS). Human primary PBMCs were isolated from whole blood from healthy donors obtained from the UCLA-CDU CFAR Centralized Core Laboratory by Ficoll Paque Plus (GE Healthcare, Uppsala, Sweden). PBMCs were cultured in RPMI-1640 supplemented with 10% FBS, 50 U/mL of interleukin-2 (IL-2) (PeproTech, Cranbury, NJ), and GPS. Primary human FL-derived $CD34^+$ HSPCs were isolated from human FL obtained from the UCLA CFAR Gene and Cellular Therapy Core Laboratory (Los Angeles, CA) or Advanced Bioscience Resources (Alameda, CA) using a $CD34^+$ microbead isolation kit (Miltenyi Biotec, Auburn, CA). Human mPB $CD34^+$ cells were isolated from granulocyte-colony stimulating factor (G-CSF)–mobilized peripheral blood leukapheresis packs purchased from ALLCELLS (Alameda, CA) using anti-$CD34^+$ monoclonal antibodies (Miltenyi Biotec) and cryopreserved in Bambanker (GC Lymphotec, Tokyo, Japan).

Lentiviral vector production

Vesicular stomatitis virus G protein–pseudotyped lentiviral vector stocks were produced by calcium phosphate–mediated transient transfection of HEK293T cells, as previously described.[55,58,59] Vector stocks were titered on HEK293T cells based on EGFP or C46 expression analyzed by flow cytometric analysis (LSRFortessa flow cytometer [BD Biosciences, San Jose, CA]; FlowJo version 10 [Tree Star, Ashland, OR]).

Lentiviral vector transduction, 6TG treatment *in vitro*

MT4-CCR5 cell line, primary PBMCs, FL-$CD34^+$ HSPC, and human G-CSF mPB-$CD34^+$ HSPC were transduced with lentiviral vectors at an MOI of 0.1 for MT4-CCR5 and an MOI of 1 for PBMC and $CD34^+$ HSPC. After transduction, cells were split into two wells and either treated with 0.3 μM of 6TG ($6TG^+$) or left untreated ($6TG^-$). The cells were reseeded with fresh media at 0.5×10^6 cells/mL every 3–4 days. The percentage of vector-transduced cells was determined by analyzing EGFP expression measured by flow cytometry at days 4, 7, 10, and 14.

FL-$CD34^+$ HSPCs (2×10^6/well) were resuspended in Yssel’s medium (GeminiBio, West Sacramento, CA) with 2% BSA (30% BSA stock, GeminiBio) and seeded in 6-well non-tissue culture–treated plates coated with 20 μg/mL RetroNectin (Takara Bio, Shiga, Japan). After 1 h of incubation at 37°C, cells were transduced with a lentiviral vector at MOIs 0.1–3 and cultured at 37°C for 3 days in RPMI-1640 with 10% FBS and supplemented with cytokine stimulations (SCF, Flt-3, and TPO) (PeproTech, Cranbury, NJ) at a concentration of 50 ng/mL. Vector transduction efficiency was evaluated by either

flow cytometry or dPCR using ThermoFisher QuantStudio 3D Digital System (Applied Biosystems, Waltham, MA).

HIV-1 inhibition assay *in vitro*

6TG-treated vector-transduced MT4-CCR5 cells (0.2×10^6) were infected with either X4-tropic HIV-1_{NL4-3} (MOI 1) or R5 tropic HIV-1_{NFNSX} (MOI 5) in a single experiment in triplicate. Levels of p24 (ng/mL) production were measured in culture supernatant collected at 4 days post-HIV-1_{NL4-3} challenge and 7 days post-HIV-1_{NFNSX} challenge ELISA. The range of quantitative p24 detection was 0.031–200 ng/mL.

6TG mediated preconditioning and *in vivo* selection in huBLT mice

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and was approved by the Institutional Animal Care and Use Committees of the University of California, Los Angeles (protocol ARC-2007-092). For huBLT mice, all of the surgeries were performed under ketamine/xylazine and isoflurane anesthesia and all efforts were made to minimize animal pain and discomfort. NSG (NOD.Cg-Prkdc scid Il2rg tm1Wjl/SzJ) mice were used to generate huBLT mice according to UCLA Humanized Mouse Core Laboratory procedures. Human fetal thymus and FL were obtained from Advanced Bioscience Resources. Fetal tissues were obtained without patient-identifying information. Written informed consent was obtained from patients for the use of tissues for research purposes. $CD34^+$ cells were isolated from FLs using anti-$CD34^+$ magnetic bead–conjugated monoclonal antibodies (Miltenyi Biotec) 1 day before transplantation. $CD34^+$ cells were transduced with lentiviral vectors overnight. NSG mice (6–8 weeks old) were preconditioned by an intraperitoneal injection of 10 mg/kg 6TG (2.5 mg/bottle, Sigma-Aldrich, St. Louis, MO, dissolved in 0.1 N NaOH). The concentration was adjusted to 10 mg/mL with 0.9% sodium chloride (UCLA Pharmacy, Los Angeles, CA). On the day of transplantation, a mixture of vector-transduced FL-$CD34^+$ cells ($\sim 0.5 \times 10^6$ cells per mouse) and $CD34^-$ cells ($\sim 4.5 \times 10^6$ cells per mouse) were semi-solidified with 5μL of Matrigel (BD Biosciences). The Matrigel-semi-solidified cell mix was implanted with a piece of thymus under the kidney capsule to create a human thymus implant. Mice were then injected with vector-transduced FL-$CD34^+$ cells ($\sim 0.5 \times 10^6$ per mouse) using a 27G needle through the retroorbital vein plexus. To perform *in vivo* selection, 6TG was reconstituted in 0.1 N NaOH, and the concentration was adjusted with 0.9% sodium chloride to 5 mg/mL 6TG was freshly reconstituted each time. A group of mice ($6TG^+$) were injected with 6TG (5 mg/kg) into the intraperitoneal cavity weekly for 8 weeks (optimized) or twice per week for 3 weeks.

Peripheral blood and tissue collection

huBLT mice were bled (100 μL) retroorbitally on week 9 posttransplant, which occurred 1 week before HIV-1 challenge, and then again on weeks 12 and 14 posttransplant. Red blood cell (RBC) lysed cells from peripheral blood were analyzed for human immune cell engraftment by monoclonal antibody staining and flow cytometry. huBLT mice were sacrificed at week 17 posttransplant and the spleen, bone marrow, lung, and human thymus implants were harvested. Tissue samples were collected in MACS tissue storage solution (Miltenyi Biotec) at necropsy and processed immediately for single-cell isolation, as described previously.[60] Isolated cells were stained for surface markers and analyzed by flow cytometry immediately. One-fourth of the stained cells were aliquoted to check the VCN.

Flow cytometry

Single-cell suspensions prepared from peripheral blood, spleen, implanted thymus, lung, or bone marrow of huBLT mice were stained with surface markers and analyzed on a LSRFortessa flow cytometer (BD Biosciences). The following antibodies were used in flow cytometry: CD45-eFluor 450 (HI30, eBioscience, San Diego, CA), CD3-APC H7 (SK7, BD Pharmingen, San Diego, CA), CD4-APC (OKT4, eBioscience), CD8-PerCP Cy5.5 (SK1, BioLegend, San Diego CA), CD19-Brilliant Violet 605 (HIB19, BD Horizon, San Diego, CA), CCR5-PECy7 (2D7, BD Pharmingen), and Countbright beads (Invitrogen, Grand Island, NY). RBCs were lysed with RBC lysis buffer after cell surface marker staining. Stained cells were fixed with 2% formaldehyde in PBS. The resulting data were analyzed by FlowJo version 10 software.

Determination of VCN per human cell by dPCR

RBCs from 25 μL peripheral blood, spleen, implanted thymus, lung, or bone marrow of huBLT mice were lysed with 5 μL of 0.2 M NaOH in a 75°C water bath for 5 min. Cell lysates were cooled in a 4°C refrigerator for 5 min, and 45 μL Tris-HCl was added to neutralize the lysates. The lysate cells were directly used in dPCR performed in Thermo Fisher (Waltham, MA) QuantStudio 3D Digital System set at 96°C for 10 min, followed by 42 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 2 min, and last extension at 60°C for 2 min. The primers and probe specific to woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) were customized by Thermo Fisher Scientific, which are primer sequence 1, 5′-CCTTTCCGGGACTTTCGCTTT-3′; primer sequence 2, 5′-GC AGGCGGCGATGAGT-3′; and probe 5′-(FAM)-CCCCCTCCCTA TTGCC-3′. The primers and probe specific to β-globin were purchased from Thermo Fisher. The average VCN was determined by multiplex dPCR of the WPRE sequence in the vector and normalized to the cell housekeeper gene β-globin.

HIV-1 infection in huBLT mice

X4-tropic HIV-1_{NL4-3} stocks were prepared by the calcium phosphate plasmid DNA transfection method, as previously described.[59] huBLT mice were injected with HIV-1_{NL4-3} (200 ng of p24 per mouse) via the retroorbital vein plexus using a 27G needle on week 10 posttransplant. A total of 100 μL of whole blood was harvested via the retroorbital vein plexus starting 2 weeks after HIV-1 injection and every 2 weeks afterward. Approximately 40 μL of plasma was separated from peripheral blood, 20 μL of which was used for viral RNA extraction. Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Germantown, MD). The RNA was eluted in 30 μL of elution buffer, and 10 μL of the elution was applied for reverse transcription using Superscript III Reverse Transcriptase (Invitrogen). dPCR was performed in the

Thermo Fisher QuantStudio 3D Digital System set at 96°C for 10 min, followed by 42 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 2 min, and last extension at 60°C for 2 min. The levels of HIV-1 RNA in the plasma of infected huBLT mice were determined by dPCR assay. The following primers and probe specific to HIV-1_{NL4-3} gag region were used: primer sequence 1, 5′-CCCTACCAGCATTCTGGACATAAG-3′; primer sequence 2, 5′-GCTTGCTCGGCTCTTAGAGTT-3′; and probe 5′-(FAM)-ACAAGGACCAAAGGAACCCT-BHQ1-3′.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad, La Jolla, CA). The Mann-Whitney *U* test was used for nonparametric testing of independent groups. Inverse correlations were analyzed using the Spearman nonparametric correlation test. Statistical significance was evaluated as *$p < 0.05$. Other significance levels are indicated as follows: **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

DATA AND CODE AVAILABILITY

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2023.12.007.

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AUTHOR CONTRIBUTIONS

D.S.A., Q.G., S.S., W.K., K.H., and N.K. conceived and designed the study. W.K., S.L., Q.G., C.Z., and O.P. collected the data. Q.G., J.Z., K.P., and A.B. analyzed the data. Q.G., J.Z., K.P., and A.B. wrote the original drafts of the paper. D.S.A. reviewed and edited the draft, supported the data analysis, and provided invaluable direction throughout the conceptualization and execution of the project. All of the authors had the opportunity to review the manuscript before submission.

DECLARATION OF INTERESTS

D.S.A. has a financial interest in CSL Behring. D.S.A. and S.S. hold US and Japanese patents US20210310007A1 and the University of California Board of Regents has licensed that intellectual property to CSL Behring. No funding was provided by these companies to support this work. All of the other authors declare no competing interests.

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