

ORIGINAL ARTICLE

Gene transfer of two entry inhibitors protects CD4⁺ T cell from HIV-1 infection in humanized miceNY Petit¹, C Baillou¹, A Burlion¹, K Dorgham¹, B Levacher², C Amiel^{1,3}, V Schneider³, FM Lemoine¹, G Gorochov^{1,4} and G Marodon¹

Targeting viral entry is the most likely gene therapy strategy to succeed in protecting the immune system from pathogenic HIV-1 infection. Here, we evaluated the efficacy of a gene transfer lentiviral vector expressing a combination of viral entry inhibitors, the C46 peptide (an inhibitor of viral fusion) and the P2-CCL5 intrakine (a modulator of CCR5 expression), to prevent CD4⁺ T-cell infection *in vivo*. For this, we used two different models of HIV-1-infected mice, one in which *ex vivo* genetically modified human T cells were grafted into immunodeficient NOD.SCID.γc^{-/-} mice before infection and one in which genetically modified T cells were derived from CD34⁺ hematopoietic progenitors grafted few days after birth. Expression of the transgenes conferred a major selective advantage to genetically modified CD4⁺ T cells, the frequency of which could increase from 10 to 90% in the blood following HIV-1 infection. Moreover, these cells resisted HIV-1-induced depletion, contrary to non-modified cells that were depleted in the same mice. Finally, we report lower normalized viral loads in mice having received genetically modified progenitors. Altogether, our study documents that targeting viral entry *in vivo* is a promising avenue for the future of HIV-1 gene therapy in humans.

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INTRODUCTION

Although there is no consensus on a definitive immune correlate of protection, there are multiple convincing examples linking human genetics and susceptibility to HIV-1 infection. The best example of a genetic predisposition protecting from HIV-1 remains the Δ32 mutation that prevents CCR5 expression at the cell surface, and thus completely protects 1% of Europeans from being infected.¹ The so-called 'Berlin patient' was grafted with CCR5-deficient bone marrow to treat his leukemia and was subsequently cured of both diseases.² Simultaneously, genetic interventions targeting chemokine receptors using DNA nucleases gave encouraging results *in vitro* and *in vivo* in humanized mice (HuMice).^{3–5} Clinical trials applying this strategy to lymphocytes or stem cells have shown that modified cells possessed a selective advantage compared with non-modified cells,⁶ which is one criterion of success for the therapy. Thus, there is a strong rationale to use gene therapy as an adjunct to current and future treatments.⁷

Maraviroc, a CCR5 chemical antagonist, is a powerful medication *in vitro* but resistant variants rapidly emerge in treated patients for complex reasons, such as mutations in the gp120-coding sequence affecting CCR5 docking.⁸ Similarly, the fusion inhibitor Enfuvirtide (a gp41 analog), which is delivered in solution to patients, rapidly becomes ineffective because gp41 mutates to escape Enfuvirtide binding.⁹ Thus, the therapeutic arsenal targeting viral entry is scarce and poorly efficient. However, strategies based on blocking entry are perhaps the most promising to rapidly restore a pool of functional T cells, the main goal to prevent AIDS.¹⁰ More recently, it was shown that HIV-1 infection needs not to be productive in CD4⁺ T cells to induce cell death by pyroptosis.¹¹ This mechanism of HIV-1-induced cell death highlights the interest of strategies aimed at preventing viral entry. We proposed developing a gene transfer

vector in which two viral entry inhibitors in combination would have a better efficacy at preventing viral entry. In support of this hypothesis, a synergistic effect of Enfuvirtide was demonstrated in cells with low levels of CCR5.¹² Importantly, viral variants that are able to escape gp41 analogs and CCR5 inhibitors at the same time have only been described *in vitro* with a drastic cost on viral fitness,¹³ illustrating the difficulty for the virus to escape both inhibitors at the same time. Using monocistronic lentiviral vectors, we previously showed a synergistic effect of the P2-CCL5 intrakine with the C46 peptide on HIV-1 infection *in vitro*.¹⁴ The P2-CCL5 intrakine, originally described as a high-affinity CCL5 (RANTES) variant,¹⁵ was later modified to incorporate an ER retention sequence, sequestering CCR5 away from the cell surface.¹⁶ The C46 peptide is the optimized membrane-bound form of Enfuvirtide and has been used in several gene therapy studies as it is effective on both CCR5- or CXCR4-tropic HIV-1, and can be accommodated in several gene transfer vectors, including lentiviral vectors.^{17–20} Here, we aimed to evaluate the *in vivo* efficacy of an optimized lentiviral vector co-expressing those two entry inhibitors. We used two preclinical models of HIV-1 gene therapy, either infusing genetically modified peripheral blood lymphocytes (PBL) in adult immunocompromised NOD.SCID.γc^{-/-} (NSG/PBL) mice or grafting genetically modified CD34⁺ hematopoietic progenitors in NSG neonates (NSG/CD34).

RESULTS

A lentiviral vector expressing two inhibitors of HIV-1 entry With the general aim to validate the combination of the C46 peptide and the P2-CCL5 intrakine for HIV-1 gene therapy *in vivo*, we used an optimized version of our previously described lentiviral vector, which efficiently inhibited HIV-1 infection

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in vitro.¹⁴ To facilitate the detection of genetically modified cells, we added the green fluorescent protein (GFP) reporter gene before the therapeutic cassette to generate the LvGFP-C46-P2 vector (Figure 1a). A vector using the same strong promoter EF1 α , but in which the therapeutic cassette was omitted, was used as a control (Figure 1a). We transduced anti-CD3/CD28-activated peripheral blood mononuclear cells to monitor transgene expression and function *in vitro* and *in vivo*. Expression of the GFP reporter molecule was well correlated with the expression of the C46 peptide (detected with the 2F5 monoclonal antibody; Figure 1b) and was also associated with a lower median fluorescence intensity of CCR5 *in vitro* (Figure 1c). Passive diffusion of the intrakine was ruled out by the observation that GFP⁻ cells exhibited similar CCR5 median fluorescence intensity than non-transduced cells (Figure 1c), suggesting that the decrease staining intensity of CCR5 in GFP⁺ cells was due to ER retention of CCR5 through interaction with the P2-CCL5 intrakine. The median

fluorescence intensity of CCR5 was also reduced twofold in genetically modified peripheral blood mononuclear cells injected *in vivo* in NSG mice (Figure 1d), reflecting the expected down-modulation of CCR5 surface expression. Thus, GFP expression was a faithful reporter of transgene expression and function, and was thus used to follow genetically modified cells *in vivo*.

Protection of genetically modified human CD4⁺ T cells from HIV-1 infection in NSG/PBL mice

As a model for HIV-1 infection of human CD4⁺ T cells *in vivo*, we first used adoptive cell transfer (ACT) in immunocompromised NSG mice (NSG/PBL). A major problem with ACT of human T cells in NSG mice is the xenogeneic graft versus host disease that develops thereafter and that invariably leads to death.²¹ We tested various ACT protocols in NSG mice and found that injection of 6 × 10⁶ activated T cells in 1-Gy-irradiated mice represented an

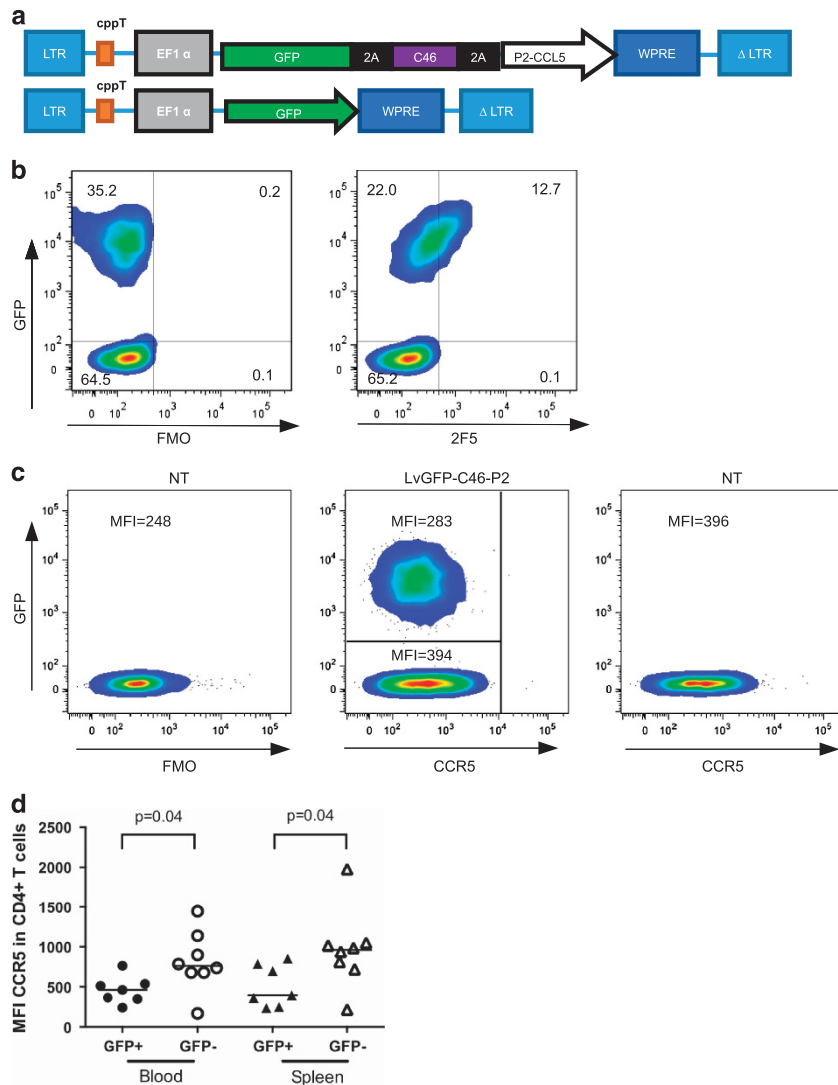


Figure 1. Lentiviral vector design and co-expression of anti-HIV-1 genes and eGFP into a lentiviral vector. **(a)** A schematic representation of the structure of the lentiviral vectors used in the present study is shown. (LTR: long terminal repeat; cppT: central polyurine tract of HIV-1; EF1 α : Elongation factor 1 promoter; C46: membrane-bound form of T20 (C46 peptide); 2A: 2A sequence of the foot-and-mouth disease virus; P2i: P2-CCL5 intrakine; WPRE: Woodchuck Hepatitis virus regulatory element; Δ LTR: U3 deleted LTR). Not to scale. **(b)** Co-expression of the C46 peptide (detected with the 2F5 mAb) and of eGFP and **(c)** co-expression of CCR5 and eGFP in human CD4⁺ peripheral blood mononuclear cell activated by CD3/CD28 beads and interleukin (IL)-2 21 to 29 days post transduction with the LvGFP-C46-P2 vector (NT: Non-transduced; FMO: fluorescence minus one; MFI: median fluorescence intensity). **(d)** *In vivo* CCR5 expression on CD45⁺CD3⁺CD4⁺ T cells in GFP⁺ and GFP⁻ cells from non-irradiated NSG mice grafted with 2 × 10⁶ LvGFP-C46-P2-transduced T lymphocytes and analyzed in the blood and the spleen 34–53 days post graft.

optimal trade-off between survival and engraftment efficiency (protocol P3 in Supplementary Figure S1). To normalize the number of genetically modified cells across experiments and vectors, we diluted transduced cells into non-transduced cells *ex vivo* prior ACT, establishing a number of GFP⁺ cells at 10% of the injected cells. Twelve days after ACT, mice were infected intravenously with the CCR5-tropic NL-AD8 HIV-1. The frequencies of CD4⁺GFP⁺ cells steadily increased in the blood of LvGFP-C46-P2-treated animals during the course of the infection to reach a plateau, where up to 95% of all CD4⁺ T cells expressed the transgene (Figure 2a). In contrast, the frequencies of GFP⁺ cells in control HIV-1-infected LvGFP-treated mice remained close to the 10% input throughout the experiment (Figure 2a). The increase in GFP⁺ cells with the LvGFP-C46-P2 vector was dependent on HIV-1 infection because it was not observed in non-infected NSG/PBL mice (Supplementary Figure S2), showing that the therapeutic

vector did not increase the proliferation of modified cells *per se*. The frequencies of GFP⁺ cells were also superior in the spleen and in the bone marrow of LvGFP-C46-P2-treated mice compared with LvGFP-treated control mice (Figure 2b). These increased frequencies translated into increased numbers of CD4⁺GFP⁺ cells in the spleen and the bone marrow of LvGFP-C46-P2-treated mice compared with LvGFP mice (Supplementary Figure S3). Altogether, the results demonstrate that LvGFP-C46-P2-transduced CD4⁺ T cells possessed a selective advantage relative to LvGFP-modified T cells.

To test the hypothesis that genetically modified cells resisted HIV-1-induced depletion, we analyzed longitudinally the frequencies of CD4⁺ cells in CD3⁺GFP⁺ and CD3⁺GFP⁻ T cells in the blood of LvGFP-C46-P2- and LvGFP-treated mice (Figure 2c). The frequencies of CD4⁺ T cells in the GFP⁻ subset rapidly dropped after HIV-1 infection, showing that non-protected CD4⁺ T cells underwent

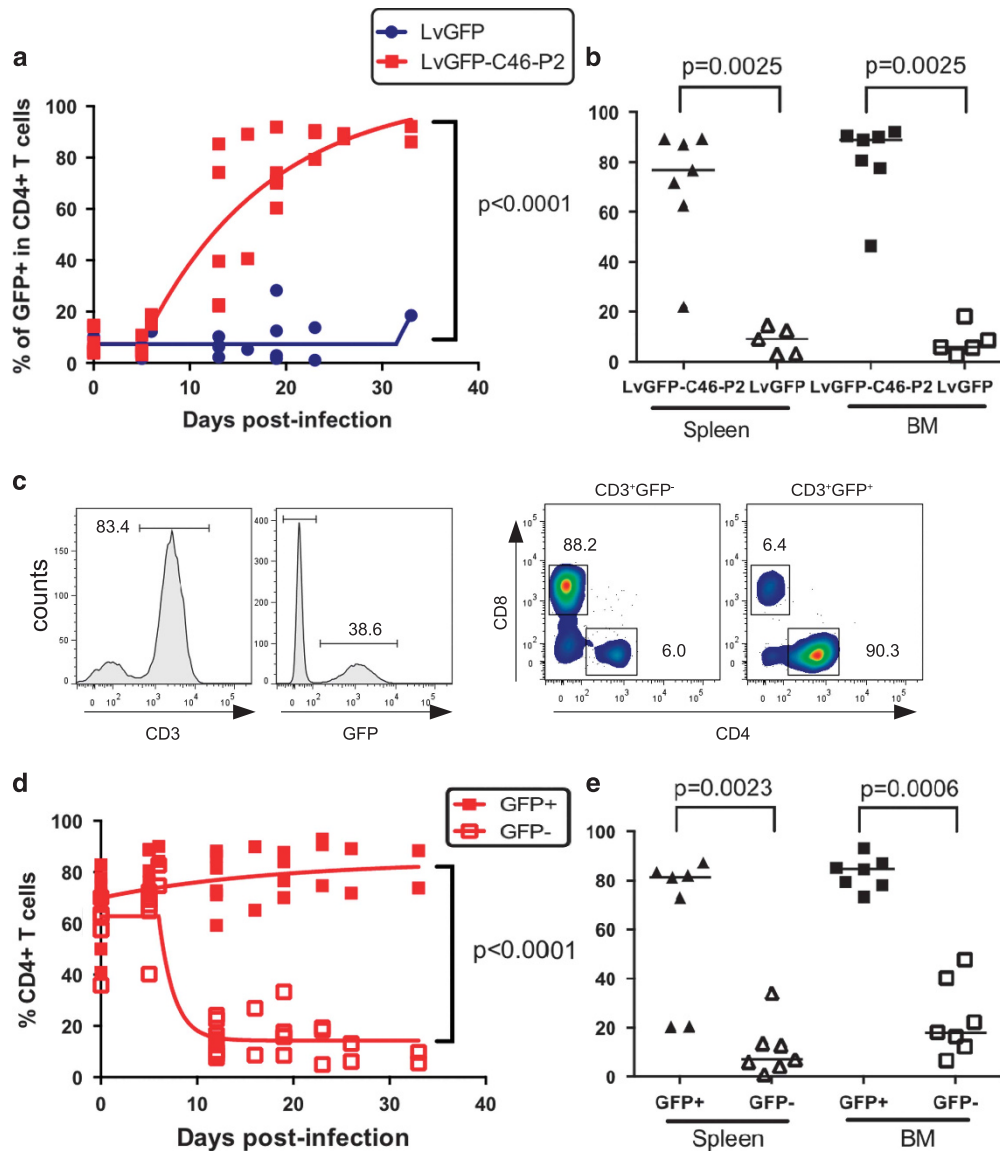


Figure 2. Protection of genetically modified human CD4⁺ T cells from HIV-1 infection in NSG/PBL mice. **(a, b)** Frequencies of GFP⁺ cells in human CD45⁺CD3⁺CD4⁺ T cells in the blood at various days after HIV-1 infection **(a)** and in the spleen or bone marrow (BM) **(b)**. **(b)** Thirty-five to forty-five days after injection of LvGFP- or LvGFP-C46-P2-modified T cells in NSG mice. **(c)** Representative histograms and dot plots showing the gating strategy to determine the frequencies of CD4⁺ T cells in GFP⁺ and GFP⁻ human CD3⁺ T cells. **(d, e)** Frequencies of CD4⁺ cells in the CD3⁺GFP⁺ and CD3⁺GFP⁻ populations were determined in LvGFP-C46-P2-injected mice in the blood at various days after infection **(d)** and in the spleen or BM **(e)** at the end of the experiment. The results are compiled from two independent experiments using the P3 ACT protocol (Supplementary Figure S1). Nonlinear regression analysis curve fits are shown. The *P*-value indicates the significant difference between the two slopes.

HIV-1-induced depletion as expected (Figure 2d). In striking contrast, the frequency of CD4⁺ T cells in the GFP⁺ fraction remained constant throughout the experiment, showing that these cells were protected from HIV-1-induced depletion. Resistance to depletion was also observed in the spleen and in the bone marrow of LvGFP-C46-P2-treated animals, with statistically significant differences in the frequencies of CD4⁺ T cells in GFP⁺ versus GFP⁻ T cells (Figure 2e). In contrast, the frequencies of GFP⁺ cells, similar to the GFP⁻ subset, steadily decreased in the blood of control LvGFP-treated mice (Supplementary Figure S4a), showing that GFP expression *per se* did not protect from HIV-1-induced depletion. A similar depletion of GFP⁺ cells was found in the spleen and in the bone marrow of control LvGFP-treated mice (Supplementary Figure S4b). Thus, CD4⁺ T cells expressing the combination of viral entry inhibitors were protected from HIV-1-induced depletion in NSG/PBL mice in the blood and in lymphoid tissues.

Resistance of genetically modified human CD4⁺ T cells to HIV-1-induced depletion in NSG/CD34 HuMice

We next wanted to confirm the potency of the vector to prevent HIV-1-induced CD4⁺ T-cell depletion in a more physiological setting. For this, we grafted LvGFP-C46-P2-transduced CD34-purified cells from the cord blood into neonatal NSG mice and monitored human cell reconstitution and transgene expression overtime. At 17 weeks post injection, 11.9±11.0% of total cells from the blood (excluding erythrocytes) were human CD45⁺CD3⁺ T cells in the animals used for the experiment. The frequencies of CD4⁺ and CD8⁺ T cells among CD3⁺ cells were less variable representing 40.0±5.8% and 47.0±5.3%, respectively (Supplementary Figure S5). Among 14 NSG/CD34 HuMice generated with LvGFP-C46-P2-modified CD34⁺ cells, only 8 had detectable GFP⁺ cells in CD4⁺ T cells 17 weeks after. Four of those

mice were infected with a CCR5-tropic HIV-1 strain, whereas four were left uninfected. Because the frequency of GFP⁺ cells was highly variable among NSG/CD34 HuMice, it was not possible to reliably measure a selective advantage in that setting. To directly assess the resistance of genetically modified CD4⁺ T cells to HIV-1-induced depletion, frequencies of CD4⁺ T cells were measured in GFP⁺ and GFP⁻ cells (Figure 3). In non-infected mice, the frequencies of CD4⁺ T cells in the blood remained similar in GFP⁺ versus GFP⁻ T cells throughout the course of the experiment (Figure 3a). As expected, frequencies of GFP⁻ cells steadily decreased in HIV-1-infected animals, whereas frequencies of GFP⁺ remained stable, showing that CD4⁺GFP⁺ T cells resisted HIV-1-induced depletion in the blood of NSG/CD34 HuMice (Figure 3b). As expected in non-infected mice, the frequencies of CD4⁺ T cells in lymphoid organs were similar in GFP⁺ or GFP⁻ subsets (Figure 3c). In contrast, frequencies of CD4⁺ T cells among GFP⁺ and GFP⁻ cells significantly differed in the lymph node, spleen and bone marrow (Figure 3d). Of note is the one mouse in which resistance to deletion was not evident in the blood did not show any sign of resistance in the lymphoid organs. Thus, gene transfer of two entry inhibitors in CD34⁺ cells conferred resistance to CD4⁺ T cells in three mice out of four analyzed.

Gene transfer of entry inhibitors has an impact on viral replication in NSG/CD34 HuMice

To assess the impact that the therapy might have on viral loads, we measured viremia in LvGFP-C46-P2-treated mice in which GFP⁺ cells were observed (*n*=4) or not (*n*=6) prior HIV-1 infection. To accommodate the various levels of human cells engraftment among the different mice (Supplementary Figure S5), viremia was corrected by the frequency of CD45⁺CD3⁺CD4⁺ T cells among total cells of the blood at the time of the analysis. Initially,

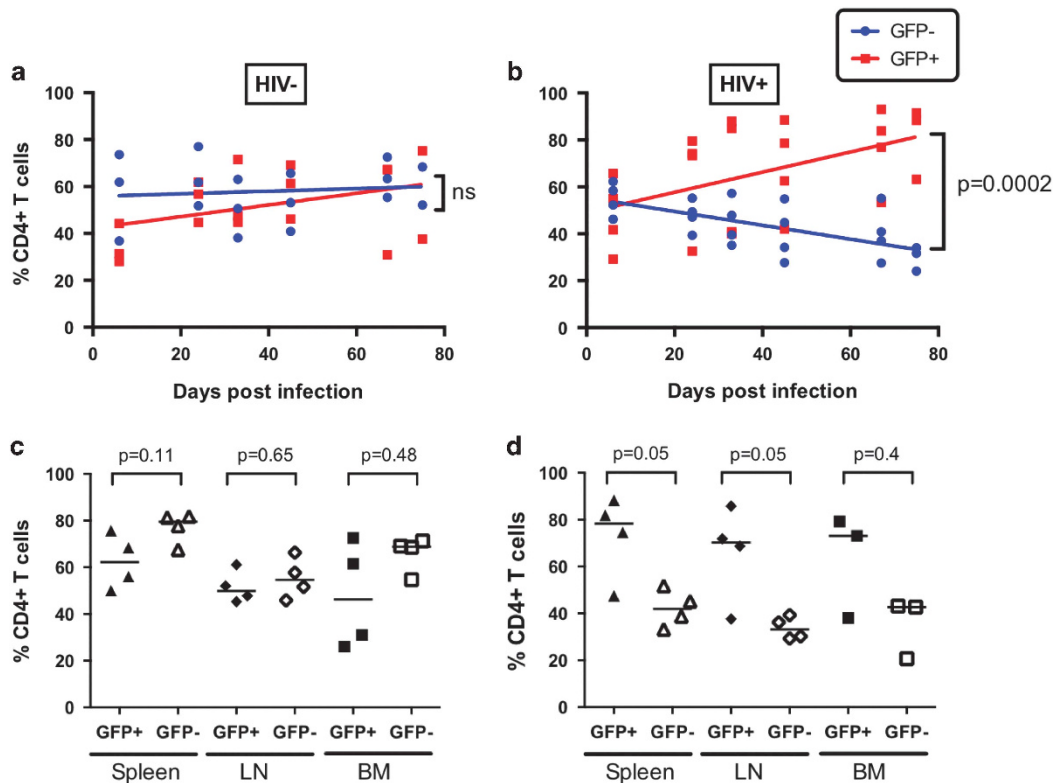


Figure 3. Resistance of genetically modified CD4⁺ T cells to HIV-1-induced depletion *in vivo* in NSG/CD34 HuMice. **(a)** Blood frequencies of CD4⁺ cells in CD3⁺GFP⁺ or CD3⁺GFP⁻ populations were determined in non-infected (HIV⁻) or **(b)** infected (HIV⁺) NSG HuMice at various time points after infection. Linear regression curve fit and *P*-values are depicted on the graphs. n.s., not significant (*P* > 0.05). **(c)** Frequencies of CD4⁺ cells into CD3⁺GFP⁺ or CD3⁺GFP⁻ populations in HIV⁻ or **(d)** HIV⁺ mice in the spleen, lymph nodes (LN) and the BM 11 weeks post infection.

normalized viremia was similar in both groups, showing that the therapy was not associated with an immediate effect on viral replication. However, we observed a tendency for lower normalized viral loads in mice bearing GFP⁺ cells compared with mice in which no GFP⁺ cells could be detected (Figure 4a). To confirm that animals with GFP⁺ cells carried less virus, we analyzed p24 expression in CD4⁺ T cells at the end of the experiment. We found that the frequencies of CD4⁺ cells expressing p24 in mice with GFP⁺ cells were lower than in mice without GFP⁺ cells and close to background staining obtained in non-infected HuMice (Figure 4b). Altogether, we conclude that NSG/CD34 HuMice reconstituted with gene-modified CD34⁺ progenitors were protected from HIV-1-induced CD4⁺ T-cell deletion and had a lower number of infected cells, corroborating with lower viral loads.

DISCUSSION

Here, we show that a lentiviral vector encoding two viral entry inhibitors confers a selective advantage to genetically modified cells *in vivo* because of their resistance to HIV-1-mediated depletion. We observed a strong and long-lasting selective advantage in the NSG/PBL model. A lower selective advantage was reported in a very similar model of NSG/PBL HuMice using a vector expressing only the C46 peptide.¹⁸ This observation suggests that two entry inhibitors might be better than one at protecting cells from HIV-1. However, a preclinical study in macaques reconstituted with progenitors expressing the C46 peptide alone showed lower viral loads correlated to a clear selective advantage.²² Moreover, recent studies showed that inhibition of CCR5 expression with short hairpin RNA was sufficient to protect CD4⁺ T cells from infection and to confer a selective advantage in chimeric bone marrow–liver–thymus HuMice.^{23,24} Thus, targeting gp41 and CCR5 have independently the potential to curb HIV-1 infection, highlighting the interest of using two inhibitors of this crucial step of HIV-1 infection in the same vector.

A strong selective advantage is not always associated with lower viral loads. In CD34-reconstituted HuMice, Walker *et al.*²⁵ reported that expression of a triple combination of anti-HIV-1 genes did not have an impact on viral replication, although a

significant selective advantage was observed. A modest but significant effect on viral loads was reported following CCR5-specific zinc finger nucleases-mediated modification in NSG/PBL HuMice.⁵ However, only one time point was analyzed in that study. A kinetics study showed that the reduction in viral loads using the same technology was much more discrete in NSG/CD34 HuMice despite a considerable selective advantage.³ Our PCR and p24 data concur to the hypothesis that selective advantage conferred by our vector had an impact on viral replication. Recently, a complete protection from HIV-1 was observed in bone marrow–liver–thymus humanized mice reconstituted with human cells modified with a vector very similar to our, encoding the C46 peptide and a short hairpin RNA targeting CCR5.¹⁹ This is the first report showing that viral replication can be totally controlled in HuMice by gene therapy without prior sorting of genetically modified cells, as recently shown for a CCR5 short hairpin RNA.²⁴ This surprising and unique result suggests that maximal efficacy of HIV-1 gene therapy might necessitate a functional immune response that is present in monkeys and bone marrow–liver–thymus HuMice but lacking or severely hampered in other HuMice models. One must keep in mind though that some HIV-1-specific polymerase chain reactions (PCRs) might amplify the vector used for gene transfer as well.²⁶ The use of HIV-1-specific PCR discriminating HIV-1 from the vector such as the one employed in our study should become the gold standard.

Considering the recent developments of nucleases that target CCR5 in CD34⁺ progenitor cells, we believe that residual expression of the molecule such as the one observed with our intrakine might allow normal hematopoiesis and circulation of modified cells while total ablation by genetic means may have an impact on these processes. Recent advances in lentiviral delivery of Zn finger nucleases might improve specific targeting of the nuclease to mature CD4⁺ T cells, a protocol that would limit bystander effects.²⁷

The selective advantage of genetically modified cells would only be obtained in the context of high levels of viral replication. Although ART interruptions have been performed in the past to provoke selective growth of modified cells in small-scale clinical trials for gene therapy,^{28,29} an interruption in therapy is not foreseeable in patients in the long term. Gene therapy might thus be particularly suitable for patients experimenting treatment failure with high viral loads.

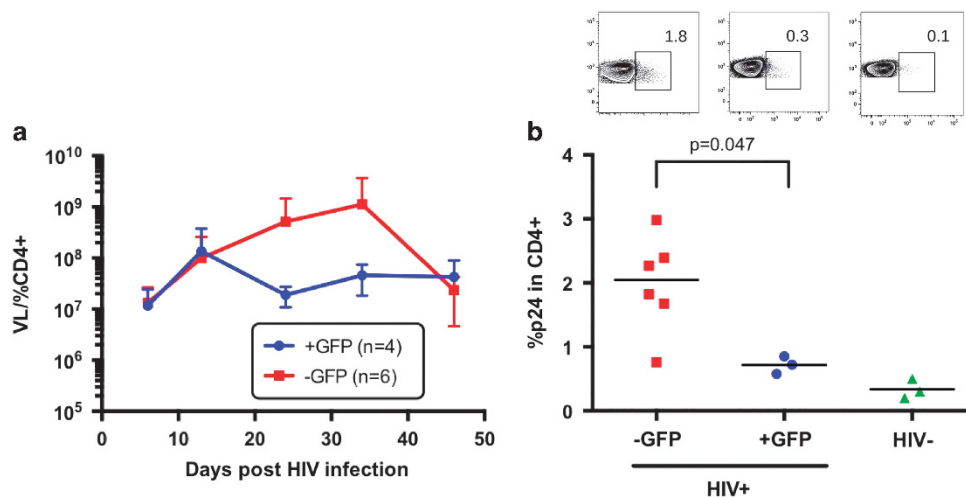


Figure 4. Gene transfer of entry inhibitors has an impact on viral replication in NSG/CD34 HuMice. **(a)** Viral load was measured using qPCR after HIV-1 infection in LvGFP-C46-P2-treated mice with undetectable (–GFP) or detectable GFP⁺ cells (+GFP) in CD4⁺ T cells before infection. Shown is the viral load value normalized by the frequency of human CD45⁺CD3⁺CD4⁺ T cells present in total cells of the blood sample for each time point. **(b)** Frequencies of p24⁺ cells in CD4⁺ T cells from the lymph node of NSG HuMice with (+GFP) or without GFP⁺ cells (–GFP) 77 days after infection with NL-AD8 HIV-1 (HIV+) or non-infected (HIV–). A representative CD4 versus p24 staining is shown above each group. One mouse from the (+GFP) group was excluded from the graph as it was not protected against HIV-1-induced depletion in the periphery.

MATERIALS AND METHODS

Lentiviral vector design and production

Third-generation self-inactivating lentiviral vectors were used in this study.³⁰ The LvGFP-C46-P2 vector was constructed by adding an eGFP gene and 2A sequence upstream of the therapeutic cassette (construction encoding the C46 peptide and P2-CCL5 analog described in Petit *et al.*¹⁴) in the backbone of a lentiviral vector carrying the EF1a promoter. As a control, the LvGFP vector expressing GFP only was used. Details on the cloning procedures are available on request. Lentiviral vectors were produced in mycoplasma-free HEK-293T cells, as described previously.³¹ Briefly, 23.3 µg of the Δ8.9 packaging plasmid, 30 µg of the vector plasmid and 10 µg of the vesicular stomatitis virus-G envelope were transfected into 15 × 10⁶ cells in T-175 flasks by calcium phosphate precipitation. Vector supernatants were collected 48 h post transfection and concentrated by ultrafiltration (Centricon Plus-70; Millipore, Molsheim, France) at 3500 g at 4 °C. Viral stocks were kept frozen at -80 °C. Viral titers were determined on HEK-293T cells with various concentrations of vector supernatants in the presence of Polybrene (8 µg ml⁻¹; Sigma-Aldrich, Saint-Quentin-Fallavier, France). Seventy-two hours after transduction, the percentage of cells expressing the transgenes was determined with flow cytometry and used to calculate a viral titer as the number of infectious particles per milliliter.

Mice and humanization

NOD Prkdc^{scid} Il2r^{tm1Wjl} (NSG) mice (strain #05557; Jackson Laboratory, Bar Harbor, ME, USA) were bred in animal facilities of Centre d'Expérimentation Fonctionnelle according to the Jackson Laboratory handling practice specific to that strain. The regional ethical committee on animal experimentation Darwin approved all mouse protocols. Primary human cells were obtained from leukapheresis samples collected from healthy donors at the Etablissement Français du Sang after informed consent. Cells were grown at a concentration of 1 × 10⁶ cells ml⁻¹ and activated in RPMI, 10% fetal calf serum, penicillin/streptomycin, interleukin-2 (Proleukin, 600 IU ml⁻¹; Novartis, Basel, Switzerland) and CD3/CD28 beads (Invitrogen, Carlsbad, CA, USA) at three beads per cell. Two days after activation, cells were transduced by spinoculation for 2 h at 1000 g at 30 °C, with the indicated lentiviral vectors at a multiplicity of infection of 6–8 in the presence of protamine sulfate (2 µg ml⁻¹, Sigma-Aldrich, Lyons, France). Three days after transduction, 1-Gy-irradiated female 8–12-week-old NSG mice were injected with 6 × 10⁶ cells. Twelve days post-ACT, mice were infected with 25 ng of p24 of NL-AD8 HIV-1 strain in a final volume of 100 µl of 1 × PBS. All mice used in this study were randomly assigned to experimental group and cages. Investigator was not blinded to the group allocation during the experiments.

Human hematopoietic progenitor cells were obtained from cord blood samples collected from healthy donors after informed consent. Mononuclear cells from human cord blood were isolated by Ficoll density gradient and centrifuged at 200 g during 13 min to remove platelets. Then, CD34⁺ progenitors were sorted with the human CD34 MicroBeads kit, according to the manufacturer's instructions (Miltenyi Biotech, Paris, France). CD34⁺ cells were incubated at a concentration of 1 × 10⁶ cells ml⁻¹ overnight into StemSpan SFEMII medium (StemCell Technologies, Grenoble, France) complemented with human recombinant cytokines (interleukin-6 and thrombopoietin at 20 ng ml⁻¹, SCF and FLT3-L at 100 ng ml⁻¹, Peprotech, Neuilly sur Seine, France) and antibiotics. Cells were transduced with the LvGFP-C46-P2 lentiviral vector in StemSpan medium in the presence of cytokines, the proteasome inhibitor MG-132 (1 µM, Sigma-Aldrich), antibiotics and protamine sulfate (8 µg ml⁻¹; Sigma-Aldrich). CD34⁺ cells underwent two rounds of transduction separated by 3-h incubation at 37 °C and 5% CO₂. For each transduction cycle, cells were centrifuged at 1000 g at 30 °C for 2 h with the lentiviral vector at a multiplicity of infection of 15. Twenty-four- to forty-eight-hour-old NSG mice were irradiated at 0.9 Gy and grafted with 0.5 × 10⁵–2.5 × 10⁵ transduced CD34⁺ cells by the intrahepatic route. Ten nanograms of the p24 NL-AD8 HIV-1 strain were injected into the retro-orbital sinus of 17-week-old mice in a final volume of 100 µl of 1 × PBS.

HIV-1 production and quantification

HIV-1 molecular clone NL-AD8 was obtained through the AIDS Research and Reference Reagent Program. HIV-1 stocks were prepared with 30 µg of plasmid transfected into 15 × 10⁶ mycoplasma-free HEK 293T cells in T-175 flasks by calcium phosphate precipitation. The supernatant was frozen at -80 °C and viral titers were quantified by p24 ELISA according to the

manufacturer's instructions (Zeptomatrix, Buffalo, NY, USA). Mice were bled on acid–Citrate–Dextrose anticoagulant and plasma HIV-1 RNA viral loads were measured using the Abbott RealTime HIV-1 RT-PCR assay (Abbott Laboratories, Des Plaines, IL, USA) that do not amplify genomic regions present in lentiviral vectors contrary to the Roche (Basel, Switzerland) Cobas PCR (our unpublished observations and De Ravin *et al.*²⁶). Owing to the small volumes of plasma from the mice, a dilution was necessary to reach the volume needed for the assay. Thus, the detection limit varied between 200 and 2000 copies ml⁻¹ depending on the initial volume of mouse plasma.

Flow cytometry

Red blood cells from whole blood were lysed with 4.5 ml of water for 15 s before adding 0.5 ml of 10 × phosphate-buffered saline (PBS). Red blood cells from the spleen or bone marrow were lysed with ACK buffer (NH₄Cl 0.15 M, KHCO₃ 10 mM, EDTA 0.1 mM). Cell suspensions were stained with an optimal quantity of antibodies at a concentration of 10⁷ cells ml⁻¹ in a final volume of 100 µl of PBS/fetal calf serum 3%. Incubation was performed in the dark at 6 °C for 20 min. The following anti-human monoclonal antibodies were used for cell surface staining: CD45 PE-CE594 (clone HI30; catalog number (cat#) 562279, BD Biosciences, le Pont de Claix, France) anti-CCR5 Alexa Fluor 647 (HEK/1/85a; cat#313712, Biolegend, San Diego, CA, USA), anti-CD4 PerCP (RPA-T4, cat#300528, Biolegend), anti-CD8 Alexa Fluor 700 (HIT8a, cat#300920, Biolegend) and CD3 PE-Cy7 (UCHT1, cat#300420, Biolegend). The human IgG1 mAb 2F5 specific for a gp41 epitope (cat#AB001, Polymun, Klosterneuburg, Austria) was used to detect the C46 peptide. The KC57-RD1 (cat#6604667, Beckman Coulter, Villepinte, France) antibody was used to detect intracellular p24 after cells were treated with permeabilization buffer (eBioscience, San Diego, CA, USA). All cell preparations were acquired on an LSRII cytometer (BD) and analyzed with the FlowJo software (Tree Star, Portland, OR, USA). The frequencies of positive cells were determined according to the fluorescence minus one staining negative control.

Statistical analysis

No statistical method was used to assess sample size needed to detect an effect. Except for the NSG/CD34 model, which is a single experiment, all the results shown in this study are compiled from two independent experiments. Two-tailed *P*-values indicated on the graphs were calculated with the Prism version 6.0 software (GraphPad Software, San Diego, CA, USA), using the unpaired Mann–Whitney test with a confidence interval of 95%. The median values are indicated by horizontal bars on the graphs. Linear and nonlinear regression analyses were performed using Prism 6.0 to determine whether slopes significantly differed. Plateau with one phase decay association or dissociation equations was used to model the data.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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AUTHOR CONTRIBUTIONS

NYP, CB, AB and GM performed experiments; BL constructed the vectors; CA and VS performed HIV-1 qPCR; KD, FML and GG conceived experiments, contributed to essential reagents and corrected the manuscript; NYP and GM conceived experiments, analyzed the data and wrote the manuscript.

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