

Lentivector knock-down of CCR5 in hematopoietic stem cells confers functional and persistent HIV-1 resistance in humanized mice

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Abstract

Gene-engineered CD34⁺ hematopoietic stem and progenitor cells (HSPCs) can be used to generate an HIV-1-resistant immune system. However, a certain threshold of transduced HSPCs might be required for transplantation into mice for creating an HIV-resistant immune system. Here, we combined CCR5 knock-down by a highly efficient miRNA lentivector with pre-transplantation selection of transduced HSPCs to obtain a rather pure population of gene engineered CD34⁺ cells. Low-level transduction of HSPCs and subsequent sorting by flow cytometry yielded >70% transduced cells. Mice transplanted with these cells

showed functional and persistent resistance to a CCR5-tropic HIV strain: viral load was significantly decreased over months, and human CD4⁺ T-cells were preserved. In one mouse, viral mutations, resulting presumably in a CXCR4-tropic strain, overcame HIV resistance. Our results suggest that HSPC-based CCR5 knock-down may lead to efficient control of HIV *in vivo*. We overcome a major limitation of previous HIV gene therapy in humanized mice studies in which only a proportion of the cells in chimeric mice *in vivo* are anti-HIV engineered. Our strategy underlines the promising future of gene engineering HIV-resistant CD34⁺ cells that produce a constant supply of HIV resistant progeny.

Importance

Major issues in experimental long-term *in vivo* HIV gene therapy have been (i) low efficacy of cell transduction at the time of implantation and (ii) transduction resulting in multiple copies of heterologous DNA in target cells. In this study, we demonstrated the efficacy of a transplantation approach with a selection step for transduced cells that allows transplantation of an enriched population of HSPCs expressing a single (low) copy of a CCR5 miRNA. Efficient maintenance of CD4⁺ T-cells and a low viral titer resulted only when at least 70% of the HIV target cells were genetically modified. These findings imply that clinical protocols of HIV gene therapy require a selective enrichment of genetically targeted cells because positive selection of modified cells is likely to be insufficient below this threshold. This selection approach may not only be beneficial for HIV patients, but also other patients requiring transplantation of genetically modified cells.

Introduction

Combined anti-retroviral therapy (cART) changed the face of HIV medicine: patients have a life-expectancy close to uninfected people (1). However, cART has major disadvantages, including adverse events, emergence of drug-resistant strains in patients with poor adherence, a need for life-long intake, psychological dependence, and cost. Thus, cART has not halted the pandemic (<http://www.who.int/hiv/en/>), and alternative therapies are needed to cure HIV.

Gene therapy has been widely discussed as a possible strategy to cure HIV and has been tested in phase I/II clinical trials. Autologous CD4⁺ T-cells (2, 3) or CD34⁺ cells (4, 5) were gene engineered to express various anti-HIV moieties, including a combination of three RNA-based anti-HIV moieties (tat/rev shRNA, TAR decoy, and CCR5 ribozyme) (4), a tat-vpr-specific anti-HIV ribozyme (5), and a conditionally replicating lentiviral vector expressing long anti-sense to HIV (3) or were gene edited by zinc-finger nucleases for CCR5 knockout (2). Gene engineering also generated HIV-specific CD4⁺ or CD8⁺ T-cells (6, 7). Overall, the effects on HIV infection were modest, but importantly, gene engineering proved to be safe in humans.

The concept of engineering an HIV-resistant immune system received new impetus from the “Berlin patient,” who was treated with hematopoietic stem cell transplantation for acute myeloid leukemia. He received bone marrow from a donor homozygous for the $\Delta 32$ CCR5 mutation, and thus, the progeny cells did not express CCR5. He was the first documented cure for HIV (8) and provided hope that eliminating CCR5 from the cell surface would be the “Holy Grail” for the cure of HIV. However, another HIV-infected patient suffering from anaplastic large cell lymphoma also received a stem cell transplant from a homozygous CCR5-null donor. Unfortunately, in that case, X4-tropic HIV strains emerged that necessitated the re-initiation of cART (9).

In view of the modest success of phase I/II clinical trials and the data from stem cell transplantation, pre-clinical studies are needed to define the best anti-HIV moieties and the minimal number of gene engineered cells required to advance gene therapy in HIV. Humanized (hu) mice, which are generated by the transplantation of CD34⁺ cells, are of particular value in this context. Hu mice excel in their multi-lineage hematopoiesis (10), are highly permissive to HIV (11), and allow for the gene engineering of human CD34⁺ cells before transplantation (12). Indeed, various anti-HIV moieties have been investigated in hu mice as gene therapy options, including cellular factors, boosting the anti-HIV-immune response, and the HIV genome itself (12). These mice were used *in extenso* to investigate the effects of targeting CCR5 by shRNA (13-15) or ZNF (16). All these studies reported a decrease in CCR5 expression in

circulating and tissue leukocytes, which were not permissive to HIV *ex vivo*, but only the study by Holt et al. reported a significant decrease of HIV RNA copy number *in vivo* (16). The other studies either did not analyse the effects on HIV infection *in vivo* (14) or demonstrated no effect on viral load (15). The results of Holt et al. revealed disruption of CCR5 in only ~20% of all CD4⁺ T-cells. The follow-up in that study was only 8 weeks. Gene engineering HSPCs with a lentiviral vector encoding the broadly neutralizing anti-HIV human antibody 2G12 showed suppression of HIV RNA but was only studied 7 days after a challenge with a virus containing the corresponding epitope (17); gene engineering an HIV-specific T-cell receptor also lowered the HIV RNA but only modestly (18). A very elegant study with CD34⁺ cells edited with an HIV-1 LTR specific Tre-recombinase showed a potent lowering of HIV RNA activity after HIV challenge in hu mice (19). All these data are promising; however, we lack a long-term follow-up of the effects of anti-HIV gene therapy in hu mice, the number of gene engineered CD34⁺ cells needed in the various studies to obtain an HIV RNA lowering effect, and a detailed characterization of the hematopoietic system. A major advance was recently presented by Barclay et al., who purified the gene engineered CD34⁺ cells via the expression of a truncated version of CD25 (20).

Various means are available for gene engineering CD34⁺ cells; each has its pros and cons. A great deal of experience exists with shRNA (21); potential cons may be its potential to trigger the innate immune system (22) and its less than absolute downregulation of the target gene. Targeted gene disruption by ZFN, Talen or Crispr/cas has the advantage of complete disruption of the gene of interest (23-25); however the modest rate of gene engineering CD34⁺ cells (26), the potential of off-target effects (27-29) and the lack of clinical experience represent substantial hurdles for wider use *in vivo*.

We recently reported a novel microRNA-based gene knock-down strategy with improved knock-down, relative to methods conventionally used (30). A triple hairpin cassette targeting CCR5 resulted in >90% CCR5 knock-down upon single-copy transduction in HeLa cells. The aim of the present study was to assess whether gene engineering CD34⁺ cells with this vector construct results in down-regulation of CCR5 in progeny cells in hu mice and whether it could protect against HIV challenge *ex vivo* as well as *in*

vivo. Since some evidence in the literature exists that the number of gene engineered CD34⁺ cells is a major determinant of the success of the anti-HIV moieties (15, 16), we also made a major effort to generate hu mice with a very high number of gene engineered CD34⁺ cells. Finally, we characterised *in extenso* the hematopoietic system subsequent to HIV infection in hu mice with gene-engineered CD34⁺ cells.

Materials and Methods

Ethics statement

The procurement and use of CD34⁺ cells from human cord blood was approved by the Cantonal Ethical Committee of Zurich (EK-1103). All adult subjects provided written informed consent. Animal care and experimental protocols were in accordance with the “Swiss Ethical Principles and Guidelines for Experiments on Animals,” and approved by the Veterinary office of the Canton of Zurich, permit 26/2011. Manipulations of mice were in accordance with the regulations of the Veterinary office of the Canton of Zurich. (<http://www.veta.zh.ch/internet/gesundheitsdirektion/veta/de/home.html>).

Lentiviral vector production and titration

Lentiviral vector stocks were generated using transient transfection of HEK 293T/17 cells with the self-inactivating vector, pCLX-R4-DEST-R2 encoding the microRNA to CCR5 (30), the psPAX2 plasmid encoding gag/pol, and the pCAG-VSVG envelope plasmid, as described (31). Lentivector titration was performed using transduction of HT-1080 cells, followed by quantification of GFP-positive (or mCherry positive) cells 5 days after infection by flow cytometry as described (31).

Generation of humanized mice

NOD.scid.IL2R^{-/-} (NSG) mice were bred and maintained in individual ventilated cages and were fed autoclaved food and water. Mice with a human immune system (humanized (hu) mice) were generated as described (32). Briefly, newborn (<5 days old) NSG mice received sub-lethal (1Gy) total body irradiation

with a Cs source, and then received 2×10^5 transduced or untransduced CD34⁺ human HSPCs with a 50- μ l Hamilton syringe via the intrahepatic (i.h.) route. For the FACS-sorted R5 knock-down animals, CD34⁺ cells were sorted post-transduction into green-fluorescent protein (GFP) positive and -negative fractions, and then, 2×10^5 CD34⁺ GFP-positive or GFP-negative cells were injected i.h. into respective cohorts. All manipulations of hu mice were performed under laminar flow. Cell suspensions of the hu mouse organs were prepared in RPMI1640 medium supplemented with 2% fetal calf serum.

HIV virus stock and infection of mice

Viral stocks were obtained by polyethylenimine (PEI)-mediated transfection (Polysciences) of 293T cells with either pYU-2 (R5 tropic) or JRCSF (R5 tropic) (provided through the NIH AIDS Research and Reference Reagent Program). At 48 hours after transfection, virus was harvested, filtered (0.45 μ m), and frozen at -80°C until use. Viral titers were determined as described (33). Briefly, TCID₅₀ (tissue culture infectious dose 50%) was determined by infecting human CD8⁺ T-cell-depleted peripheral blood mononuclear cells (PBMCs) from three donors that were stimulated by adding IL-2, phytohaemagglutinin (PHA) and anti-CD3 beads (Dynal 11131D, Life Technologies). Mice were infected intraperitoneally (i.p.) with either HIV YU-2 or JRCSF at 2×10^5 TCID₅₀ per mouse. HIV RNA plasma levels were measured by RT-PCR (AmpliPrep/COBAS TaqMan HIV-1 Test, Roche) at various times after infection.

Flow cytometry

The cells in whole blood were counted in a Beckman Cell Counter. Cell suspensions were labelled with anti-human monoclonal antibodies (mAb) targeting the following cell-surface markers: CD45-PerCP, CD3 ACP, CD4-Pe Cy7, CD8-BVa, CCR5-PE, CD34-APC, CD45RA-APC, and CCR7-PE (all from BD Biosciences or Biolegend). Washing and reagent dilutions were done with FACS buffer (PBS containing 2% fetal calf serum and 0.05% sodium azide). All acquisitions were performed on a Cyan ADP (Beckman Coulter) flow cytometer. Data were analyzed with FlowJo software (Ashland, OR). Cellular debris and dead cells were excluded by their light-scattering characteristics. Transduced CD34⁺ cells were sorted according to intrinsic GFP expression as measured by a BD FACSAria™ III cell sorter.

HIV challenge *ex vivo*

Spleens of five hu mice transplanted with HSPCs gene engineered with the microRNA to CCR5 were dissociated through a nylon mesh, and red blood cells were lysed with ACK buffer for 3 minutes (LONZA). Cells were sorted ($\geq 99\%$ pure) with an ARIA sorter (BD Bioscience) into GFP-positive and -negative cells, and were subsequently activated for 24 hours with PHA (4 mg/ml) in RPMI culture medium supplemented with IL-2 (100 U/ml) and 10% fetal calf serum. Thereafter cells were infected with a TCID of 3.3×10^5 /ml of YU-2 for 6 hours and washed three times. The supernatant of the last wash was used as the base line p24 antigen level measured by an in house ELISA (34). Virus spread was then monitored at day 1, 4, 6, 8 and 10 post-infection.

Analysis of HIV envelope sequences

Nucleic Acid Extraction: For viral envelope sequencing, total nucleic acid was extracted from 60 μ L of mouse plasma on an EasyMag extractor (BioMerieux, Switzerland) according to the manufacturer's instructions. The elution volume was 50 μ L.

Reverse Transcription and PCR: For cDNA synthesis, 9 μ L of extracted nucleic acid was reverse-transcribed in a total reaction volume of 20 μ L at 42°C for 30 min. using a shorted, sequence-specific primer MSR5 (35) and the PrimeScript One Step RT-PCR kit (Takara Bio Europe/SAS, France). After heat inactivation at 96°C for 5 min. amplification primers were added to the reaction mixture, and DNA corresponding to positions 5956 to 8535 in isolate HXB2 (Accession K03455, M38432) was amplified for 20 cycles. Nested PCR in a total reaction volume of 40 μ L with Phusion Hot Start II DNA polymerase (Thermo Scientific, Switzerland) was carried out to amplify, respectively, gp120 (positions 6817 to 7812) and gp41 (positions 7789 to 8382).

DNA Sequencing: Before sequencing, amplified DNA was treated with Illustra ExoStar 1-step reagent (Fisher Scientific, Switzerland). For cycle sequencing, the BigDye Terminator v3.1 Cycle Sequencing Kit (LifeTechnologies, Switzerland), and specific sequencing primers were used. Twenty-five cycles of heat denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec, and synthesis 60°C for 4 min were carried out

on a 2720 Thermal Cycler (Life Technologies, Switzerland). Samples were further processed by ethanol precipitation, followed by capillary electrophoresis on a 3130xl Genetic Analyzer (Applied Biosystems, Switzerland). The sequences were assembled and edited with SeqMan Pro from the Lasergene 11 package (DNASStar).

Statistical analyses

The statistical analyses were performed using GraphPad Prism 5.04 (GraphPad Software). Data were subjected to either unpaired t-tests or paired t-tests, as indicated in the figure legends. The *P* values obtained were considered significant when $*P < 0.05$. The statistical outlier analysis was performed using the GraphPad Outlier calculator with an alpha of 0.01 (<http://graphpad.com/quickcalcs/Grubbs1.cfm>).

Results

Transplanting CD34⁺ cells with partial CCR5 knock-down does not hinder HIV replication

We previously developed a highly efficient microRNA called “mirGE” that allows efficient knock-down by single-copy transduction (30). Here we explored the potential of a mirGE lentivector targeting CCR5 to produce an HIV-resistant immune system in hu mice. The construct consists of a triple hairpin, and the vector cassette contains GFP driven by the same promoter as the miRNA that allows transduced cells to be identified directly. To minimize possible cellular perturbations from multiple vector inserts, we established a protocol that gave us a transduction rate of 20–30%. This transduction rate was based on previous work and should correlate with single-copy integration (36). In a first series of experiments, mice were transplanted with mirGE-transduced CD34⁺ cells without further manipulation (R5 knock-down mice); the CD34⁺ cells were a mixture of transduced (20–30%) and untransduced (70–80%) cells (data not shown). As controls, we used either mice transplanted with CD34⁺ cells transduced with a control GFP lentivector (control-transduced mice)(30) or with untransduced CD34⁺ cells (untransduced mice). Upon infection with a R5-tropic HIV (YU-2), the percentage and absolute number of GFP-positive CD4⁺ T-cells were increased in R5 knock-down cohorts and not in control-transduced cohorts (Fig. 1a, and Fig. 3g and

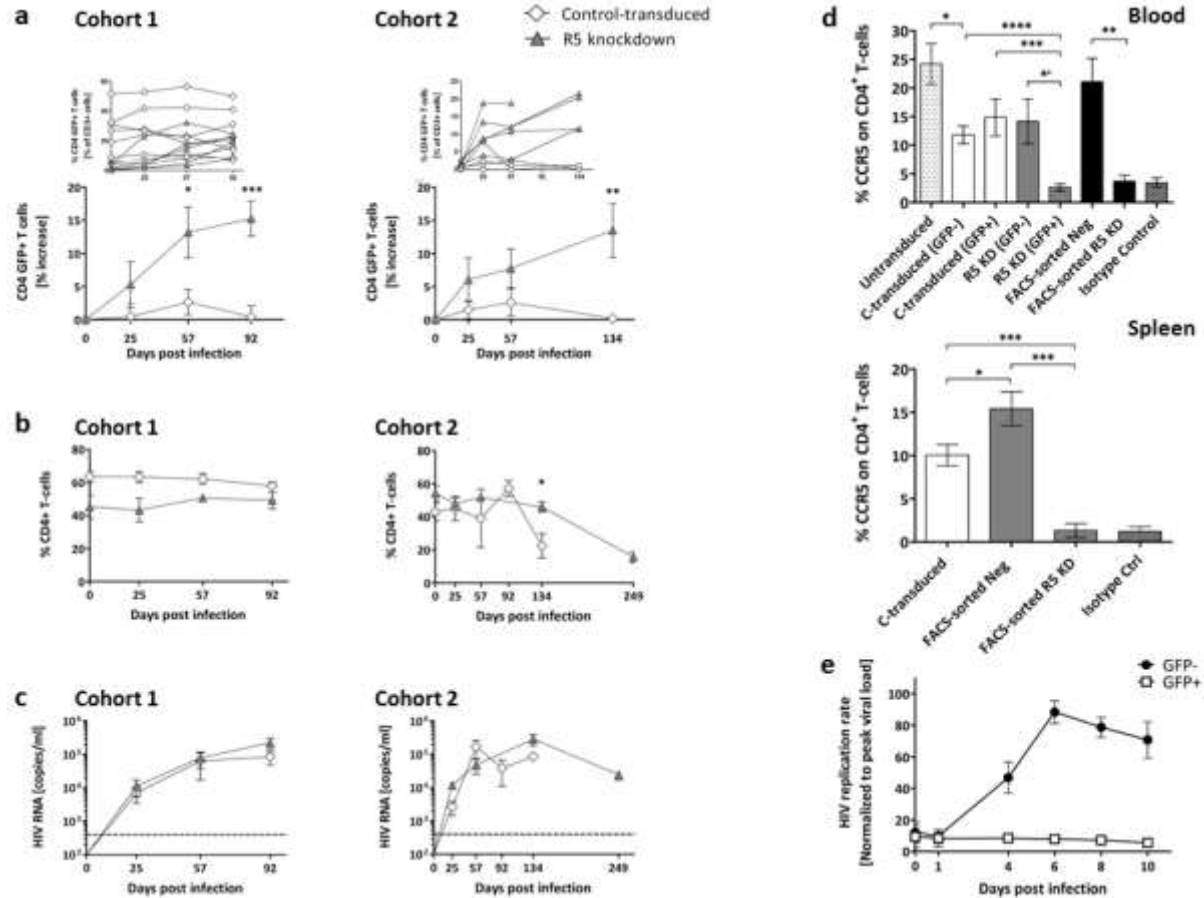


FIG 1 Homeostatic expansion of GFP-positive CD4⁺ T-cells in R5 knock-down mice despite sustained R5-tropic (YU-2) HIV infection. (a) The percentage change of GFP-positive CD4⁺ T-cells in the peripheral blood of control-transduced and R5 knock-down mice. Control-transduced mice (Cohort 1, n=8; Cohort 2, n=4) and R5 knock-down mice (Cohort 1, n=6; Cohort 2, n=5). Mean ± sem.; **P* = 0.0152, ***P* = 0.0034, ****P* = 0.0002. *P* values determined by two-tailed unpaired *t*-test. Insets show the individual mice. (b) Frequency of total CD4⁺ T-cells (percentage of total CD3 T-cells) for the control-transduced and R5 knock-down cohorts 1 and 2. Mean ± sem.; **P* = 0.0146. *P* values determined by two-tailed unpaired *t*-test. (c) HIV RNA copies per ml of blood plasma collected for the control-transduced and R5 knock-down mice cohort 1 and cohort 2 over 92 and 134 days, respectively. Time of termination was chosen at random for the various groups. The dashed line indicates 400 copies/ml, detection limit of the HIV RNA assay. Mean ± sem. (d) Percentage CCR5 expression on total CD4⁺ T-cells in peripheral blood and spleen of various cohorts of mice. Blood: mean ± sem.; **P* = 0.0237, **P* = 0.0121, ***P* = 0.0035, ****P* = 0.0007, *****P* = 0.0001. Spleen: mean ± sem.; **P* = 0.041, ****P* = 0.0003. *P* values determined by two-tailed unpaired *t*-test. (e) HIV replication is inhibited *ex vivo* in GFP-positive sorted splenocytes from R5 knock-down mice. Splenocytes were isolated from R5 knock-down mice 20 weeks after CD34⁺ cell injection. Splenocytes were sorted into GFP-positive (n=5) and GFP-negative (n=5) fractions at 99% purity and challenged with R5 tropic HIV (YU-2). The amount of HIV production in the culture supernatant was monitored by HIV p24 ELISA. Viral loads were normalized to the peak viral load in each condition (mean ± sem).

h). The CD4⁺ cell population, however, remained the same over the observation period of 92 days (cohort 1), or showed a CD4⁺ T-cell loss only at day 134 days (cohort 2) (Fig. 1b). We explain this increase in GFP⁺ HIV-resistant CD4⁺ cells as the result of preferential expansion at the cost of untransduced CD4⁺ T-cells, while the lymphoid system tries to keep the lymphoid T-cell number constant. However, HIV replication was similar in R5 knock-down mice and control-transduced mice over the observation period at 92 and 134 days (Fig. 1c). Analysis of absolute numbers of CD4⁺ T-cells (cohort 2) indicated that the control-transduced mice lost CD4⁺ T-cells; whereas, they were more or less maintained in the R5 knock-down mice (data not shown).

In the R5 knock-down mice, CCR5 was down-regulated in the GFP-positive CD4⁺ T-cells, but CCR5 was detected on the GFP-negative CD4⁺ T-cells in blood and spleen (Fig. 1d). In the control-transduced mice, CCR5 was detected on GFP-negative and -positive CD4⁺ T-cells. We verified the efficacy of our gene engineering approach by separating transduced from untransduced splenocytes (R5 knock-down mice) by FACS and infected the populations *ex vivo* with R5 tropic HIV. GFP-positive splenocytes had no HIV replication (Fig. 1e).

These results suggest that CCR5 knock-down efficiently protects CD4⁺ T-cells from HIV infection, while CCR5-expressing CD4⁺ T-cells are eradicated. In our mice, despite HIV challenge, at least a proportion of the ~70–80% untransduced hematopoietic stem cells survive and continue to produce HIV-permissive CD4⁺ T-cells, which sustains high HIV titers. It is still not entirely clear what effect HIV infection has on CD34⁺ cells and to what extent they are depleted, if at all (37).

Transplantation of purified CCR5 knock-down CD34⁺ cells results in mice with “pure” populations of transduced cells *in vivo*

The lack of resistance to HIV infection was likely due to the chimerism of transduced and untransduced CD34⁺ cells in our initial experiments. Therefore, we sorted the CD34⁺ cells after transduction into CCR5 knock-down, GFP-positive and -negative fractions obtaining a >90% pure population of GFP-positive CD34⁺ cells. Mice transplanted with GFP-sorted cells were called “FACS-sorted R5 knock-down mice.”

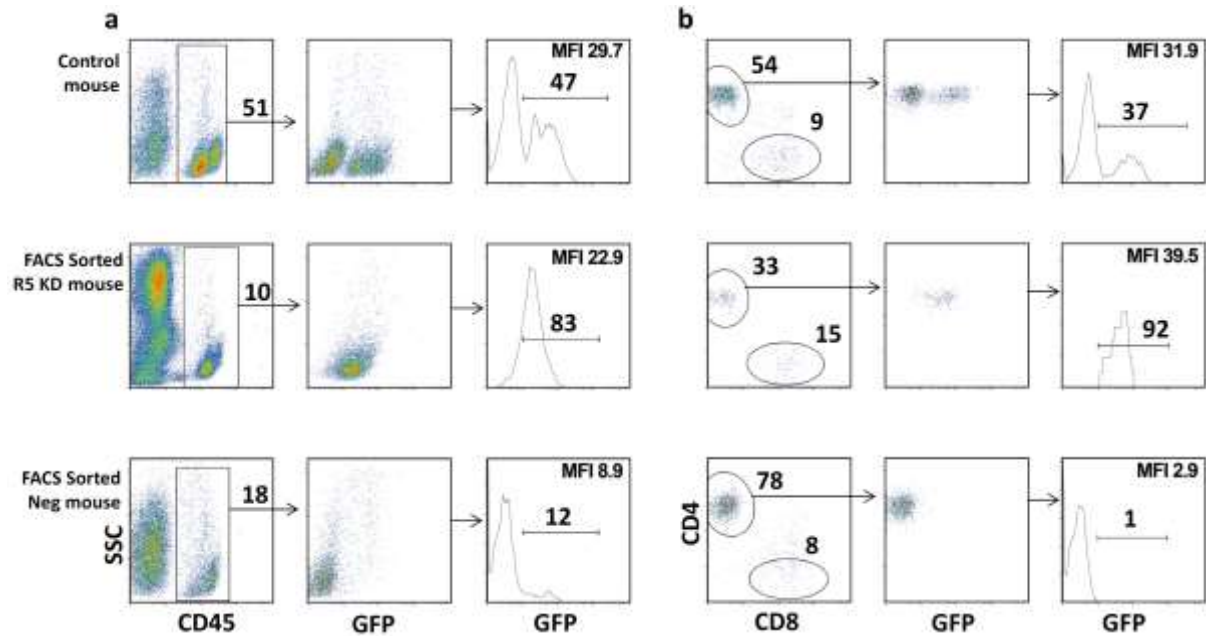


FIG 2 GFP-positive CCR5 knock-down sorted CD34⁺ HSPCs produced mice with “pure” populations of transduced cells *in vivo*. (a) FACS plots showing the percentage human engraftment CD45⁺ (percentage of live cells) and GFP-positive CD45⁺ (percentage of human CD45) cells for representative control-transduced, FACS-sorted R5 knock-down and FACS-sorted negative mice before HIV infection. (b) FACS plots showing the percentage CD4⁺ T-cells (percentage of CD3⁺), CD8⁺ (percentage of CD3⁺) and GFP-positive CD4⁺ T-cells (percentage of CD4⁺) in the peripheral blood before HIV infection of the same mice as in (a). For all cell subset analysis, the sub-gating was done as follows, total live cells, CD45⁺, CD3⁺, CD4⁺, CD8⁺. Mean fluorescence intensity (MFI) for GFP is indicated.

Analysis of the peripheral blood in six of the FACS-sorted R5 knock-down animals showed a single GFP-positive peak for human CD45⁺ and CD4⁺ T-cells, suggesting that only transduced CD34⁺ cells engrafted in these mice (Fig. 2); the level of GFP-positive cells was a major criterion for successful gene engineering and engraftment. Mice transplanted with the GFP-negative fraction were called “FACS-sorted negative mice.” They developed CD4⁺ and CD8⁺ T-cell populations with no GFP expression (Fig. 2). In contrast, the control-transduced mice had two distinct GFP-negative and -positive populations for CD45⁺ and CD4⁺ T-cells (Fig. 2). A summary of mice used in this study is indicated in (Table 1). Mice with less than 5% human engraftment in the peripheral blood before HIV infection were excluded.

Table 1: Mice used in the study

	%GFP ⁺ CD34 ⁺ cells; [Pre-transplantation]	Total mice	% Engraftment (>5% hCD45 ⁺)	% GFP ⁺ CD4 ⁺ T-cells (>70%)	% GFP ⁺ CD4 ⁺ T-cells (20-70%)	% GFP ⁺ CD4 ⁺ T-cells (>20%)
Untransduced	n/a	15	11	n/a	n/a	n/a
Control-transduced	15-40	17	12	0	5	7
R5 knock-down	17-20	15	11	0	1	10
FACS GFP-positive	12-32	20	10	6	4	0
FACS GFP-negative	n/a	26	15	n/a	n/a	n/a

Transplanting purified CCR5 knock-down CD34⁺ cells dramatically lowered viral load and protected HIV target cells *in vivo*

The FACS-sorted R5 knock-down mice had markedly lower viral loads than the FACS-sorted negative mice over 28 weeks (Fig. 3a). Peak viremia for the FACS-sorted R5 knock-down mice was on average 4.2×10^3 copies/ml, and FACS-sorted negative mice had 3.5×10^5 copies/ml. Viral loads for the untransduced, control-transduced, R5 knock-down, and FACS-sorted negative mice were similar. The FACS-sorted R5 knock-down mice had lower viral loads than all cohorts.

CD4⁺ T-cells (percentage of total CD3⁺ T-cells) from the FACS-sorted negative mice declined steadily upon infection (day 0: 55%; day 134: 20%) (Fig. 3b). In contrast, the FACS-sorted R5 knock-down mice showed a steady increase in CD4⁺ T-cells (day 0: 33%; day 196: 65%). Furthermore, the absolute numbers of CD4⁺ T-cells increased for the FACS-sorted R5 knock-down mice but decreased for the FACS-sorted negative mice (data not shown).

At euthanasia, for the FACS-sorted R5 knock-down mice, 70% of CD4⁺ T-cells in the blood and 83% of CD4⁺ T-cells in the spleen were GFP-positive (Fig. 3c-d). In the control-transduced and R5 knock-down groups, this was on average 20% and 18% in the blood and 21% and 20% in the spleen, respectively (Fig. 3c,d). Similarly, the CD4⁺/CD8⁺ T-cell ratios in the blood and spleen (end/pre-infection) were very low in the various groups except for the FACS-sorted R5 knock-down group (Fig. 3e,f). Absolute numbers of GFP-positive CD4⁺ T-cells expanded significantly upon HIV challenge in the FACS-sorted R5 KD mice

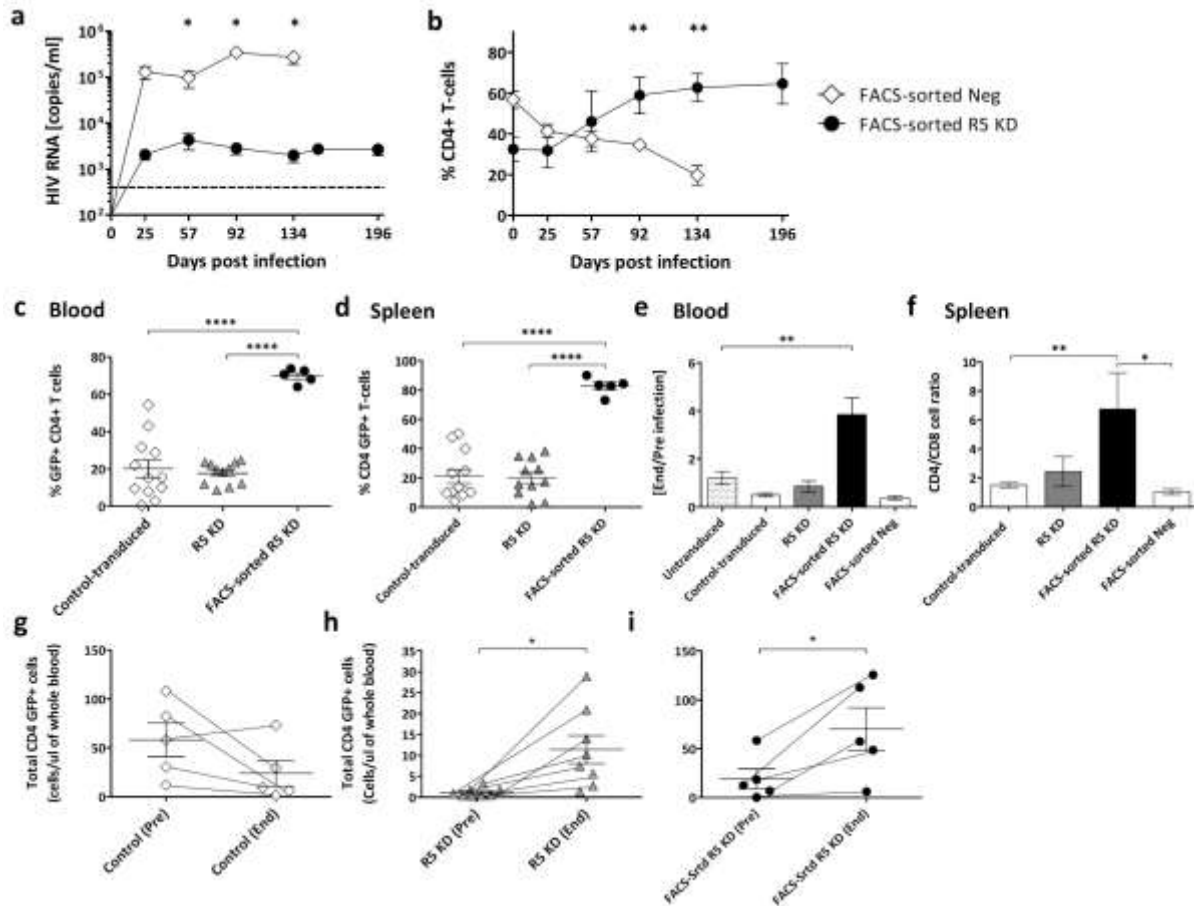


FIG 3 Sustained HIV viral load inhibition in FACS-sorted R5 knock-down mice. (a) HIV RNA copies per ml in blood plasma from FACS-sorted R5 knock-down $n=5$ (YU-2) and FACS-sorted negative $n=15$ (YU-2 $n=9$ or JRCSF $n=6$) mice collected over 134 and 196 days, respectively. The viral load detection limit is indicated by the dashed line (400 copies/ml). Mean \pm sem.; Day 57 $*P = 0.0486$, day 92 $*P = 0.0188$, and day 134 $*P = 0.0391$. P values determined by a two-tailed unpaired t -test. (b) Percentage CD4⁺ T-cells of FACS-sorted R5 knock-down ($n=5$) and FACS-sorted negative ($n=15$) mice. Mean \pm sem.; Day 92 $**P = 0.0017$ and day 134 $**P = 0.0018$. P values determined by two-tailed unpaired t -test. (c) Percentage GFP-positive CD4⁺ T-cells in the peripheral blood at termination. Mean \pm sem.; $****P = 0.0001$. P values determined by two-tailed unpaired t -test. (d) Percentage GFP-positive CD4⁺ T-cells in the spleen at termination. Mean \pm sem.; $****P = 0.0001$. P values determined by two-tailed unpaired t -test. (e) Change of CD4⁺/CD8⁺ T-cell ratio in the peripheral blood, comparing the CD4⁺/CD8⁺ T-cell ratio of each cohort end/pre-infection. Mean \pm sem.; $**P = 0.0026$. P values determined by two-tailed unpaired t -test. (f) CD4⁺/CD8⁺ T-cell ratio at termination in the spleen of various cohorts of mice. Mean \pm sem.; $**P = 0.0059$, $*P = 0.039$. P values determined by two-tailed unpaired t -test. (g-i) Absolute numbers of GFP⁺ CD4⁺ T-cells or total CD4⁺ T-cells/ μ l of blood of representative mice from the control-transduced ($n=5$), R5 knock-down ($n=8$) and FACS-sorted R5 knock-down ($n=5$) cohorts. Mean \pm sem.; $*P = 0.0194$, $*P = 0.0369$, respectively. P values determined by paired t -test.

(Fig. 3i), and CCR5 expression was down-regulated in the blood and spleen of the FACS-sorted R5 knock-down mice (Fig. 1d).

In summary, transplantation of GFP-positive sorted CD34⁺ cells produced mice with high levels of gene-engineered CCR5 knock-down CD4⁺ T-cells *in vivo*. This resulted in long-term inhibition of HIV replication *in vivo* and preservation of HIV target cells in the blood and spleen.

Outlier analysis and follow-up of mice that did not meet acceptance criteria

The FACS-sorted R5 knock-down mice typically controlled the virus long-term (titers < 10⁴ copies/ml) while maintaining a high level of GFP-positive CD4⁺ T-cells in the blood (Fig. 4a). In contrast, a single FACS-sorted R5 knock-down mouse (#954) showed an unexpected decline in GFP-positive CD4⁺ T-cells with high HIV copy numbers, >10⁵ copies/ml on days 57 and 92 (Fig. 4a). We performed an outlier analysis (see methods section), and at four different times, viral loads detected in animal #954 were statistical outliers (Fig. 4a). Based on this, animal #954 was not included in the mean values and was analyzed separately (see below). At termination, this mouse had much lower splenic engraftment of total human splenocytes with 31% human cells compared to the FACS-sorted R5 knock-down mice which had 54% (mean ± sem: 11%). GFP-positive CD4⁺ T-cells were barely detected in the spleen of mouse #954 (6%), while the FACS-sorted R5 knock-down mice had, on average, 83% (mean sem ± 2%) GFP-positive CD4⁺ T-cells in the spleen (Fig. 3d).

The inclusion criterion we defined as successful reconstitution for FACS-sorted R5 knock-down mice was 70% GFP⁺ CD4⁺ T-cells in the peripheral blood before infection. Four mice did not meet these criteria despite being transplanted with GFP-positive sorted CD34⁺ cells (Fig. 4b). Mouse #1113 had no protection against HIV and had a limited expansion of GFP-positive CD4⁺ T-cells (23% on day 0 to 43% on day 137). Mice #958 and #1115 had massive expansions of GFP-positive cells reaching close to 100% of the total CD4⁺ T-cell population which went in parallel with a decrease in the viral load (Fig. 4b). For mouse #1608, the dynamics of GFP-positive CD4⁺ T-cell recovery and viral load were slower, and GFP-positive CD4⁺ T-cell recovery on day 134 was less extensive (Fig. 4b). These three mice (#958, #1115,

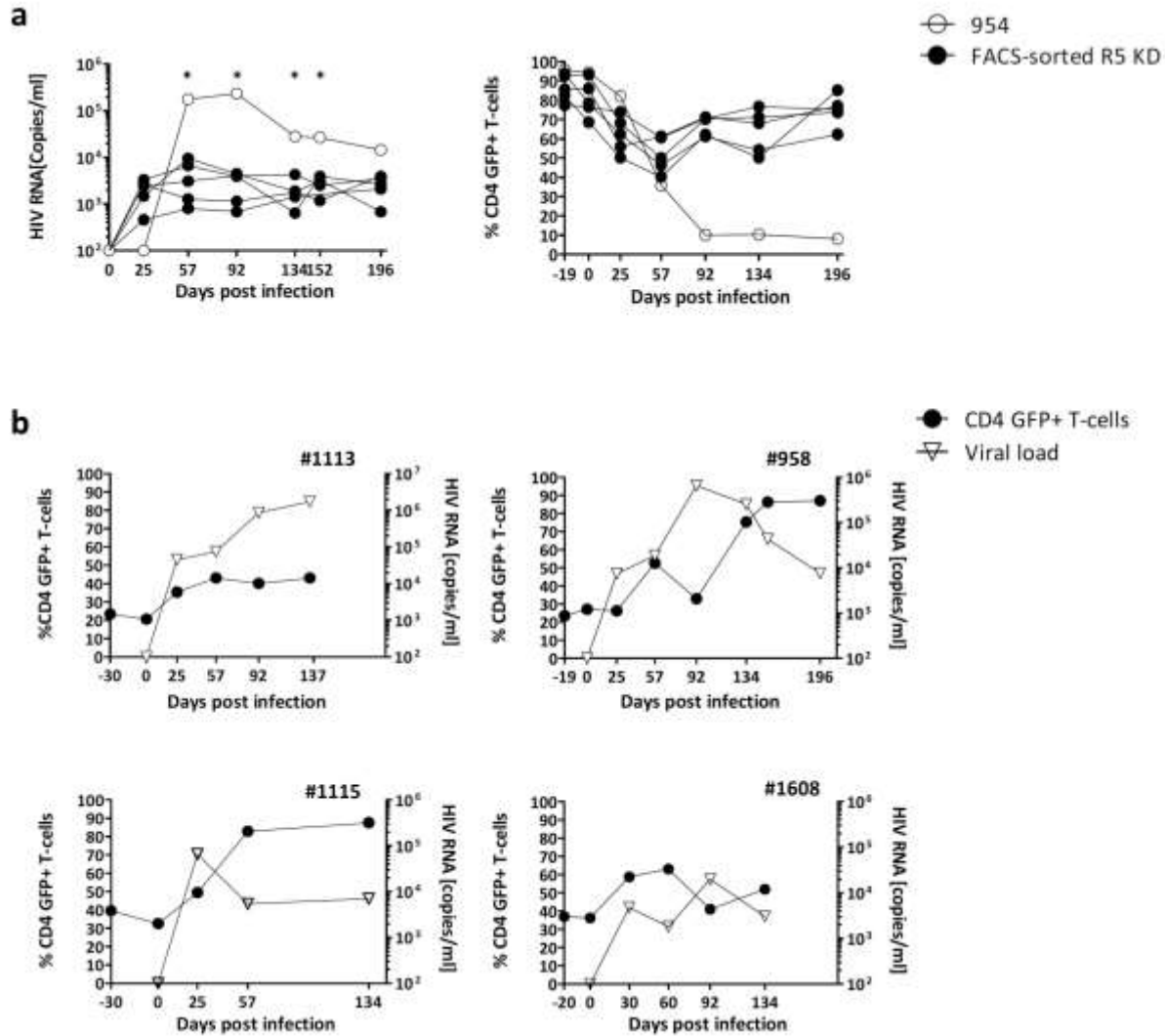


FIG 4 Outlier analysis and examples of viral load control due to homeostatic expansion of transduced cells. (a) Left panel, viral load of the FACS-sorted R5 knock-down mice (YU-2) and viral load of “outlier FACS-sorted R5 knock-down mouse #954” (YU-2). Right panel, percentage GFP-positive CD4⁺ T-cells of the FACS-sorted R5 knock-down mice and mouse #954. Percentage GFP-positive CD4⁺ T-cells as a percentage of total CD4⁺ T-cells. *P* values determined by GraphPad Outlier calculator. **P* < 0.001. (b) Viral load and percentage GFP-positive CD4⁺ T-cells of three individual FACS-sorted R5 knock-down mice (mouse #1113, JRCSF, #958, YU-2, #1115, JRCSF and #1608, JRCSF) that were excluded due to not reaching the inclusion criteria of over 70% GFP-positive CD4⁺ T-cells before HIV infection..

#1608) maintained a high CD4/CD8 ratio, and the percentage of GFP-positive CD4⁺ T-cells in the blood increased upon infection from 30 to 80, 49 to 82 and 37 to 52% respectively. These animals also had high frequencies of GFP-positive CD4⁺ T-cells in the spleen of 59, 60 and 83% respectively.

Strikingly, we observed this level of expansion (Fig. 4b) of HIV-resistant GFP-positive CD4⁺ T-cells cells and a concomitant inhibitory effect on HIV only in mice transplanted with CCR5 knock-down GFP-sorted CD34⁺ cells (these animals had 30±4% GFP⁺ CD4⁺ T-cells on day 0). This degree of expansion was not seen in R5 knock-down mice (which had 5±2% GFP⁺ CD4⁺ T-cells before HIV infection). Based on these results, we estimate that at least 20% of CD4⁺ T-cells need to be CCR5 repressed to observe homeostatic expansion and relevant effects on viremia.

Preserved engraftment and preferential expansion of central memory T-cells in FACS-sorted R5 knock-down mice upon HIV infection

Engraftment as reflected in peripheral blood decreased in all control cohorts but increased in the FACS-sorted R5 knock-down mice (Fig. 5a). This effect on total engraftment was even more impressive in the spleen. FACS-sorted R5 knock-down mice had 10 times more human cells than control cohorts (Fig. 5b).

We evaluated the CD4⁺ and CD8⁺ effector (CD45RA^{pos}; CCR7^{neg}), effector memory (CD45RA^{neg}; CCR7^{neg}), naive (CD45RA^{pos}; CCR7^{pos}) and central memory (CD45RA^{neg}; CCR7^{pos}) T-cell subsets in the blood and spleen of the FACS-sorted R5 knock-down and representative FACS-sorted negative mice (Fig. 5c-f). In the peripheral blood of the FACS-sorted negative mice, the frequency of central memory CD4⁺ T-cells was significantly decreased, and the CD8⁺ central memory T-cell subset was unchanged (Fig. 5c,e). In contrast, central memory CD4⁺ and CD8⁺ T-cells were increased in the FACS-sorted R5 knock-down mice. Similarly, more CD4⁺ and CD8⁺ central memory T-cells were present in the spleens of the FACS-sorted R5 knock-down mice (Fig. 5d,f). We observed no differences between the cohorts for effector and effector memory cells (data not shown). Notably, however, there was a trend towards a decrease in naive CD4⁺ and CD8⁺ T-cells in all cohorts (data not shown).

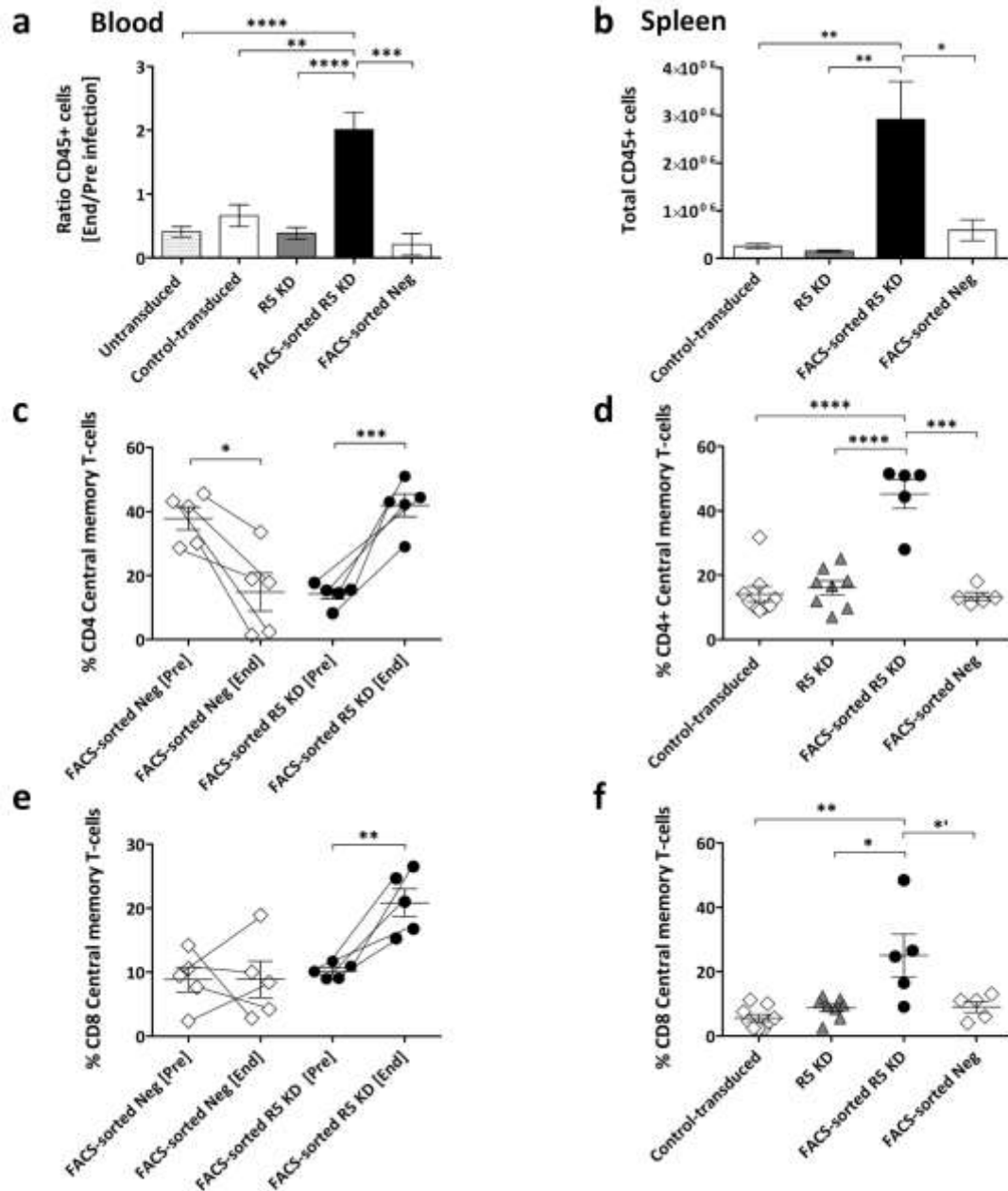


FIG 5 Increased engraftment and central memory T-cells in blood and spleen of FACS-sorted R5 knock-down mice. (a) Change in level of peripheral blood engraftment expressed as the ratio of total CD45+ end/pre-infection. Mean \pm sem.; $^{*}P = 0.0031$, $^{***}P = 0.0006$, $^{****}P < 0.0001$. *P* values determined by two-tailed unpaired *t*-test. (b) Absolute numbers of human cells (CD45+) in the spleen at termination. Mean \pm sem.; $^{*}P = 0.0229$, $^{**}P < 0.0019$. *P* values determined by two-tailed unpaired *t*-test. (c, e) Percentage of CD4⁺ and CD8⁺ central memory T-cells in the peripheral blood, pre-infection and at the end time point. Mean, sem.; $^{*}P = 0.0153$, $^{**}P = 0.0042$, $^{***}P = 0.0004$. *P* values determined by paired *t*-test. (d, f) Percentage CD4⁺ and CD8⁺ central memory T-cells in the spleen at termination. Mean \pm sem.; $^{*}P = 0.0472$, $^{*}P = 0.0106$, $^{**}P = 0.0022$, $^{***}P = 0.0001$, $^{****}P < 0.0001$. *P* values determined by two-tailed unpaired *t*-test.

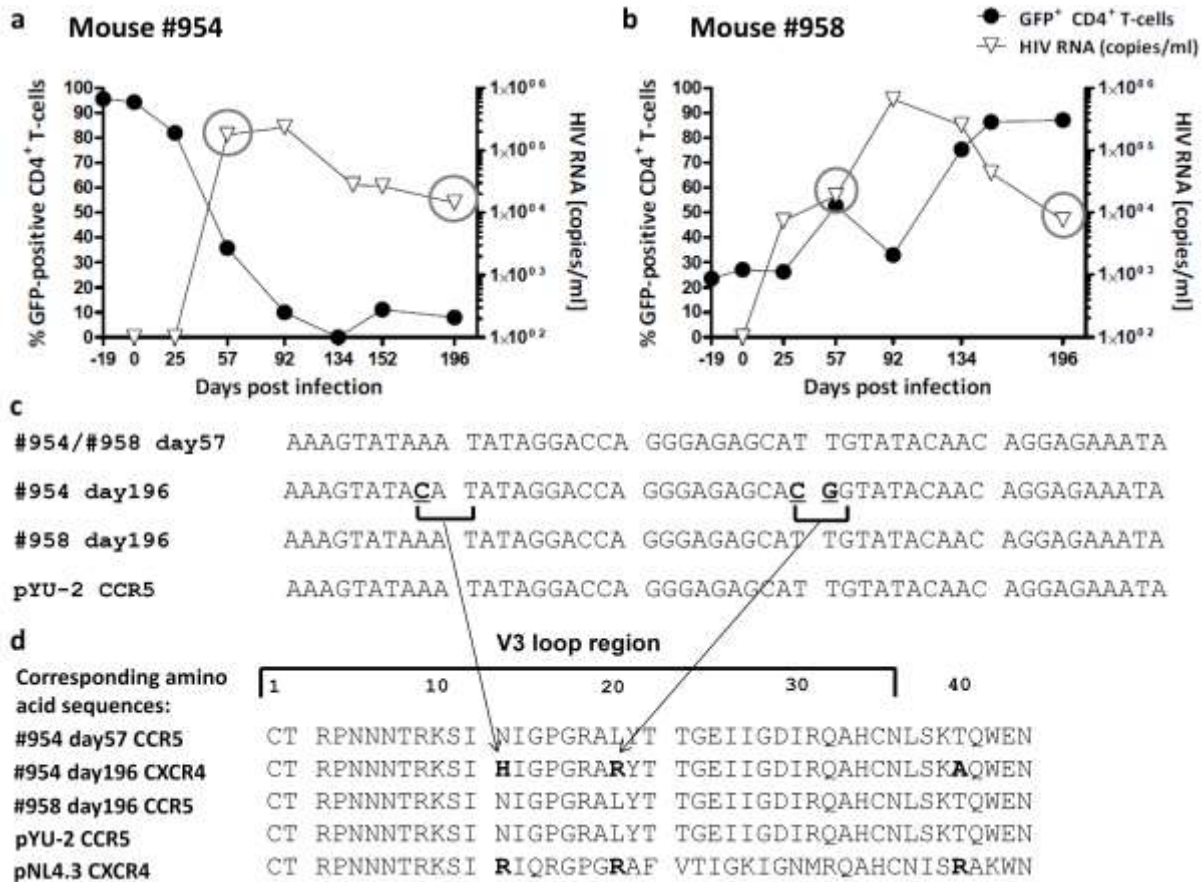


FIG 6 Population sequencing of HIV in plasma: evidence for gene-therapy failure. (a, b) Plots showing the percentage of GFP-positive CD4⁺ T-cells in peripheral blood (as a percentage of CD3⁺ T-cells) on the left Y axis and HIV viral load on the right Y axis for mice #954 and #958, respectively. Mouse #954 had a high and sustained viral load overtime with a complete loss of GFP-positive CD4⁺ T-cells. Mouse #958 experienced an expansion of GFP-positive CD4⁺ T-cells from less than 30% on day 19, to over 80% on day 196. (c) On day 57, both animals had a homogenous HIV population in the peripheral blood, sequencing data of HIV envelope V3 loop is consistent with an R5 tropic HIV strain. On day 196, animal #954, which experienced a complete loss of GFP-positive CD4⁺ T-cells (blood and spleen), had detectable mutations within the V3 loop of HIV. For mouse #958, no mutations were detected in the V3 loop on day 196, indicating the presence of a homogenous HIV population. (d) Amino acid changes to basic amino acids H and R. According to a Geno2Pheno (<http://www.geno2pheno.org/>) analysis of the obtained sequence in (c), there is only 18% confidence that the virus at day 196 of mouse #954 was not an X4 variant.

Evidence of shift from R5 to X4 tropic strain in one mouse

As described above (Fig. 4), FACS-sorted R5 knock-down mice had a low viral load and maintained high levels of CCR5 knock-down CD4⁺ T-cells. Mouse #954 was clearly an outlier: it had high viral titers and

lost GFP-positive CD4⁺ T-cells (Fig 6a). We hypothesized that this mouse might have had a tropism shift of the virus from R5 to X4. We performed HIV population sequencing (from plasma) on days 57 and 196. As a control, we analyzed mouse #958 (Fig. 6b). Sequencing revealed no mutations in mouse #958. Mouse #954 had mutations in the V3 loop of the HIV envelope sequence (Fig. 6c), resulting in amino acid substitutions to basic amino acids as indicated (Fig. 6d). Substitutions in the V3 loop to basic amino acids have been reported to result in a switch from R5 to X4 tropism (38).

Discussion

Here we investigated a lentiviral vector-based, CCR5-targeting miRNA as a tool for engineering an HIV-resistant human immune system. We show that i) the miRNA-based vector was very efficient in down-regulating CCR5 from T-cells and prevented their infection by HIV *ex vivo*, ii) only mice that were transplanted with a pre-selected population of transduced CD34⁺ cells and maintained gene engineered CD4⁺ T-cells had a dramatically reduced viral load (functional cure), and iii) the HIV-infected mice transplanted with miRNA CCR5 gene-engineered CD34⁺ cells showed a dramatic expansion of memory T-cells (i.e., the miRNA edited T-cells were mainly of this phenotype). Thus, we provide here pre-clinical proof-of-concept for gene engineering of an HIV-resistant immune system through the use of vector-mediated miRNA expression and the need for a certain threshold of gene engineered CD34⁺ cells for functional cure of HIV.

While gene engineering HIV-resistant cells is a viable option to cure of HIV, major issues remain to be solved. These include finding the best anti-viral moiety or combination, the most efficacious way to gene engineer the CD34⁺ cells, and the threshold of gene engineered CD34⁺ cells needed for functional cure.

Lentivirus-based transduction has been supplemented by gene-targeting methods, such as ZNF-or Talen nucleases, or the Crispr/Cas system for gene editing (23-25). However, off-target effects of these methods are still unknown and gene engineering in primary cells is only modestly effective (26). And even though no adverse events have been reported, there is less experience in clinical trials with gene-targeting methods than with lentivirus-based transduction. Thus, we opted for lentivirus-based gene engineering

(39-41). Furthermore, we are the first to engage in miRNA technology for knocking down the HIV co-receptor CCR5 in CD4⁺ T-cells via gene engineering CD34⁺ cells. miRNAs closely mimic naturally occurring pri-miRNAs and, thus, are less likely to cause oversaturation of the RNA interference pathway and to affect cellular homeostasis than the widely used shRNAs (42, 43). However, miRNAs are thought to have a lower capacity to down-regulate target genes than shRNAs. Here we used a miRNA we developed with optimized features that efficiently knock-down target genes upon single-copy vector transduction (30). *Ex vivo* sorted cells were indeed resistant to a challenge with CCR5-tropic strains. However, bulk transplantation of transduced CD34⁺ cells into mice resulted in a human lymphoid system that replicated HIV similar to untransduced hu mice but preserved CD4⁺ T-cell counts. Similar data have been reported (15).

We hypothesized that the majority of HSPCs needs to be gene engineered to see an effect on the HIV load; otherwise, the HIV permissive CD4⁺ T-cells that originated from the untransduced CD34⁺ cells would “outnumber” the HIV-resistant cells. Thus, we used GFP to allow for efficient sorting of transduced CD34⁺ cells before transplantation. By doing so, we found that, to achieve long-term suppression of viral load, more than 70% of CD34⁺ transplanted should be gene engineered. Walker et al. obtained an average engraftment (\pm STD) level of anti-HIV vector transduced cells of 17.5 \pm 8% in the peripheral blood and argued that these numbers of cells were insufficient to see any decrease in plasma viremia (15). Furthermore, a very recent publication from the same group sorted the gene engineered CD34⁺ cells with a truncated version of CD25 before their transplantation into 2–5-day-old NRG mice (20). They found that mice transplanted with tCD25 purified CD34⁺ cells had normal multi-lineage hematopoiesis similar to mice transplanted with untransduced CD34⁺ cells. Upon HIV challenge, tCD25 transplanted mice did not suffer from HIV induced CD4⁺ T-cell depletion as did the untransduced mice and tCD25 mice had a 1.5 log inhibition in plasma viremia, compared to mice with untransduced CD34⁺ cells. Our data nicely complement the data provided by Walker et al. and Barclay et al. and underline the importance of the number of transduced cells that are required for efficient HIV gene therapy. Notably, three hu mice transplanted with purified gene engineered CD34⁺ cells showed at baseline ~40% GFP⁺ cells which

expanded substantially upon HIV infection; the expansion went along with a decrease in viral load. These three mice were reminiscent of the data reported by Holt et al where disruption of CCR5 by zinc-finger nucleases was achieved in ~20% of CD34⁺ cells and resulted in viral repression over time (16).

Obviously, in humans, GFP-based sorting would not be an option, given the xenogeneic nature of the protein. However, novel strategies for sorting of transduced CD34⁺ cells based on the expression of truncated cellular surface receptors, such as CD25 (20), the epidermal growth factor receptor (44) or the nerve growth factor receptor (45), are very promising for achieving high numbers of engrafted gene engineered cells. An alternative approach to pre-transplantation sorting would be *in vivo* selection of transduced cells (46, 47). Regrettably, current *in vivo* selection methods use potentially carcinogenic compounds, such as mycophenolate, methotrexate or alkylating agents (i.e., O6-benzylguanine/bis-chloroethylnitrosourea), that offset their use in a disease, such as HIV, that is amenable to an efficient and well-tolerated cART. We want to emphasize that, in our gene-engineering efforts, we aimed for single lentiviral copy integration. The two recent phase I clinical trials used gene engineering protocols that resulted in vector copy numbers of ranging from 2-4 per genome of bone marrow cells prior to transplantation without documenting any adverse events over an observation period of >20 months (39, 40). Thus, ensuring CD34⁺ transduction might present another alternative for increasing the number of gene engineered CD34⁺ cells. These protocols appear not to affect the long-term engraftment negatively in these phase I clinical trials.

In fact, we do not know the number of gene engineered HSPCs needed to render the immune system resistant to HIV. As outlined above, we aimed for a rather pure population of gene engineered HSPCs as proof-of-pre-clinical concept. However, we observed HIV lowering effects in some mice with 20-40% of engraftment – similar data as reported by Holt et al. (16). HIV certainly by killing untransduced cells via its cytopathic effects will promote the expansion of HIV resistant cells. To what extent the HIV-resistant cells will foster an efficient HIV-specific immune response and thereby constrain HIV remains unknown. Whether additional factors contribute to HIV lowering effects remain unknown.

White blood cell counts from HIV-infected mice generated with FACS sorted R5 KD cells showed an expansion of central memory CD4⁺ and CD8⁺ T-cells, while all other groups showed a progressive loss of these CD4⁺ memory T-cells and no change in CD8⁺ T-cells. This pattern was also evident when looking at the splenocytes. These memory CD8⁺ T-cells might have contributed to the control of HIV. There was a decrease in the frequency of naive CD4⁺ and CD8⁺ T-cells in the peripheral blood in both FACS-sorted negative and FACS-sorted R5 KD mice, whereas in the spleen, the naive CD4⁺ and CD8⁺ T-cells in the FACS-sorted R5 KD mice tended to be higher (data not shown). The expansion of central memory T-cells is reminiscent of the immune reconstitution seen in HIV-infected patients on ART (48)

HIV is known for its high mutational rate. In this respect, we observed one mouse (#954) with an escape mutation. Despite high levels (day 0) of engraftment of CCR5 knock-down cells, this mouse had a high viral load and a complete loss of circulating CD4⁺ T-cells. Population sequencing of the V3 loop indicated a likely shift to X4 tropism which might explain the uncontrolled infection. The mutations were detectable in the blood only at relatively late time points. This might be due to an initial compartmentalized replication of the X4-tropic strains in the thymus. We previously showed that X4-HIV NL4-3 severely depleted the thymus, whereas YU-2 only had minor effects (49). However, we do not know whether the potential emergence was due to the CCR5 knock-down or was just a coincidence. Indeed, emergence of CXCR4-tropic strains may occur without any immune or drug pressure in hu mice infected with CCR5-tropic strains (50). In any case, CCR5 knock-down should be done in concert with another strategy to constrain HIV (i.e., including another anti-HIV moiety, combining with efficient antiretrovirals, or boosting the immune response in parallel to transplantation). Indeed, the solidness of successful gene engineering by the expression of more than one antiviral moiety may prevent HIV evolution (51). Gene engineering could be combined with conventional ART: combining two treatment modalities was efficient in cell lines (52), as induction therapy (53) or with anti-PD1 antibodies that decrease viral load and increase the level of CD4⁺ T-cells in HIV-infected mice (54). In any case, gene engineering efforts cannot promote more virulent HIV strains, neither for the individual patient nor for the general population.

In summary, our results provide the first preclinical proof of concept that transplantation of miRNA CCR5 knock-down CD34⁺ cells can lead to long-term control of HIV viremia. Translation of our results to the clinical setting is relatively straightforward, but will require the implementation of existing strategies for pre/post-transplantation selection compatible with human use. At this point, our strategy demonstrates long-term viral control, but not yet a cure. However, while a cure remains the ultimate goal, long-term viral control independent of antiretrovirals is a relevant intermediate step, worth translating to the clinical setting. We believe that a definitive cure of HIV might indeed come from a combination of different approaches such as CCR5 knock-down combined with drug therapy, vaccination or second gene therapy target.

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Author Contributions

R.M. designed and performed experiments, analyzed data and wrote the paper. R.F.S. and K.H.K. designed the experiments, supervised the work, analyzed the data and wrote the paper. M.S.P. was involved in the initial conceptualization of the study, supervised aspects of the work and helped to write the paper. A.A. helped to coordinate the work with the mice, transplantation of new-born mice, processing cord blood and write the paper. P.S. helped supervise aspects of the work and designed certain experiments. V.J. helped to write the paper and analyze data. G.G.H., L.D., and M.A.R., helped to

terminate experiments and process cord bloods. S.I. helped in processing of cord bloods. S.R. provided the expertise and obtained the viral loads for HIV-infected mice. M.G.M. gave highly valuable input in the entire study.

Conflict-of-interest disclosure: The authors declare no conflicts of interest.

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