

Safety and Efficacy of a Lentiviral Vector Containing Three Anti-HIV Genes—CCR5 Ribozyme, Tat-rev siRNA, and TAR Decoy—in SCID-hu Mouse-Derived T Cells

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Gene therapeutic strategies show promise in controlling human immunodeficiency virus (HIV) infection and in restoring immunological function. A number of efficacious anti-HIV gene constructs have been described so far, including small interfering RNAs (siRNAs), RNA decoys, transdominant proteins, and ribozymes, each with a different mode of action. However, as HIV is prone to generating escape mutants, the use of a single anti-HIV construct would not be adequate to afford long range-viral protection. On this basis, a combination of highly potent anti-HIV genes—namely, a short hairpin siRNA (shRNA) targeting rev and tat, a transactivation response (TAR) decoy, and a CCR5 ribozyme—have been inserted into a third-generation lentiviral vector. Our recent *in vitro* studies with this construct, Triple-R, established its efficacy in both T-cell lines and CD34 cell-derived macrophages. In this study, we have evaluated this combinatorial vector *in vivo*. Vector-transduced CD34 cells were injected into severe combined immunodeficiency (SCID)-hu mouse thy/liv grafts to determine their capacity to give rise to T cells. Our results show that phenotypically normal transgenic T cells are generated that are able to resist HIV-1 infection when challenged *in vitro*. These important attributes of this combinatorial vector show its promise as an excellent candidate for use in human clinical trials.

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INTRODUCTION

Highly active anti-retroviral therapy (HAART) utilizing a combination of drugs has proven to be effective in controlling human immunodeficiency virus (HIV) disease progression and in decreasing overall mortality rates in parts of the world where HAART is accessible. However, systemic toxicity and the generation of drug-resistant mutants during prolonged treatment remain major issues

to be addressed.^{1–3} Alternative therapeutic strategies are continuously being sought to overcome these limitations. In this regard, gene therapy approaches to render virus-susceptible cells resistant to HIV-1 infection hold considerable promise. In contrast to many viral infections, which are primarily acute, with severe pathology seen within a few days, HIV disease lends itself as an ideal target for novel therapeutic strategies.⁴ First, it is a chronic disease with a long incubation period before serious life-threatening symptoms manifest, thus providing ample opportunity for effective clinical intervention. Second, the primary viral target cells are of hematopoietic origin and are continuously generated from hematopoietic precursor cells originating in the bone marrow.^{5,6} Therefore, treatment modalities can focus on hematopoietic stem cells (HSCs) as a single therapeutic target, with viral resistance being inherited continuously in progeny T cells and monocytes/macrophages.

On the basis of the potential of intracellular immunization strategies, a number of approaches have utilized both viral and cellular molecules as therapeutic targets. The anti-HIV molecules can include ribozymes, RNA decoys, small interfering RNAs (siRNAs), species-specific restriction factors such as TRIM5alpha, transdominant proteins, antisense RNA, and aptamers, to name a few.^{7–27} These constructs have targeted both viral regulatory molecules such as tat and rev and cellular molecules that are involved in viral entry, namely CCR5 and CXCR4. The effectiveness of these approaches in suppressing viral replication varies depending on the viral target and/or the inhibitory strategy. Of these strategies, an approach using anti-HIV siRNAs that function via an endogenous RNA interference pathway has been promising both *in vitro* and *in vivo*.^{9,11–16,18,19,25,28,29} However, since the mechanism of RNA interference is highly sequence specific and the HIV genome is prone to high mutation rates, the generation of viral escape mutants is a potential problem during prolonged siRNA treatment.^{30–32} To overcome this problem, it is necessary to employ a combination of siRNAs targeted to different conserved regions of the HIV genome and/or incorporate siRNAs that act on cellular molecules such as CCR5. Alternatively, siRNAs can be utilized in combination with other

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inhibitory molecules, such as RNA decoys and ribozymes, that function differently.^{11,24}

To obtain sustained transgene expression and anti-viral protection in differentiated HIV-susceptible end-stage cells such as T cells and macrophages, CD34 HSCs are used for gene transduction.^{7-11,16,17,24,25,33,34} Delivery of transgenes into these cells with high efficiency and retention of their expression has been problematic in the past using conventional retroviral vectors owing to their inability to enter quiescent cell types.³⁵ However, the use of lentiviral vectors has overcome some of these limitations, and more recent work has used these vectors for gene delivery.^{36,37} With the goal of minimizing the potential for generating viral resistance, we have experimented with a combinatorial lentiviral vector that incorporates three different anti-HIV RNA inhibitory constructs targeted to a cell surface receptor, CCR5, and two HIV regulatory proteins, *tat* and *rev*.¹¹ The CCR5 co-receptor is essential for the entry of R5 tropic viral strains involved in primary infection.³⁸ In a certain population of individuals, a 32-base-pair deletion in the genome results in a lack of CCR5 expression.³⁹⁻⁴¹ As individuals homozygous for this gene deletion are apparently healthy and show significant resistance to HIV infection and disease progression, CCR5 is an ideal target for gene therapy. Previous *in vitro* and *in vivo* studies have documented the effectiveness of CCR5 inhibition in preventing HIV infection.^{10,11,16,20,21,24,42} HIV *tat* and *rev* proteins are viral regulatory molecules essential in the HIV life-cycle. siRNAs targeted to destroy their respective viral transcripts proved to be highly efficacious in suppressing viral growth.^{10-12,19} As a different strategy, a transactivation response (TAR) RNA decoy has been found to neutralize the activity of the HIV *tat* protein, thus restricting viral transcription.²² The combinatorial lentiviral construct we recently tested harbored a U6 Pol-III promoter-driven short hairpin siRNA (shRNA) targeting *rev* and *tat* messenger RNAs, a U6 transcribed nucleolar-localizing TAR RNA decoy, and a VA1-derived Pol-III cassette that expresses an anti-CCR5 ribozyme.¹¹

Evaluation of this construct *in vitro* in T-cell lines and in macrophages derived from CD34⁺ cells showed long-term expression and anti-viral protection in a more than additive fashion compared with the individual constructs tested alone. Transduced cells were protected even at a higher multiplicity of infection (MOI) of challenge, with no viral breakthrough being evident during the *in vitro* evaluation (~2 months).¹¹ Integral to the practical utility of this combinatorial construct for its *in vivo* use in the human is the question of its safety with respect to the transduced hematopoietic cells. In *in vitro* differentiation assays, it was found that vector-transduced cells differentiated normally into transgenic macrophages. Furthermore, the transgenic macrophages appeared to be functionally normal in their phagocytic capacity and their ability to up-regulate a co-stimulatory molecule, B7, involved in antigen presentation.¹¹ Owing to its harboring three different anti-HIV constructs in a single vector backbone and its capacity to afford long-term anti-viral protection and to reduce the risk of viral escape mutants being generated, this combinatorial construct shows great promise for testing in human clinical trials.¹¹

Although the above data showed the efficacy, safety, and normal differentiation of transgenic macrophages *in vitro*, it is not clear whether the vector-transduced CD34 cells have the capacity

to generate HIV-resistant T cells and whether these cells will be normal. In this regard, the severe combined immunodeficiency (SCID)-hu mouse xenograft model that harbors a normally functioning human thymus provides an *in vivo* system to evaluate the thymopoietic capacity of gene-transduced CD34 cells.^{33,34} Here we show that combinatorial "Triple-R" vector-transduced HSCs undergo normal lineage-specific differentiation, giving rise to phenotypically normal transgenic T cells resistant to HIV infection. These data further support the use of this vector for clinical applications.

RESULTS

Triple-R vector-transduced CD34 cells can reconstitute SCID-hu mouse thymic grafts

For the combinatorial Triple-R vector to be effective in a clinical setting, it is important that vector-transduced CD34 cells undergo proper lineage-specific differentiation *in vivo* into T cells in the human thymus. Therefore, one primary objective of these studies is to evaluate this differentiation. SCID-hu mouse thymic grafts provide an ideal environment for CD34 cells to undergo thymopoiesis and give rise to T cells at various stages of development. CD34 cells were transduced with the control HIV7-green fluorescent protein (HIV7-GFP) vector or Triple-R vector, and transduction efficiency was evaluated by fluorescence-activated cell sorting (FACS) for enhanced GFP (EGFP) expression. The levels of transduction were 66.2% for the HIV7-GFP control vector and 73.0% for the Triple-R vector (Figure 1a). Even though similar MOIs were used for control vector and Triple-R transductions, a

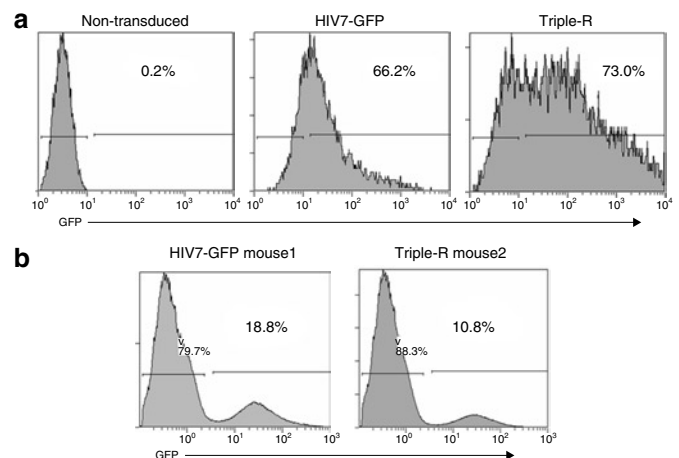


Figure 1 Lentiviral vector transduction efficiency in CD34 cells and enhanced green fluorescent protein (EGFP) expression by thymocytes derived in severe combined immunodeficiency (SCID)-hu mice thy/liv grafts. **(a)** Purified CD34 cells were transduced with either control human immunodeficiency virus-7 green fluorescent protein (HIV7-GFP) or Triple-R vector and analyzed by fluorescence-activated cell sorting (FACS) for EGFP expression to determine transduction efficiency. The percentage of EGFP-positive cells is indicated for each vector. Non-transduced cell control is also shown. **(b)** Vector-transduced CD34 cells were injected into the SCID-hu mice thy/liv grafts and allowed to differentiate into thymocytes. At 6 weeks after engraftment, the cells were harvested and analyzed by FACS for EGFP expression. Representative examples from two injected mice are shown. The percentage of EGFP-positive cells is indicated in each panel.

slight variation in transduction efficiency was observed; this is not inconsistent with what we have previously seen with different batches of vector preparations. The transduced CD34 cells were directly injected into thymic grafts. After allowing 6 weeks for cell engraftment and differentiation, the mice were killed and the thymocytes were analyzed by FACS for EGFP expression to determine the levels of *in vivo* cell reconstitution. Both control and Triple-R vector-transduced cells were able to thrive and engraft, although the levels of engrafted cells varied from mouse to mouse. The levels of reconstitution for the control HIV7-GFP vector cell-injected mice ranged from 17.9 to 22.0%; the levels ranged from 8.7 to 22.2% for Triple-R vector cell-injected mice (Table 1). Representative FACS plots for EGFP⁺ cells obtained from transgenic CD34 cell-injected mice are shown in Figure 1b. The average engraftment for all mice was 20.1% for control HIV7-GFP and 15.2% for Triple-R. In both cases, the values of engraftment reached as high as 22%. EGFP expression retained by transgenic cells *in vivo* until the time of killing at 6 weeks is indicative of persisting cell engraftment, vector retention, and stability.

Triple-R vector transgenic thymocytes are resistant to HIV-1 challenge

The above data showed that Triple-R vector-transduced CD34 cells successfully engrafted the human thymic grafts and differentiated into T lymphocytes at various stages of development. To determine whether these transgenic thymocytes were resistant to viral infection, they were challenged with X4-tropic NL4-3 HIV-1 at an MOI of 0.01. Thymocytes were first sorted on the basis of EGFP expression to obtain enriched populations of transgenic cells and then expanded by culturing in the presence of phytohemagglutinin (PHA) and interleukin-2 (IL-2) before viral challenge. As shown

in Figure 2, HIV resistance amounting to >1 log was observed in Triple-R vector transgenic thymocytes compared with non-transduced and HIV7-GFP vector-transduced cells. These results confirm that transgenic thymocytes exhibited viral resistance.

Phenotypic analysis of transgenic thymocytes

The presence of foreign transgenes in lineage-specific differentiating cells can have varied effects that influence developmental steps and adversely affect phenotypic properties.^{43,44} For example, even when thymocytes are generated *in vivo*, the transgene may have differential effects on different sub-populations of thymocytes. To evaluate these differences, multi-parametric FACS analysis was performed on *in vivo*-derived thymocytes after staining for various characteristic cell surface markers. Results are shown in Table 1. The majority of cells, as expected from the thymic grafts, were positive for CD3, a pan-T cell marker, with HIV7-GFP-transduced cells showing levels of 90.2–92.0%, which is similar to the level for Triple-R vector transduced cells, at 88.3–92.4%. Figure 3a is a representative histogram depicting CD3 expression. Further phenotypic analyses were carried out by gating on CD3⁺ cells to ensure only T cells were evaluated. In addition, as non-transduced EGFP-negative cells are also present in each of the thymic grafts, we took advantage of these cells as internal controls for comparison with EGFP⁺ transgenic cells. All three thymocyte sub-populations bearing either CD4 or CD8 single-positive cells (mature thymocytes) or CD4 and CD8 double-positive cells (immature thymocytes), which form the majority, were observed in normal ratios for internal control non-transduced, HIV7-GFP-transduced, and Triple-R vector-transduced thymocytes (Table 1). As shown in the FACS plots in Figure 3b, a normal distribution of all three cell populations was found. Similar profiles were observed

Table 1 Multicolor FACS analysis of SCID-hu-derived transgenic thymocytes

Mouse ^a	CD3	CD4	CD8	CD4/CD8	CCR7/CD45RA	CXCR4 ^b	CD28 ^b	GFP%
HIV7GFP1(-)	92.0	18.1	7.6	73.4	33.9	960	3,730	0
HIV7GFP1(+)		12.6	5.4	81.4	32.7	1,070	3,780	18.8
HIV7GFP2(-)	90.2	18.5	6.6	74.0	29.8	840	3,360	0
HIV7GFP2(+)		15.4	5.4	78.7	30.3	950	3,510	17.9
HIV7GFP3(-)	91.1	18.9	7.0	73.3	31.6	1,030	3,340	0
HIV7GFP3(+)		15.1	5.5	78.8	32.3	1,150	3,350	21.5
HIV7GFP4(-)	90.4	20.4	8.8	69.7	31.5	960	3,560	0
HIV7GFP4(+)		15.1	6.1	78.1	29.5	1,100	3,440	22.0
Triple-R1(-)	92.4	22.1	23.7	46.6	50.1	1,110	2,900	0
Triple-R1(+)		16.0	11.1	70.3	38.5	1,440	3,110	8.7
Triple-R2(-)	89.7	14.0	8.2	75.4	27.4	840	2,560	0
Triple-R2(+)		12.1	7.3	78.9	27.3	970	2,640	10.8
Triple-R3(-)	90.9	16.9	8.6	71.8	30.3	990	2,730	0
Triple-R3(+)		14.2	7.4	76.3	30.2	1,100	2,800	15.8
Triple-R4(-)	88.3	19.7	8.5	68.5	26.8	710	2,440	0
Triple-R4(+)		18.4	7.6	71.1	27.4	790	2,630	10.7
Triple-R5(-)	90.7	16.6	10.4	70.3	27.6	820	2,530	0
Triple-R5(+)		13.6	8.1	76.4	27.9	920	2,650	22.2
Triple-R6(-)	90.6	15.0	9.5	72.9	26.9	830	2,510	0
Triple-R6(+)		13.3	8.1	76.3	27.3	970	2,730	10.4
Triple-R7(-)	91.7	16.0	10.2	71.3	27.8	900	2,590	0
Triple-R7(+)		13.3	8.5	76.1	27.1	1,030	2,590	15.7
Triple-R8(-)	90.7	21.6	12.1	62.2	28.7	850	2,960	0
Triple-R8(+)		18.9	10.8	67.2	29.9	950	3,160	12.3

^aThymocytes were harvested from severe combined immunodeficiency (SCID)-hu thymic grafts and analyzed for normal phenotypic markers by multi-parametric fluorescence-activated cell sorting (FACS) analysis. For each mouse, both green fluorescent protein (GFP)-negative (-) and GFP-positive (+) cells were analyzed. The percentage of positive cells for each respective cell surface marker is shown. ^bCXCR4 and CD28 expression was measured by mean fluorescence intensity.

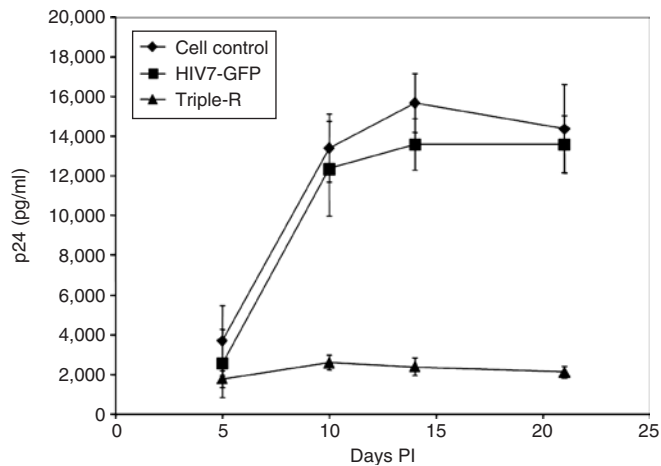


Figure 2 Human immunodeficiency virus-1 (HIV-1) resistance by *in vivo*-differentiated transgenic thymocytes. Vector-transduced CD34 cells were injected into severe combined immunodeficiency-hu thymic grafts and allowed to differentiate for 6 weeks to derive T cells *in vivo*. After cell harvest, thymocytes were sorted by fluorescence-activated cell sorting for enhanced green fluorescent protein (EGFP) expression (>90% purity). The sorted cells were expanded by culturing *in vitro* and challenged with the T-cell-tropic HIV-1 NL4-3, at a multiplicity of infection of 0.01. Culture supernatants were analyzed by p24 enzyme-linked immunosorbent assay at different days after infection to determine viral replication.

for triple-transgenic thymocytes and non-transduced and HIV7-GFP control cells.

Naïve T cells, before encountering specific antigens, express two cell surface markers, namely CCR7 and CD45RA, whose expression decreases upon stimulation. Thymic T cells are predominantly naïve, and such cells were observed in all cell groups showing similar expression of CCR7 and CD45RA (Table 1). Representative FACS profiles of both non-transduced and transduced cell populations are shown for HIV7-GFP and Triple-R vector thymocytes (Figure 3c). The presence of CD28 and CXCR4, as seen on normal thymocytes, was also analyzed. Levels of these two markers were found to be similar for Triple-R thymocytes, non-transduced, and HIV7-GFP-transduced thymocytes. The levels of these surface molecules are shown as mean fluorescence intensity (Table 1). As seen from these results, development and cell surface marker expression by either HIV7-GFP control vector-transduced or Triple-R vector-transduced cells *in vivo* was similar to that seen with non-transduced EGFP-negative cell populations. Thus, expression of the three RNA inhibitory molecules during CD34 differentiation *in vivo* did not adversely affect cell differentiation.

Absence of gene silencing during *in vitro* culture of transgenic thymocytes

Transgene silencing may occur during cell differentiation into end-stage cells. The data above showed that differentiating cells in SCID-hu grafts retained reporter transgene expression as assessed by EGFP from samples collected 6 weeks after engraftment. However, we also wanted to determine whether *in vivo*-differentiated cells continue to express the reporter transgene after PHA and IL-2 stimulation *in vitro*. Accordingly, EGFP expression was determined at various post-stimulation days after the cells were biopsied from the thymic grafts. Both PHA-stimulated and unstimulated cells

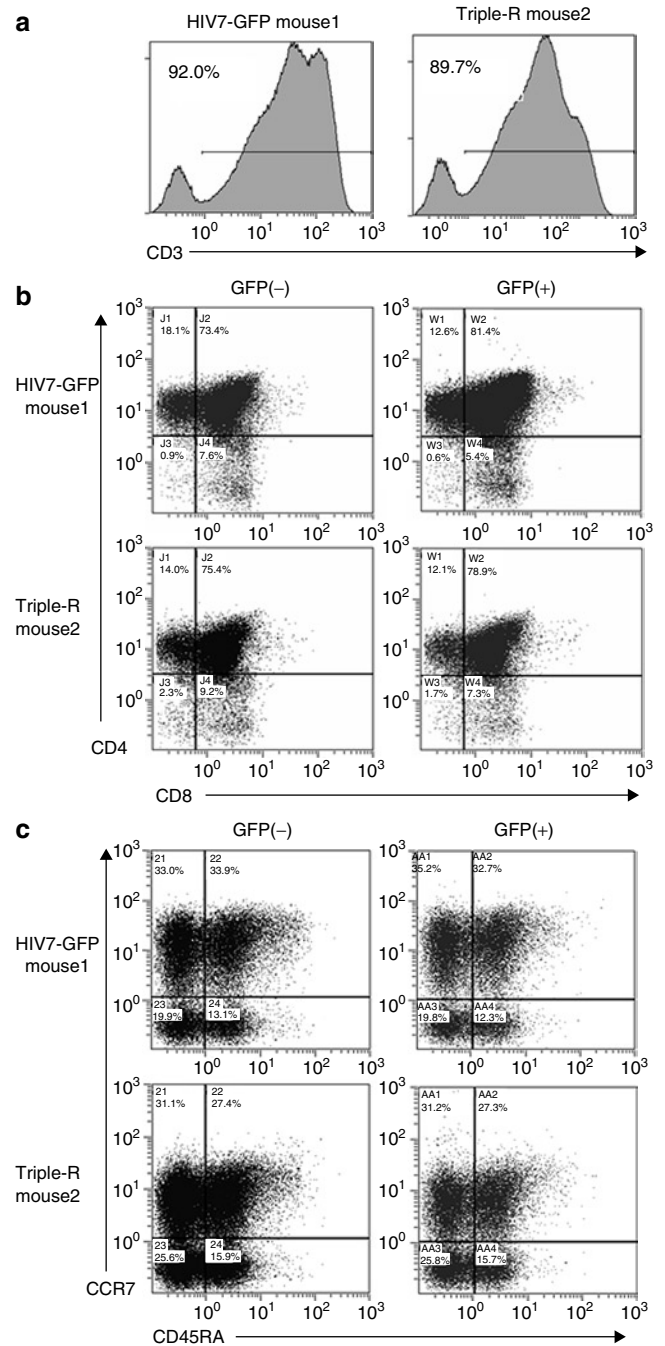


Figure 3 Multi-parametric fluorescence-activated cell sorting (FACS) analysis of severe combined immunodeficiency-hu graft-derived thymocytes. Single cell suspensions of *in vivo*-differentiated thymocytes were stained with antibodies against CD3, CD4, CD8, CD45RA, and CCR7 and subjected to FACS analysis. Representative FACS plots of cells from control vector-transduced and Triple-R vector-transduced injected mice are shown. (a) Total cell populations stained with CD3. (b) Cells stained with CD4 and CD8 gated from enhanced green fluorescent protein positive (EGFP⁺) and negative (EGFP⁻) populations. (c) Cells stained with CD45RA and CCR7 gated from EGFP⁺ and EGFP⁻ thymocyte populations. HIV7, human immunodeficiency virus 7.

were monitored. As seen in Figure 4, no substantial loss of EGFP expression was observed. By day 14 after stimulation, only a slight decrease in EGFP expression was observed for GFP-alone control thymocytes in both unstimulated (HIV7-GFP1 and 2) (Figure 4a)

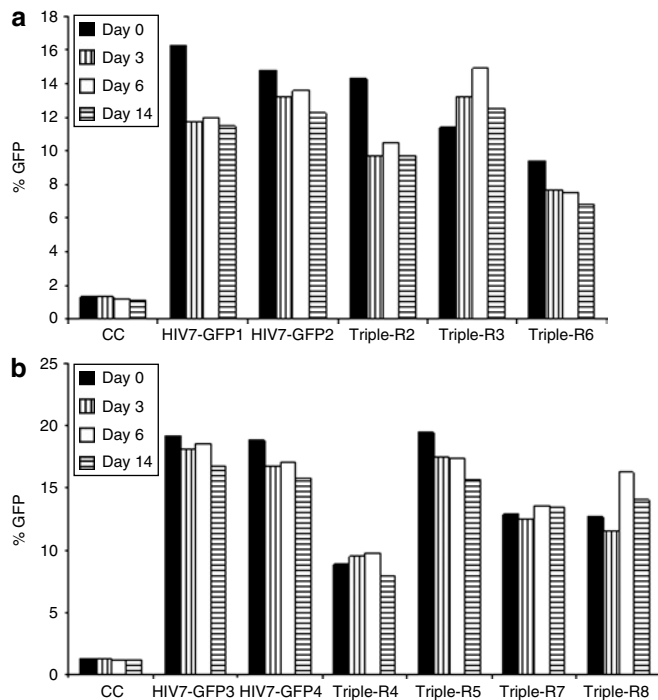


Figure 4 Vector expression analysis in *ex vivo*-cultured thymocytes. To evaluate continued vector expression in *ex vivo*-cultured cells, total thymocyte populations were cultured *in vitro* in (a) the absence or (b) the presence of phytohemagglutinin stimulation. Cell samples at various days of culture were analyzed for enhanced green fluorescent protein (EGFP) expression. The percentages of EGFP-expressing cells at different times after culture are indicated. HIV7, human immunodeficiency virus 7.

and PHA-stimulated (HIV7-GFP3 and 4) (Figure 4b) cultures. As for Triple-R vector transgenic thymocytes, cells from four of the seven mice analyzed showed a minimal decrease in EGFP expression (Triple-R2, 6, 4, and 5), whereas cells from the other three mice showed a slight increase in expression (Triple-R3, 7, and 8) (Figure 4). No noticeable differences in loss of EGFP expression were observed between PHA-stimulated and unstimulated cell groups, suggesting that no gene silencing was occurring.

DISCUSSION

Many effective gene therapeutic constructs with different mechanisms of action have been tested individually in both T-cell lines and HSCs. A select number of these constructs have also been through phase I and II clinical trials.^{43,44} Owing to the complexity of the HIV lifecycle and the high mutation rate of the HIV genome, it has become clear that the use of a single anti-HIV gene therapeutic construct would not be adequate to afford extended viral protection. Our recent studies showed that different anti-HIV constructs can be combined into a single lentiviral vector to provide additional benefit by reducing the chances of generating viral escape mutants when compared with deploying individual constructs.¹¹ In addition, potential off-target effects of siRNA-based transgenes could affect cell physiology.^{45,46} Thus, it is important that combinatorial constructs that have shown efficacy *in vitro* be evaluated *in vivo* before human clinical trials, as it is not clear whether genetically modified HSCs with such constructs will develop into phenotypically normal T cells. Using the

SCID-hu mouse human thymopoiesis model, here we have shown that CD34 HSCs transduced with a combinatorial lentiviral vector harboring an anti-CCR5 ribozyme, an shRNA against *tat/rev*, and a TAR decoy developed normally into HIV-resistant T cells *in vivo*. Previous detailed characterization established the intracellular expression of each of the constructs from its respective promoter, more than additive long-term anti-viral effect, and functionality in differentiated macrophages.¹¹

For stem cell gene therapy to be effective, anti-HIV transgenes need to be retained and expressed throughout progenitor cell development. First, the transduced CD34 cells were analyzed *in vivo* in *thy/liv* SCID-hu mice to see whether these cells engrafted, retained the vector, and any differences existed in outcome between the control HIV7-GFP vector-treated cells and Triple-R vector-treated cells. Engraftment levels ranged between 17.9 and 22.0% for HIV7-GFP and between 8.7 and 22.2% for Triple-R vector-transduced cells, as assessed by FACS for the presence of EGFP-expressing cells in the reconstituted grafts (Table 1). Although individual engraftment levels varied, in both cases the levels reached 22%. The percentages of EGFP expression in differentiated thymocytes in the graft are considerably lower than the initial 66.2–73.0% EGFP expression (initial transduction levels before injection) seen in their respective CD34 cells. The levels of EGFP-expressing cells also varied among mice. It was previously determined that such a variation is due to differences in sizes of the grafts that were injected, which also reflected varied original numbers of endogenous thymocytes present in the graft at the time of injection. Thus, although similar numbers of CD34 cells were injected into each of the grafts, percentages of EGFP-positive cells 6 weeks after injection varied according to their relative proportion to endogenous thymocytes, which generally constitute the majority of cells in the graft. Expression of the EGFP reporter gene by differentiated thymocytes confirmed the retention and expression of the vector. Transgenic thymocytes were then analyzed by flow cytometry to evaluate the proportion of normal T cell sub-populations. For this evaluation, a multi-parametric FACS analysis was employed in which levels of CD3, CD4, and CD8 markers on both EGFP-negative and EGFP-positive cells were determined. As is typical for a normally functioning thymus, the majority of cells (>88%) stained positive for the T-cell marker, CD3. Characteristic sub-populations of thymocytes consisting of CD4⁺, CD8⁺, and CD4⁺/CD8⁺ cells were detected at expected levels in all cell groups, namely non-transduced, HIV7-GFP, and Triple-R vector-transduced cells. This suggested that the vector did not adversely influence or skew the thymocyte subset development. Naïve T cells express the cell surface markers CD45RA and CCR7. Similar normal expression of these markers was seen for transgenic thymocytes, control non-transduced, and HIV7-GFP transduced cells. The co-stimulatory molecule CD28 and the HIV co-receptor CXCR4 were also seen at normal levels in Triple-R thymocytes and the controls. These data, combined, demonstrated that phenotypically normal thymocytes could be derived from combinatorial vector-transduced CD34 HSCs. Thus, constitutive expression of the foreign transgenes had no apparent untoward effect on normal thymocyte development.

After maturation in the thymus, naïve T cells migrate into the periphery. They proliferate and develop into effector cell types

such as cytotoxic T cells in response to antigenic stimulation. It is possible that vector sequences could be rearranged or silenced during cellular proliferation after mitogenic stimulation. Our experiments evaluating this question showed that the *ex vivo*-cultured thymocytes continued to express the vector reporter transgene upon PHA stimulation. This confirms the functionality of the vector in proliferating cells. Another main objective of these studies is to determine whether the *in vivo*-differentiated transgenic thymocytes are able to resist HIV challenge. *Ex vivo* challenge of Triple-R transgenic thymocytes demonstrated significant viral resistance (>1 log inhibition) compared with control cells. Taken together, the above data established the proof of principle that a combinatorial lentiviral vector construct harboring three different anti-HIV genes can be effectively used for stem cell transduction and derivation of HIV-resistant T cells.

The unique feature of our lentiviral vector is the incorporation of three different RNA inhibitory molecules that show protection against HIV infection. These constructs, a ribozyme, an shRNA, and an RNA decoy operate by different mechanisms of action and are directed to inhibit HIV infection at the levels of both viral pre- and post-entry steps. Thus, naïve cells will be protected against initial viral infection and, in the case of viral break-in, will still be able to resist active viral replication and subsequent spread. These properties of this vector make it an attractive candidate for proceeding with clinical trials. As the presence of the foreign protein EGFP in the present construct is likely to invoke immunity in the human subject, possibly leading to immunological rejection of vector-harboring cells, a modified version of the vector without the EGFP reporter gene is being tested for use in clinical trials.

MATERIALS AND METHODS

Combinatorial lentiviral vector design and production. A third-generation HIV-derived lentiviral vector containing an EGFP reporter gene was used in this study, HIV7-GFP.³⁷ The vector backbone contains two *cis*-acting elements to enhance vector performance, namely the central DNA flap consisting of a central polypurine tract and central termination sequence, to facilitate the nuclear import of the viral pre-integration complex, and the woodchuck hepatitis virus post-transcriptional regulatory element to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts (Figure 5a).³⁷ The construction of the combination Triple-R lentiviral vector along with the production of high-titer vectors was described previously.¹¹ In brief, the shRNA component targeting rev/tat is placed under the control of a U6 promoter, whereas the TAR decoy is embedded in the U16 small nucleolar RNA backbone for nucleolar localization and is also driven by a U6 promoter. The CCR5 ribozyme is under the control of an adenoviral VA1 promoter (Figure 5b). Lentiviral vectors were generated in 293T cells. In all, 15 µg of the packaging construct, pCHGP-2, 15 µg of HIV7-GFP (control empty vector) or Triple-R (combination) transfer vector, 5 µg of vesicular stomatitis virus glycoprotein (pseudotyping envelope), and 5 µg of cytomegalovirus-Rev were transfected into cells cultured on 100-mm plates by calcium phosphate transfection. Vector supernatants were collected at 24, 36, 48, and 60 hours after transfection and concentrated by ultracentrifugation. Vector titers were obtained by transduction of 293T cells and measured for EGFP expression by FACS. The titers are 4×10^8 and 3×10^8 transduction units for the HIV7-GFP control and Triple-R vectors, respectively.

Isolation and transduction of CD34 HSCs. CD34 HSCs were purified from human fetal liver using monoclonal antibody-conjugated immunomagnetic beads (Miltenyi Biotech, Auburn, CA). The purity of CD34

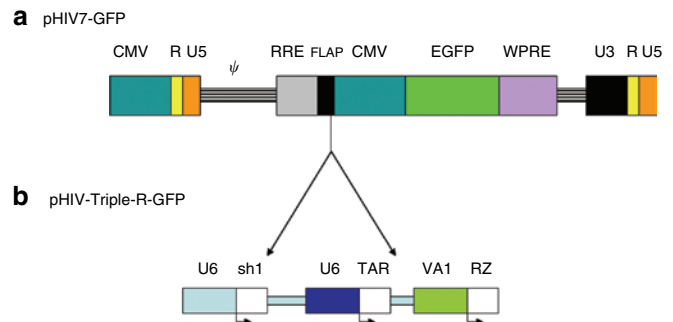


Figure 5 Human immunodeficiency virus-1 (HIV-1)-based combinatorial lentiviral transfer vector. **(a)** Control self-inactivating vector, pHIV7-enhanced green fluorescent protein (EGFP), contains an EGFP reporter gene driven by the cytomegalovirus (CMV) promoter. **(b)** To derive the combinatorial Triple-R transfer vector, expression cassettes of a tat/rev short hairpin small interfering RNA, transactivation response (TAR) decoy, and CCR5 ribozyme were inserted upstream of the EGFP reporter gene. RRE, rev-responsive element; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element.

cells obtained was routinely >97% (data not shown). Cells were cultured in Iscove's medium containing 10% fetal bovine serum and 10 ng/ml of each of the cytokines IL-3, IL-6, and stem cell factor. For transductions, CD34 cells were incubated with the respective vectors (MOI 15), HIV7-GFP and Triple-R, overnight in the presence of 5 µg/ml polybrene. Two rounds of transductions were performed on two consecutive days. Transduction efficiencies ranged from 66.2 to 73.0% for HIV7-GFP and Triple-R, respectively.

Reconstitution of human thymic grafts of SCID-hu mice with transduced CD34 cells. To evaluate the capacity of vector-transduced CD34 cells to develop into T cells, an SCID-hu mouse *in vivo* model that supports thymopoiesis was used.^{33,34} All protocols using mice were approved by the institutional animal care and use committee. Transduced CD34 cells ($\sim 2 \times 10^6$ cells per mouse) were injected directly into the SCID-hu thymic grafts as described.^{33,34} Six weeks after injection, after the cells had been allowed to engraft and differentiate, the mice were killed and the grafts were collected. Single cell suspensions were made and later analyzed for EGFP expression by FACS to evaluate engrafted cell reconstitution levels. To determine the presence of characteristic phenotypic cell surface makers, the thymocytes were further analyzed by multi-parametric FACS.

Ex vivo HIV-1 challenge of transgenic thymocytes. To determine whether Triple-R transgenic thymocytes were resistant to HIV-1 infection, challenge assays were performed with an X4-tropic HIV-1 strain, NL4-3. Thymocytes biopsied from thymic grafts were sorted according to CD3 and EGFP expression and subsequently stimulated with IL-2 (10 ng/ml) and PHA (1 µg/ml). Three days after stimulation, 2.5×10^5 cells were challenged in triplicate with NL4-3 HIV-1 at an MOI of 0.01. On various days after infection, cell culture supernatants were sampled and assayed for viral antigen using a p24 antigen enzyme-linked immunosorbent assay kit (Beckman Coulter, Fullerton, CA).

Multi-parametric FACS analysis of transgenic thymocytes. To determine whether transgenic thymocytes differentiated in the SCID-hu thymic grafts were phenotypically normal, various cell surface markers were analyzed. Cells were stained with respective antibodies to CD3, a pan-T cell marker, CD4, and CD8 to detect sub-populations of T-helper, T-cytotoxic, and immature double-positive cells. CCR7 and CD45RA antibodies were used to detect naïve thymocytes. Cells were also stained for CD28, a co-stimulatory molecule involved in antigen presentation, and CXCR4, a critical co-receptor used by HIV for attachment and entry. The following

antibodies were used for analysis: PE-Texas Red-CD3, Alexa Fluor 405-CD8 (Caltag, Burlingame, CA), APC-CY7-CD4, PE-CY7-CCR7, APC-CD45RA, PE-CD28, and PE-CY5-CXCR4 (BD Biosciences, San Jose, CA). Stained cells were analyzed on a Beckman Coulter ARIA flow cytometer. For evaluation of transgenic thymocytes, cells expressing CD3 and EGFP were analyzed. CD3-positive and EGFP-negative cells present in the graft were used as non-transduced internal cell controls for these analyses.

Ex vivo analysis of transgene expression. To determine whether reporter transgene expression was stable during *ex vivo* culture and mitogen stimulation, *in vivo*-differentiated thymocytes were cultured *in vitro*. Cells from both control vector and Triple-R vector mice were cultured in the presence of IL-2 (10 ng/ml) and PHA (1 µg/ml) or IL-2 (10 ng/ml) alone. EGFP expression was analyzed by FACS on various days after stimulation.

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