

Long-Term Inhibition of HIV-1 Infection in Primary Hematopoietic Cells by Lentiviral Vector Delivery of a Triple Combination of Anti-HIV shRNA, Anti-CCR5 Ribozyme, and a Nucleolar-Localizing TAR Decoy

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Combinatorial therapies for the treatment of HIV-1 infection have proven to be effective in reducing patient viral loads and slowing the progression to AIDS. We have developed a series of RNA-based inhibitors for use in a gene therapy-based treatment for HIV-1 infection. The transcriptional units have been inserted into the backbone of a replication-defective lentiviral vector capable of transducing a wide array of cell types, including CD34⁺ hematopoietic progenitor cells. The combinatorial therapeutic RNA vector harbors a U6 Pol III promoter-driven short hairpin RNA (shRNA) targeting the *rev* and *tat* mRNAs of HIV-1, a U6 transcribed nucleolar-localizing TAR RNA decoy, and a VA1-derived Pol III cassette that expresses an anti-CCR5 ribozyme. Each of these therapeutic RNAs targets a different gene product and blocks HIV infection by a distinct mechanism. Our results demonstrate that the combinatorial vector suppresses HIV replication long term in a more-than-additive fashion relative to the single shRNA or double shRNA/ribozyme or decoy combinations. Our data demonstrate the validity and efficacy of a combinatorial RNA-based gene therapy for the treatment of HIV-1 infection.

Key Words: lentiviral vector, shRNA, CCR5, Ribozyme, TAR, RNA decoy, HIV/AIDS gene therapy, transgenic macrophages

INTRODUCTION

The emergence of multiple drug-resistant strains of HIV-1 and the side effects of a drug-based therapy make alternative approaches for AIDS treatment necessary [1–3]. An alternative or adjuvant to chemotherapy is gene therapy. For treatment of HIV-1 infection, *ex vivo* manipulation of hematopoietic stem cells with therapeutic genes is a viable option. However, gene therapy efforts have long been hampered by the difficulty of delivering therapeutic genes into target cells, especially nondividing cells, including hematopoietic stem cells. Lentiviral vectors do not require cell division to enter the cell nucleus and maintain long-term and sustained expression of the genes carried by the vector [4–9]. These attributes make these vectors an attractive choice for gene delivery [10]. Small interfering RNAs (siRNAs) can selectively direct cleavage of both cellular and viral transcripts

[11]. The siRNAs can be designed to target any RNA, including those encoded by viruses such as HIV-1 [12–16]. We as well as others have demonstrated that lentiviral vectors can be used to deliver siRNA encoding genes efficiently into hematopoietic cells [17–19]. To date, the majority of HIV-1 gene therapy protocols have utilized a single therapeutic gene. For RNAi there are several reports demonstrating that HIV-1 and poliovirus escape mutants can arise with amazing efficiency [20,21]. We expect that by suppressing replication with a combination of therapeutic genes targeting different viral products and steps in the viral life cycle, the probability of viral escape mutants will be greatly reduced and the potency will be increased by possible synergy among the different gene therapy agents. Previously, we have shown that lentiviral vectors expressing an anti-CCR5 ribozyme, a TAR RNA decoy, or both, as well as short hairpin RNAs

(shRNAs) targeting HIV-1 *rev/tat* or *rev*, exhibit anti-HIV-1 activity [18]. Each of these therapeutic agents targets a different component required for HIV replication. Both *tat* and *rev* mRNAs encode proteins essential for HIV-1 replication [22,23]. The β -chemokine receptor CCR5 is a coreceptor essential for macrophage-tropic (M-tropic) HIV-1 infection [24,25]. A homozygous 32-base deletion in the CCR5 gene confers strong resistance to HIV-1 infection [26,27]. Knockdown of this coreceptor by a chimeric VA1 anti-CCR5 ribozyme has resulted in down-regulation of CCR5 and resistance to M-tropic HIV-1 infection in target cells [18,28]. The transcription-responsive (TAR) element is located in the 5' UTR of HIV-1. Binding of Tat to TAR is essential for efficient transcription of all classes of HIV RNAs [29,30]. Previous work from our laboratory has shown that both a retroviral and a lentiviral vector-delivered nucleolar-localizing TAR RNA decoy resulted in marked inhibition of HIV-1 replication [18,31]. In the present study we have created a triple combination of an anti-*tat/rev* shRNA, the chimeric VA1 anti-CCR5 ribozyme, and the nucleolar-localizing TAR RNA decoy expressed from a single lentiviral vector backbone. The expression of each therapeutic RNA is under the control of an individual Pol III promoter. Our results show that coexpression of all three antivirals following lentiviral-mediated transduction of CD34⁺-derived monocytes provides enhanced, long-term, inhibition of HIV-1 infection relative to single or double combination. The data provided support the validity of combining different antiviral modalities in a gene therapy setting to obtain the strongest suppression of HIV-1 replication.

RESULTS

Construction of Lentiviral Vector Containing Multiple Anti-HIV-1 Genes

The lentiviral vector backbone pHIV-7-GFP [32] (Fig. 1A) is a self-inactivating vector, which expresses an enhanced green fluorescent protein (EGFP) under the control of the human cytomegalovirus (CMV) promoter. The shRNA, shI, was designed to target simultaneously the *tat* and *rev* mRNAs [16,18]. Transcription of the shRNA sequences is under the control of the human U6 snRNA Pol III promoter, with six consecutive uracils serving as the transcriptional terminator (Fig. 1B). The TAR RNA decoy was inserted into a U16 snoRNA sequence for nucleolar localization (Fig. 1C) [31] and expressed under the control of the human U6 promoter. The anti-CCR5 ribozyme was designed to cleave the CCR5 transcript at a GUC cleavage site located 23 nucleotides downstream of the AUG codon [28] (Fig. 1D). The ribozyme is part of a chimeric construct that includes a modified form of the adenoviral VA1 RNA and is expressed as a cytoplasmically localizing Pol III transcript [33]. The U6 shI RNA, U6-U16 TAR decoy, and the chimeric VA1 CCR5 ribozyme

expression cassettes were inserted into pHIV-7-GFP as depicted in Fig. 1.

Production of High-Titer Vectors

After packaging and concentration we determined vector titers by transduction of HT1080 cells followed by fluorescence-activated cell sorting (FACS) analyses of EGFP⁺ cells. We routinely use 5 μ g of Rev-expressing plasmid in the cotransfection reaction for the packaging. Following a 200-fold concentration of the culture supernatant, at least 2×10^8 transduction units per milliliter was achieved for the vector expressing an unrelated shRNA or the parental HIV-7 vector. However, under the same conditions, the titers of the vectors containing the shRNA targeting *tat/rev* were about fourfold lower than those of the control vectors (Fig. 2), perhaps owing to the potential for the anti-*tat/rev* shRNA produced by the vector to cleave both the vector primary transcripts and the Rev packaging transcript. Although these lower titers were still sufficient for our experiments, higher titers are more desirable. We found that we could increase the titers of the shRNA-encoding vectors to the levels of HIV-7 by doubling the amount of Rev plasmid to 10 μ g (Fig. 2).

Expression of the Therapeutic RNAs in Target Cells

We evaluated the transduction efficiency of the various vectors in the human CEM T cell line. At a multiplicity of infection (m.o.i.) of 10, the transduction efficiency was over 90% as determined by FACS analysis for EGFP expression (data not shown). To ensure that each therapeutic gene delivered by a lentiviral vector was expressed in target cells, we carried out Northern gel analyses on total RNAs extracted from transduced CEM cells. As shown in Fig. 3, double and triple combinations of the U6-shRNA/U6-U16TAR decoy and VA1-CCR5 ribozyme were all expressed at readily detectable levels. Since each of the blots is from a population of cells it is difficult to determine whether there is promoter interference of the Pol III promoters among themselves. Nevertheless, we do observe transcription from all three constructs in all combinations tested. These results demonstrate that it is feasible to express stably at least three Pol III-promoted RNA-based therapeutic agents inserted tandemly in a single lentiviral vector backbone.

Inhibition of HIV-1 Replication in CD34⁺ Progenitor Cells Transduced with Combinatorial Anti-HIV-1 RNAs

CD34⁺ cells have multilineage differentiation potential. The derivatives include HIV-1-infectable T cells, monocytes, and macrophages. Therefore, hematopoietic stem cells are an attractive target for anti-HIV gene therapy. We have tested the lentiviral vector transduction of isolated CD34⁺ cells from umbilical cord blood samples

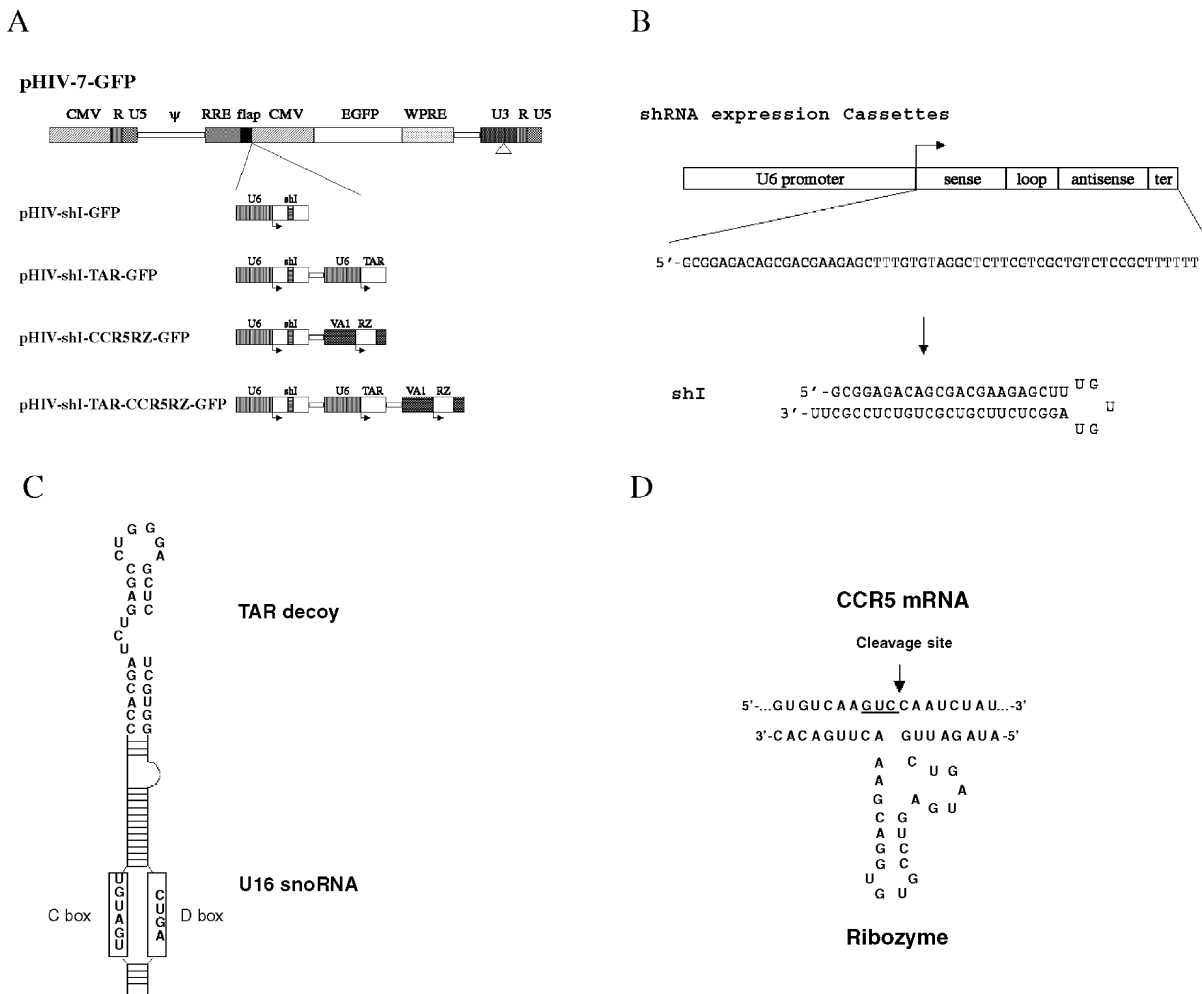


FIG. 1. (A) The lentiviral vectors. The lentiviral vector backbone pHIV-7-GFP contains a hybrid 5' LTR in which the U3 region is replaced with the CMV promoter, the packaging signal (Ψ), the RRE sequence, the flap sequence, the woodchuck posttranscriptional regulatory element (WPRE), and the 3' LTR in which the *cis* regulatory sequences are completely removed from the U3 region. The genes of interest along with their independent Pol III promoters are inserted upstream of the CMV promoter of EGFP in the pHIV-7-GFP vector. U6, U6 promoter; shI, shRNA against a common *rev/tat* exon; TAR, U16-TAR RNA decoy; VA1, VA1 promoter; RZ, anti-CCR5 ribozyme. Arrows indicate the transcriptional orientation. (B–D) The therapeutic genes and their targets. (B) The expression cassette of the shRNA targeting *rev/tat* is composed of the U6 Pol III promoter, the sense and antisense sequences of the shRNA separated by a 9-base loop, and a terminator with 6 thymidines. The putative shRNA after transcription in target cells is shown. (C) The TAR RNA decoy in the U16 snoRNA. The stem–bulge–loop configuration of the TAR decoy is shown at the top. The TAR decoy is inserted into the apical stem of the U16 snoRNA sequence, allowing its nucleolar localization [31]. C box and D box are the U16 nucleolar localization signals. (D) The anti-CCR5 hammerhead ribozyme and the CCR5 sequence complementary to the ribozyme. The ribozyme binds to the substrate with its two arms and cleaves immediately 3' of the GUC codon as indicated by the arrow. The ribozyme is inserted within the VA1 coding region as previously described [28].

enriched using anti-CD34 antibody-conjugated magnetic beads. Eleven days posttransduction, we analyzed these cells by FACS for EGFP expression. As shown in Fig. 4, using a single round of transduction at an m.o.i. of 40, the vector harboring the anti *tat/rev* shI had a transduction efficiency comparable to that of the parental HIV-7 vector alone. Although the vectors expressing double or triple therapeutic genes showed lower transduction efficiencies than the control vector, they all rendered greater than 20% transduction effi-

ciency based upon EGFP expression. We sorted cells transduced with the various vectors by FACS for EGFP expression. Northern gel analyses for the shRNA/VA anti-CCR5 ribozyme/nucleolar TAR decoy RNAs revealed that the expression levels of these genes were maintained 3 months posttransduction (Fig. 3B).

We propagated the transduced CD34⁺ cells under cell culture conditions that allow differentiation into CD4⁺ monocytes. Following a period of incubation we used them for HIV-1 challenge assays. We infected equal

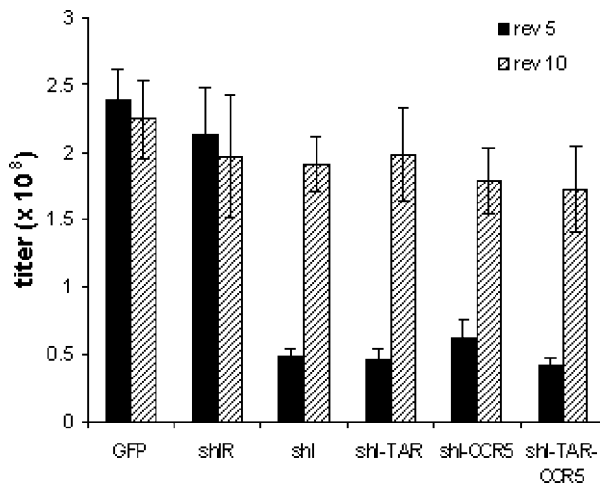


FIG. 2. Titers of lentiviral vectors with various inserts. The vectors were produced by cotransfection of 293 T cells with all required plasmids, among which pCMV-*rev* was at either 5 (solid bars) or 10 μ g (hatched bars). HT1080 cells were transduced with various packaged vectors as indicated. Forty-eight hours after transduction, the cells were analyzed by FACS for EGFP expression. The titers were represented as the number of EGFP⁺ cells per milliliter of concentrated vectors. The values are averages from three independent experiments.

numbers of transduced cells with JR-FL, an M-tropic strain of HIV-1, at multiplicities of infection of 0.01 and 0.05. We collected the culture supernatants at different time points postinfection and determined HIV-1 secreted p24 antigen levels. At the lower multiplicities, each of the cell populations transduced with the anti-HIV constructs had reduced p24 levels relative to the pHIV-7 vector

control (Fig. 5A). Cells transduced with double inserts, which were combinations of the anti-*rev/tat* shRNA and TAR decoy or anti-CCR5 ribozyme, provided stronger inhibition of HIV-1 replication than was obtained with the shRNA alone. Most importantly, the vector with the triple inserts resulted in the most potent inhibition of HIV-1 replication, yielding 2 logs or more reduction of p24 levels at day 28 postinfection. For cells infected with the higher dose of HIV-1 (m.o.i. of 0.05), at day 7 postinfection each of the therapeutic vectors showed reduced p24 production relative to the control vector (Fig. 5B), but by day 28 only the triple construct remained inhibitory, with no increase in p24 antigen levels throughout the time course of the experiment. These results suggest that the combination of three different antiviral RNAs provides more than additive inhibition of HIV-1 infection.

After the final collection of supernatants for p24 antigen assays, we collected cells for FACS analyses to determine the percentage of EGFP⁺ cells and additionally extracted DNA for real-time quantitative PCR analysis to determine the average copy number of the integrated vectors per cell. At the time of these analyses the cells had been cultured for 7 weeks following the original FACS sorting and 4 weeks post-HIV-1 infection. As shown in Table 1, the EGFP⁺ cells ranged from about 30% to almost 100%. Real-time quantitative PCR results revealed that under our transduction conditions, the population of cells contained 1 to 2 copies on average of the integrated vectors (Table 1). For the parental HIV-7 vector the average copy number per cell was

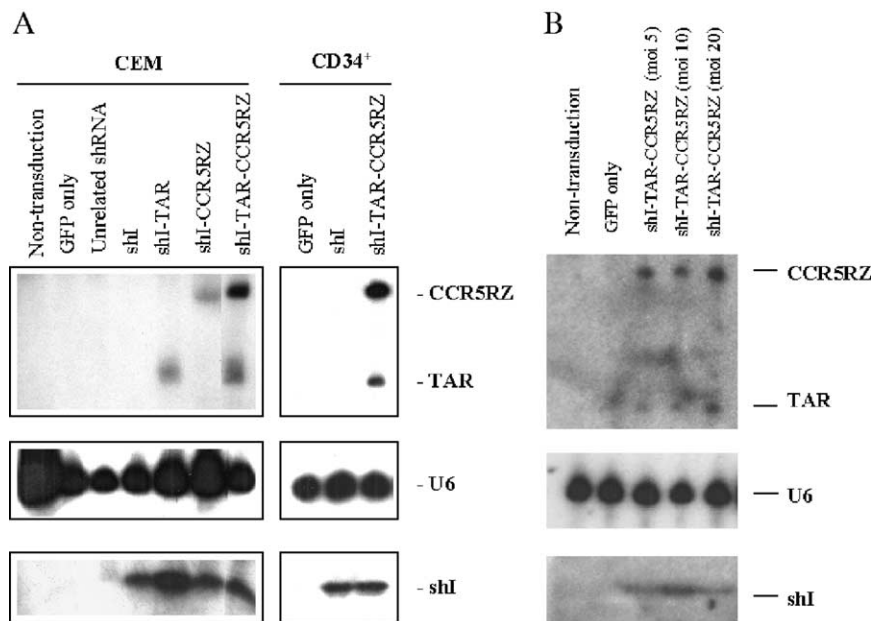


FIG. 3. Expression of the therapeutic RNAs in the target cells. (A) CEM cells and CD34⁺ cells were transduced with the lentiviral vectors harboring the expression cassettes for the anti-*tat/rev* shRNA (shI), U16TAR decoy, and anti-CCR5 ribozyme. The CD34⁺ cells were sorted for EGFP⁺ cells. Two weeks after transduction (for CEM cells) or sorting (for CD34⁺ cells), total RNA was extracted from the CEM cells and the EGFP⁺ population of the CD34⁺ cells, electrophoresed on an 8% polyacrylamide gel with 7 M urea, blotted onto a nylon membrane, and hybridized with a ³²P-labeled probe for the antisense sequence of the shI or mixed probes for the U16TAR decoy and anti-CCR5 ribozyme. A U6 snRNA complementary probe was used as an internal control. RNA extracts from untransduced cells and cells transduced with the GFP parental vector or a vector expressing an unrelated shRNA were used as negative controls. The amounts of RNA loaded into the gel were 15 μ g for CEM cells and 7.5 μ g for CD34⁺ cells. (B) CD34⁺ cells were transduced with the vectors at the indicated m.o.i. After sorting, EGFP⁺ cells were cultured for 9 weeks. The conditions for RNA extraction and Northern blotting were the same as in (A), except that the amount of RNA was 1.5 μ g in each sample.

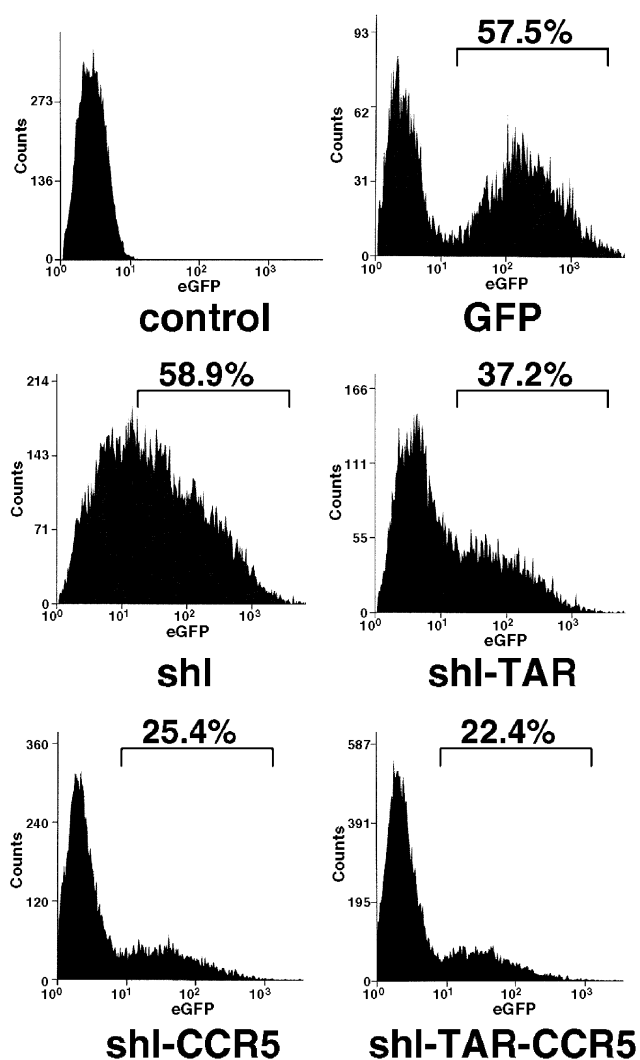


FIG. 4. Transduction efficiency of the vectors in CD34⁺ cells. CD34⁺ cells were transduced with various lentiviral vectors at an m.o.i. of 40. Eleven days after transduction, FACS analyses were performed to detect EGFP⁺ cells. The x axis indicates the intensity of fluorescence. The y axis indicates the number of cells.

about 4, and the transduction efficiency was about 60% (Fig. 4).

We initially FACS sorted and cultured the transduced cells. Seven weeks postsorting the HIV-7 EGFP⁺ cell population was close to 100%. For vectors containing the Pol III expression cassettes the EGFP⁺ cell population declined somewhat during long-term culture. Cells transduced with the vector expressing the combination of the shRNA, decoy, and ribozyme maintained over 80% EGFP expression following long-term culture but expression of the Pol III transcripts did not diminish with time (Fig. 3B and data not presented). We attribute the loss of EGFP expression in the triple construct vectors to silencing of the CMV promoter during long-term culture. The num-

ber of vector copies per cell was 1 to 2, which is ideally what we would like to obtain for clinical applications in a hematopoietic cell gene therapy setting.

The problem of viral resistance to anti-HIV drug therapies is clearly a concern for gene therapy as it is for chemotherapy. We and others have observed that single point mutations in the target region for siRNAs can give rise to RNAi-resistant viral mutants. We have therefore assayed for HIV-1 breakthrough mutants following long-term incubation of CD34⁺-derived monocytes expressing the triple construct. We challenged triple construct- or HIV-7 vector-transduced cells with JR-FL at an m.o.i. of 0.002 and incubated them for 42 days (Fig. 6A). The triple construct provided 4 logs of inhibition of p24 antigen production at this time point. Aliquots of the supernatants from both cultures were deproteinized and the viral RNAs were amplified by RT-PCR using primers flanking the shRNA target sequence. The RNA from the HIV-7 infection was readily amplified, but in contrast no RNA from the triple construct-transduced cell supernatants could be amplified (Fig. 6B). We continued the incubation up to day 56. At this time point we were able to PCR amplify viral RNA only from the triple construct, as the single-shRNA-expressing cells were no longer viable. We sequenced the DNA surrounding the shRNA I target site, and no mutations could be found (data not presented). As an additional experiment we rechallenged naïve cells transduced with the HIV-7 vector, shRNA I, or the triple construct with their respective day 28 viral supernatant from an original challenge. Although the overall p24 values are lower than we usually observe, perhaps owing to some loss of viral titer due to storage of the supernatants or some other property of JR-FL, the inhibition profile was as we predicted, with the triple vector showing no infection, whereas some replication takes place in the single-shRNA-transfected cells and more in the unprotected cells (Fig. 6C). While the studies performed here are not the most stringent for determination of resistant mutants [34], these data taken together suggest that inhibition of HIV replication is most potent in the triple-construct-expressing cells, and this in turn may provide strong protection from the emergence of shRNA I-resistant mutants.

Macrophage-Specific Functional Assays of Transduced, Differentiated Cells

Activated macrophages up-regulate the expression of B7 costimulatory molecules and present antigen to memory and effector T cells. Macrophages normally express low levels of B7. However, upon activation with various stimuli, B7 is up-regulated on the cell surface. We wanted to determine if macrophages derived from the triple construct vector-transduced CD34⁺ cells function normally in up-regulating the B7 costimulatory molecule in response to LPS stimulation. Accordingly, we stimulated

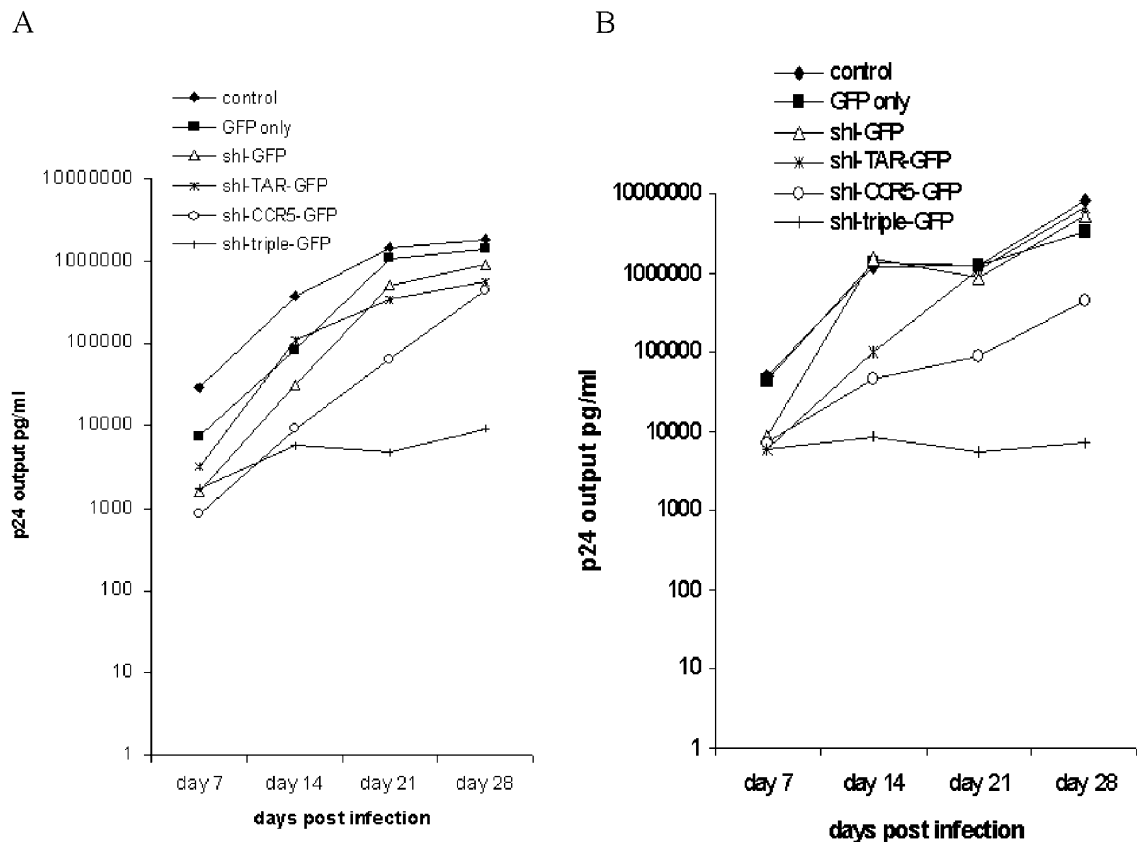


FIG. 5. Inhibition of HIV-1 replication in CD34⁺ cells expressing the anti-*rev/tat* shRNA, TAR decoy, and anti-CCR5 ribozyme. The CD34⁺ cells were transduced with the vectors indicated. The EGFP-expressing cells were collected by FACS and infected with HIV-1 strain JR-FL at m.o.i. of (A) 0.01 and (B) 0.05. The culture supernatants were collected weekly up to 4 weeks. The concentration of HIV-encoded p24 antigen was determined by ELISA.

nontransduced and HIV-7 vector- and triple construct lentiviral vector-transduced CD34⁺-derived macrophages with LPS and analyzed them by FACS 24 h later. The results in Fig. 7A showed that B7 up-regulation in the triple construct vector-transduced macrophages is similar

to that in the nontransduced and HIV-7-transduced macrophages.

Integral to the antigen presentation is the ability of macrophages to phagocytose foreign material. To determine if the transgenic macrophages are capable of normal phagocytosis, we fed tetramethylrhodamine-conjugated *Escherichia coli* (K-12) Bioparticles to the untransduced and transduced macrophages as described under Materials and Methods. We used transduced Magi-CXCR4 cells, representing nonphagocytic cells, as negative controls. As with B7 expression, the phagocytosis activity of the triple construct vector-transduced cells was similar to that observed with the control macrophages (Fig. 7B).

TABLE 1: Reporter gene expression and vector integration in CD34⁺ cells post-HIV challenge^a

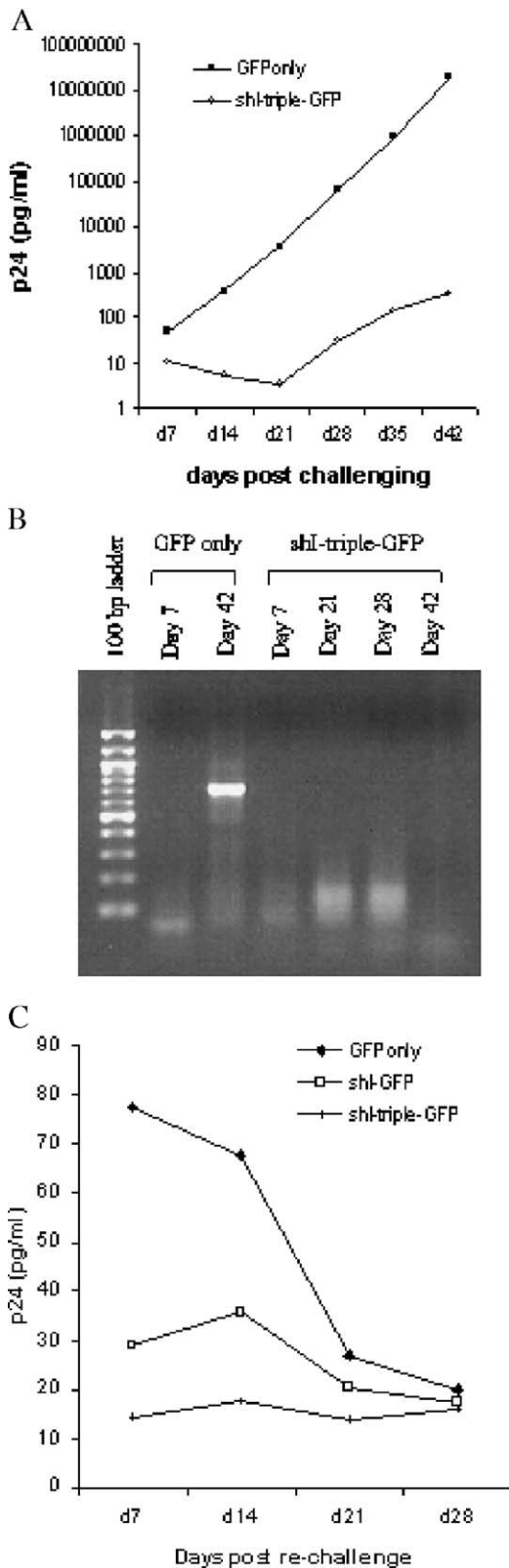
Vector	GFP ⁺ cells (%)			Copies of vector/cell ^b
	Before challenge	M.o.i. 0.01	M.o.i. 0.05	
GFP	99.5	96.4	97.8	3.57 ± 1.15
sh1	76.5	31.5	26.0	1.41 ± 0.43
sh1-TAR	85.8	63.9	67.2	0.94 ± 0.31
sh1-CCR5RZ	87.2	56.7	81.7	0.76 ± 0.38
sh1-TARCCR5RZ	92.1	85.4	81.5	1.37 ± 0.35

^a CD34⁺ cells were transduced with various vectors and sorted for EGFP⁺ cells. The EGFP⁺ cells were challenged with HIV-1 at an m.o.i. of 0.01 or 0.05. Four weeks after challenge, the percentage EGFP⁺ cells was determined by FACS.

^b The copy number of the integrated vector per cell was determined by real-time quantitative PCR with primers complementary to the WPRE sequence in the vector normalized by the albumin gene. The values for copies/cell are averages (±SD) of three independent experiments.

DISCUSSION

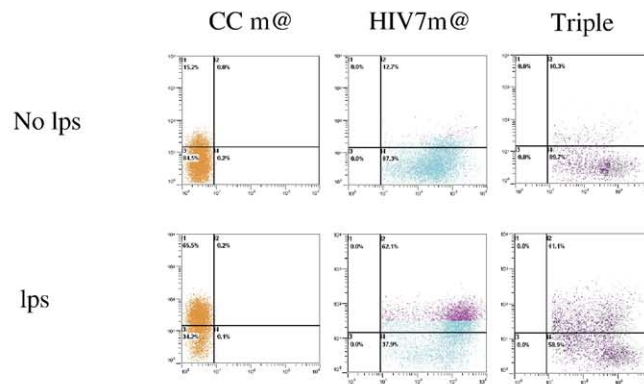
Lentiviral vectors are able to transduce nondividing cells, including primary T cells and hematopoietic stem cells [9,19,35,36], which are potential target cells for anti-HIV gene therapy. In the present study we have tested the utility of a lentiviral vector for transduction of combina-



tions of anti-HIV RNAs into hematopoietic progenitor cells. In this context, RNA interference is a potent anti-HIV agent that is highly promising for the treatment of HIV infection [8,16,37–41]. Despite the potency of RNAi as an anti-HIV agent, the sensitivity of RNAi to mismatches in the target region can lead to the emergence of RNAi-resistant mutants of HIV [21,42,43]. As with standard chemotherapy, the use of combinations of RNA-based inhibitors represents a rational approach for minimizing this problem. We propose here that a combination of different therapeutic RNAs inhibiting different viral and/or cellular targets and steps in the viral life cycles should be the most efficacious way to treat this infection in a gene therapy setting. In previous studies we demonstrated that each of the therapeutic agents used in combination in this study, the anti-*tat/rev* shRNA, the anti-CCR5 ribozyme [19], and the nucleolar-localizing TAR RNA decoy [31], has anti-HIV-1 activity. In this study, we have combined these three inhibitors as separate Pol III expression units within a lentiviral vector backbone. Despite the potential inhibitory activity of the anti-*tat/rev* shRNA to both the vector transcript and the *rev* packaging transcripts, we were able to obtain titers of the triple construct vector that approach those obtained with the parental vector HIV-7. Moreover we have demonstrated that transcripts from all three Pol III units are readily detected by Northern gel analyses in both transduced CEM T lymphocytes and CD34⁺-derived monocytes. In CD34⁺ cells at least 20% transduction efficiency was achieved following a single round of transduction. When the transduced cell population was challenged with HIV-1, all constructs containing anti-HIV-1 genes showed reduction in HIV-1 p24 antigen expression compared with the parental vector-transduced cells. Despite the early inhibitory action of the single and double combinations, some viral breakthrough occurred in all cases, whereas the triple-construct-transduced cells maintained several logs of inhibition of p24 expression relative to the parental HIV-7 vector. Furthermore, when the challenge dose of HIV-1 was raised fivefold to an m.o.i. of 0.05, only the triple construct vector-transduced

FIG. 6. Detection of HIV-1 in CD34⁺ cell culture following long-term viral infection. (A) Detection of HIV-1 p24 antigen. CD34⁺ cells were transduced with the vector expressing the triple therapeutic genes or the parental vector pHIV-7 at an m.o.i. of 10. After sorting, EGFP-expressing cells were collected by FACS and infected with the HIV-1 strain JR-FL at an m.o.i. of 0.002. The culture supernatants were collected at the days indicated and the concentration of HIV-encoded p24 antigen was determined by ELISA. (B) Detection of HIV-1 viral genomes in culture supernatants. Viral RNA was extracted from the culture supernatants from the experiment depicted in (A) at the selected time points. The RNA was amplified by RT-PCR with primers flanking the anti-*rev/tat* shRNA target sequence. (C) Detection of HIV-1 in CD34⁺ cells after rechallenge. Untransduced CD34⁺ cells were challenged with the day 28 supernatants collected from the previous challenge. The supernatants from the rechallenged cultures were collected up to 4 weeks and the concentration of HIV-1 p24 antigen was determined by ELISA.

A. B7.1 Upregulation



B. Macrophage phagocytosis assay

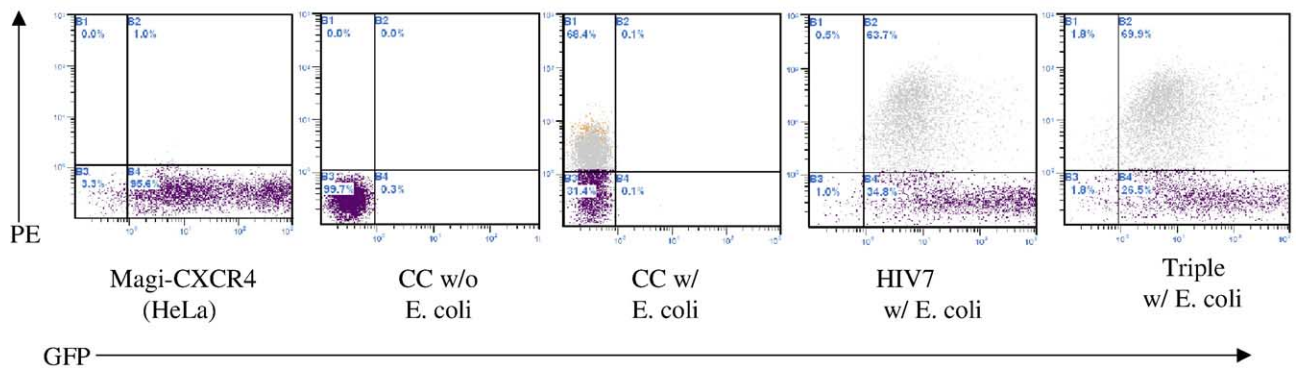


FIG. 7. (A) B7.1 up-regulation of LPS-stimulated CD34⁺-derived macrophages. Nontransduced, HIV-7-transduced, and triple construct lentiviral vector-transduced macrophages were stimulated with LPS. Twenty-four hours poststimulation, cells were stained with PE-Cy5-conjugated anti-B7.1 antibody and analyzed by FACS. These data are representative of triplicate experiments. (B) Phagocytosis of fluorescently labeled *Escherichia coli* by CD34⁺-derived macrophages. *E. coli* Bioparticles were added directly to the cultured cells. Twenty-four hours later, cells were analyzed by FACS. Graph A, control macrophages without Bioparticles. Graphs B–E show cells incubated with *E. coli* Bioparticles. Graphs B, transduced Magi-CXCR4 (nonphagocytic cell control); C, nontransduced; D, HIV-7 transduced; and E, triple construct lentiviral vector-transduced macrophages. Labeled *E. coli* Bioparticles were analyzed in the PE channel. These are representative of triplicate experiments.

cells showed suppression of HIV-1 replication over the 4-week time course of the HIV challenge. This suppression was correlated with expression of the therapeutic RNAs over the same period. These results strongly demonstrate that the combined anti-HIV functions of the shRNA, TAR decoy, and ribozyme are more than additive. Additionally our data demonstrate that lentiviral vectors can be readily engineered to incorporate and deliver multiple Pol III expression cassettes, allowing for combinatorial targeting of HIV-1.

It has been reported that some shRNAs may activate the interferon pathway [44,45]. We have carefully examined the potential activation of the interferon- α and - β pathways in triple-construct-transduced CD34⁺-derived

monocytes and macrophages and have not been able to detect any interferon production nor activation of interferon-regulated genes (M. Robbins *et al.*, submitted for publication). Macrophage functional assays for B7 up-regulation in response to LPS and phagocytosis of bacteria were also unperturbed in the triple construct vector-transduced macrophages derived from CD34⁺ cells (Fig. 7).

An important observation relative to the safety of the triple construct vector is that potent inhibition of HIV-1 replication was achieved from only 1 or 2 copies of integrated triple vector per CD34⁺ cell. An advantage of our lentiviral vector design is that each of the antiviral RNAs, the shRNA, ribozyme, and RNA decoy, inhibits

HIV-1 via a different mechanism. To our knowledge this is the first report that a single lentiviral vector is able to deliver three different types of functional anti-HIV-1 genes to primary hematopoietic progenitor cells. For therapeutic application we have further modified our vector by eliminating the Pol II EGFP expression cassette, leaving the therapeutic RNAs as the only products generated by the vectors. This vector is currently being prepared for a clinical trial using autologous hematopoietic stem cells obtained from AIDS/lymphoma patients and bone marrow transplantation.

MATERIALS AND METHODS

Plasmid construction. The lentiviral vector backbone pHIV-7-GFP is shown in Fig. 1. The short hairpin RNA, shI, was designed for targeting a shared sequence of *rev* and *tat* (site I) [16] (Fig. 1A). The short hairpin RNA expression cassette was constructed by PCR amplification of pTZ U6 + 1 with one primer complementary to the sequence upstream of the U6 promoter and another primer that covers the antisense, loop, and sense of the shRNA and the 3' end of the U6 promoter. A *Bam*HI site was included in the 5' end of both primers. In addition, a *Not*I site was added next to the *Bam*HI site in the downstream primer. The PCR products were digested with *Bam*HI and ligated into the *Bam*HI-linearized pHIV-7-GFP. The resulting construct is designated pHIV-shI-GFP. The U6-U16TAR RNA decoy (Fig. 1B), VA1-CCR5 ribozyme (Fig. 1C), and combined sequence of these two expression cassettes were amplified by PCR using pHIVU16TAR-CCR5RZ-GFP [18] as the template. All the PCR primers have a *Not*I site at the 5' end. These PCR products were digested with *Not*I and inserted into the *Not*I sites in pHIV-shI-GFP, resulting in pHIV-shI-TAR-GFP, pHIV-shI-CCR5RZ-GFP, and pHIV-shITAR-CCR5RZ-GFP. All PCR products were verified by DNA sequencing. In the final construct the shRNA sequence and the U6 promoter for the U16TAR decoy are only separated by the combined lengths of the *Not*I and *Bam*HI restriction sites. The U16TAR decoy sequence and the VACC5 ribozyme are separated by a 100-bp polylinker sequence.

Lentiviral vector production. The lentiviral vectors carrying the appropriate inserts were cotransfected with pCHGP-2, pCMV-G, and pCMV-Rev into 293 T cells using the calcium phosphate precipitation procedure as described previously [18]. The culture supernatants were collected, filtered with a 0.2- μ m SCFA filter (Nalge Nunc, Rochester, NY, USA), concentrated by ultracentrifugation, and stored at -80° C until use. The vector titers were determined by transduction of HT1080 cells and assayed for EGFP expression using flow cytometry.

Cell culture and vector transduction. The human T cell line CEM was maintained in RPMI 1640 medium supplemented with 10% FBS. CEM cells were transduced with lentiviral vectors as described previously [18]. At an m.o.i. of 8, greater than 90% of the cells expressed EGFP as determined by FACS analyses. For transduction of CD34⁺ cells, the CD34⁺ stem cells were enriched from umbilical cord blood by anti-CD34 antibody-coupled magnetic beads (Miltenyi Biotech, Auburn, CA, USA). The purity of CD34⁺ cells was above 90% as determined by FACS analysis. The CD34⁺ cells were cultured in Iscove's modified Dulbecco's medium supplemented with 20% BIT9500 (StemCell Technologies, Vancouver, BC, Canada), 40 μ g/ml human low-density lipoproteins, 10^{-4} M 2-mercaptoethanol, 100 ng/ml SCF, 100 ng/ml flt3 ligand, 10 ng/ml TPO (PeproTech, Rocky Hill, NJ, USA), 20 ng/ml IL-3, 20 ng/ml IL-6 (R&D Systems, Minneapolis, MN, USA). The lentiviral vector stock was adjusted to an m.o.i. of 40 in 200 μ l culture medium and loaded onto RetroNectin (Takara Mirus, Madison, WI, USA)-coated 24-well plates. After incubation at 32°C for 4 h, the vector supernatant was removed and the wells were washed with PBS. The prestimulated CD34⁺ cells were added to the wells at 5×10^4 /ml in the growth medium.

RNA preparation and Northern blotting analyses. Total RNA was extracted using the RNA STAT-60 reagent (Tel-Test, Inc., Friendswood, TX, USA) according to the manufacturer's protocol. Total RNAs were separated by electrophoresis in an 8% polyacrylamide gel containing 7 M urea and electroblotted onto a Hybond-N nylon membrane (Amersham, Arlington Heights, IL, USA). The membrane was hybridized with γ -³²P-labeled oligodeoxynucleotide probes complementary to the anti-CCR5 ribozyme, the U16TAR decoy, or the antisense sequence of the shRNAs. A probe specific for U6 snRNA was used as an internal control.

HIV-1 challenge and p24 antigen assay. The CD34⁺ cells were transduced with various lentiviral vectors. Eleven days after transduction, the cells were sorted by FACS and EGFP⁺ cells were collected. After recovery from sorting, 5×10^5 EGFP⁺ cells were exposed overnight to the JR-FL strain of HIV-1 at the indicated m.o.i. The infected cells were washed four times with HBSS and the cells were maintained in the culture medium for CD34⁺ cells as described above. The culture supernatant was collected on a weekly basis. The p24 antigen analyses were performed using a Coulter HIV-1 p24 antigen assay (Beckman Coulter, Brea, CA, USA) according to the manufacturer's instructions.

Real-time quantitative PCR. The CD34⁺ cells transduced with the lentiviral vectors and challenged with HIV-1 were lysed in the lysis buffer (50 mM KCl, 10 mM Tris-HCl, pH 8.0, 1.25 mM MgCl₂, 0.45% NP-40, 0.45% Tween 20, 0.75 mg/ml proteinase K). The samples were incubated at 37°C overnight followed by heat activation at 95°C for 10 min. The WPRE sequence in the lentiviral vector integrated in the genomic DNA was amplified using the iCycler iQ Real Time Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green I and the primers 5'-CCG-TTGTCAAGCAACGTG-3' and 5'-AGCTGACAGGTGGTGGCAAT-3' [46]. The albumin gene is a single-copy sequence in the human genome and a segment of this was also amplified with primers 5'-TGAACATACGTTCCCAAAGAGTTT-3' and 5'-CTCTCCTTCTCAGAAAGTGCATAT-3' for use as an internal control. The PCR conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 20 s and 64°C for 1 min.

Detection of HIV-1 genomic RNA from cell culture supernatants. Viral RNA was extracted from HIV-1-infected CD34⁺ cells with the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. RT-PCR was performed using oligonucleotide primers flanking the anti-*rev/tat* shRNA target sequence. The primer sequences were 5'-AGCCACA-CAATGAATGGACAC-3' (sense) and 5'-TCTGTAGCACTACAGATCATCA-3' (antisense). cDNA synthesis was carried out using AMV reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL, USA) at 42°C for 15 min. After *Taq* DNA polymerase (Eppendorf, Westbury, NY, USA) was added, the PCR was initiated at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s.

Assays for up-regulation of B7.1 and phagocytosis in activated, transduced macrophages. Functional assays were performed on CD34⁺ cells purified from fetal liver. The purity of the CD34⁺ cells was routinely greater than 93%. CD34⁺ cells were transduced with HIV-7 or the triple construct vector on 2 consecutive days as described above. Transduction efficiencies for HIV-7 and the triple constructs were 65 and 87%, respectively. Transduced CD34⁺ cells were plated into Methocult medium (StemCell Technologies) to derive myeloid colonies. Fifteen days post-transduction, myeloid colonies were seeded in six-well plates at a cell density of 10^6 cells per well in cytokine medium to derive macrophages. Subsequent experiments to determine the functionality of transgenic macrophages were then performed. To monitor up-regulation of the B7 costimulatory molecule in response to LPS stimulation, control non-transduced, HIV-7-transduced, and triple construct lentiviral vector-transduced CD34⁺-derived macrophages were stimulated with LPS (10 μ g/ml) (Sigma-Aldrich, St. Louis, MO, USA). Twenty-four hours post-stimulation, macrophages were stained with PE-Cy5-conjugated anti-B7.1 antibody (BD Biosciences, San Jose, CA, USA). To determine the up-regulation of B7 in transduced cells, FACS analysis was done on EGFP-gated cells.

To evaluate if the transduced macrophages could carry out phagocytosis, tetramethylrhodamine-conjugated *E. coli* (K-12) Bioparticles (Invi-

trogen, Carlsbad, CA, USA) were added to the cell culture medium. Two hours later, cells were washed five times with PBS and incubated in fresh medium. Twenty-four hours later, cells were analyzed by FACS for EGFP expression and ingested *E. coli*. Tetramethylrhodamine could be detected in the FL2 channel as phycoerythrin. Transduced Magi-CXCR4 cells, representing nonphagocytic cells, were used as negative controls.

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