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Inhibition of HIV-1 Infection by Lentiviral Vectors Expressing Pol III-Promoted Anti-HIV RNAs

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A primary advantage of lentiviral vectors is their ability to pass through the nuclear envelope into the cell nucleus thereby allowing transduction of nondividing cells. Using HIV-based lentiviral vectors, we delivered an anti-CCR5 ribozyme (CCR5RZ), a nucleolar localizing TAR RNA decoy, or Pol III-expressed siRNA genes into cultured and primary cells. The CCR5RZ is driven by the adenoviral VA1 Pol III promoter, while the human U6 snRNA Pol III-transcribed TAR decoy is embedded in a U16 snoRNA (designated U16TAR), and the siRNAs were expressed from the human U6 Pol III promoter. The transduction efficiencies of these vectors ranged from 96–98% in 293 cells to 15–20% in primary PBMCs. A combination of the CCR5RZ and U16TAR decoy in a single vector backbone gave enhanced protection against HIV-1 challenge in a selective survival assay in both primary T cells and $CD34^+$-derived monocytes. The lentiviral vector backbone-expressed siRNAs also showed potent inhibition of p24 expression in PBMCs challenged with HIV-1. Overall our results demonstrate that the lentiviral-based vectors can efficiently deliver single constructs as well as combinations of Pol III therapeutic expression units into primary hematopoietic cells for anti-HIV gene therapy and hold promise for stem or T-cell-based gene therapy for HIV-1 infection.

Key Words: lentiviral vector, CCR5, ribozyme, TAR, RNA decoy, siRNAs, HIV-1 gene therapy

INTRODUCTION

Combinations of antiviral agents (or highly active antiretroviral therapy, HAART) have profoundly suppressed levels of plasma viral loads with improved survival and outcomes of patients infected with HIV-1 [1–3]. However, the side effects, complicated dosing schedules, and multidrug-resistant variants remain problematic [4–7], suggesting that additional or adjuvant therapies need to be considered as well. Gene therapy is still an attractive and potentially powerful approach for treatment of HIV-1 infection. The attraction lies predominantly with the *ex vivo* accessibility of hematopoietic cells and hematopoietic precursor cells for genetic manipulation. Thus vector delivery is not complicated by immune responses and accessibility to the appropriate target cells. A number of monoagent gene therapy clinical trials for HIV-1 have already been attempted, without adverse consequences [8–13]. In contemplating the next step in applying gene therapy for the treatment of HIV-1 infection, the use of combinatorial strategies that attack different targets and steps in the viral life cycle is the most realistic, since viral resistance is a major concern. To make this a reality, we have been developing several different RNA-based approaches for inhibition of HIV-1. These include ribozymes, decoys, and small interfering RNAs (siRNAs). The challenge is to achieve efficient delivery and expression of the genes encoding these antiviral RNAs in the appropriate hematopoietic cells.

Retroviral vectors derived from murine leukemia virus (MuLV) have long been favored in gene delivery and human gene therapy protocols for their efficient integration into the genome of the target cells and the accompanying expression of the therapeutic genes they harbor [14–16]. However, these vectors require cell division for efficient gene transfer, which greatly limits their application in nonproliferating cells, such as hematopoietic stem cells [15]. In contrast, replication-defective lentiviral vectors derived from human immunodeficiency virus type 1 (HIV-1) are able to transduce a wide array of quiescent cell types with sustained long-term expression of the transgenes [17–20]. These properties suggest the potential advantages of using lentiviral vectors for human gene therapy [21]. Many HIV-1-targeted gene therapy protocols have been developed in recent years [8,9,11–13,22–24].

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The majority of these trials target a single therapeutic gene. If a combination of therapeutic genes targeting different viral products and steps in the viral life cycle is used, the probability of virus escape mutants will be greatly reduced. Furthermore, there is the possibility of synergy between the different gene therapy agents, as in the combined use of TAR and RRE decoys [25]. In this study, a VA1-CCR5 ribozyme chimera [26]and a chimeric U16TAR decoy [27] have been combined into a single lentiviral vector (pHIV-7-GFP) for transduction into peripheral blood mononuclear cells (PBMCs) and $CD34^+$ cells and subsequent testing for the inhibition of HIV-1 infection.

The β-chemokine receptor CCR5 is a coreceptor for macrophage-tropic (M-tropic) HIV-1 infection [28,29]. A homozygous 32-bp deletion in the CCR5 gene (CCR5Δ32/CCR5Δ32) that results in loss of surface expression of this receptor confers strong resistance to HIV-1 infection [30,31]. Individuals heterozygous for the deletion (CCR5Δ32/CCR5) exhibit slower disease progression [32,33]and better responses to HAART [4]. These findings suggest that CCR5 is a good target gene for anti-HIV-1 gene therapy. Our laboratory has developed a hammerhead ribozyme specifically targeting the CCR5 message at a site that is unique among the chemokine family members [26]. This ribozyme resulted in about a 70% down-regulation of the CCR5 receptor in HOS-CD4.CCR5 cells and resulted in resistance to M-tropic HIV-1 infection in PM1 cells [26].

The Tat protein is essential for HIV transcription [34]. Tat executes its transcriptional activation function upon interaction with the HIV TAR element in concert with cellular factors cyclin T1 and CDK9 [35]. It has previously been demonstrated that a retroviral vector-delivered polymeric TAR RNA decoy is capable of inhibiting HIV-1 replication [36]. Colocalization of decoys and target proteins in a subcellular compartment should enhance their efficacy, such as has been demonstrated for ribozymes [37,38]. Since HIV-1-encoded Tat localizes to the nucleoli of human cells [27,39–42], delivery of a TAR decoy into this compartment could enhance the antiviral activity of this decoy. We have previously demonstrated that the small nucleolar RNA (snoRNA) U16 can serve as a vehicle for nucleolar delivery of highly functional anti-HIV ribozymes and decoys [27,37]. In the present study, we have constructed lentiviral vectors capable of simultaneously expressing the chimeric VA1 anti-CCR5 ribozyme (CCR5RZ) and the U16-embedded TAR decoy (U16TAR). We report here that the combination of CCR5RZ and the U16TAR decoy results in the greatest protection from HIV-1 challenge in a T-cell-selective survival model.

RNA interference (RNAi) is a powerful mechanism for targeted transcript degradation [43]. The use of RNAi for inhibition of HIV-1 infection has been exploited by a number of researchers [44–53]. Our long-term goal is to combine siRNAs with other RNA-based anti-HIV-1 agents for the treatment of HIV-1 infection in a gene therapy setting. As a first step in this process, we have incorporated either siRNA- or short hairpin RNA (shRNA)-encoding genes into the backbone of our lentiviral vector. We report here that transduction of these vectors into primary PBMCs resulted in potent inhibition of HIV-1 in acute challenges.

Overall, our results demonstrate that either single or combined Pol III transcription units can be functionally expressed in primary human hematopoietic cells, thereby setting the stage for future gene therapy applications in the treatment of HIV-1 infection.

RESULTS

Construction of the Anti-CCR5 Ribozyme, TAR RNA Decoy, and siRNA-Expressing Vectors

The anti-CCR5 ribozyme was designed to cleave the CCR5 transcript at a GUC cleavage site located 23 nt downstream of the AUG codon [26] (Fig. 1A). The sequence targeted by the ribozyme does not share homologies with other β-chemokine receptor family members. The anti-CCR5 ribozyme is part of a chimeric construct that includes a modified form of the VA1 RNA. The VA1 gene itself is a Pol III promoter and the VA1 RNA is cytoplasmically localized [26,27,60]. Expression of the U16TAR sequence is driven by the human U6 promoter. The anti-CCR5 ribozyme and U16TAR decoy expression cassettes were cloned into the lentiviral vector pHIV-7-GFP, which also expresses an enhanced green fluorescent protein (EGFP) reporter gene under the control of the human CMV promoter (Fig. 2A). The anti-CCR5 ribozyme and U16TAR constructs either were inserted into separate vectors or were combined within a single vector backbone. Each Pol III promoter was inserted in either a forward or a reverse orientation with respect to the CMV promoter for EGFP, resulting in eight different vectors (Fig. 2B). The site I siRNA targets both *rev* and *tat* transcripts, while the site II siRNA is for *rev* only [52]. In pHIV-si(I)-GFP and pHIV-si(II)-GFP the sense and antisense siRNA sequences are under the control of separate U6 promoters, while in pHIV-sh(II)-GFP a single U6 promoter controls the expression of a short hairpin RNA [described in 55]. In the lentiviral vectors the transcriptional orientations for antisense and sense sequences and the shRNA are the same as for the CMV–EGFP construct.

After packaging, we transduced the lentiviral vectors, into HT1080 cells to assay their titers based on EGFP expression. As shown in Table 1, somewhat reduced titers were evident for all vectors harboring the Pol III-promoted RNAs (VA1-CCR5RZ, U6-U16 TAR, and siRNA genes), but relatively high titers were maintained when the U6 or VA1 promoter was in the same orientation as the CMV promoter. However, when the VA1 or U6 promoter was in the reverse orientation with respect to the CMV promoter, the vector titers were decreased further. Following concentration of the vectors by centrifugation,

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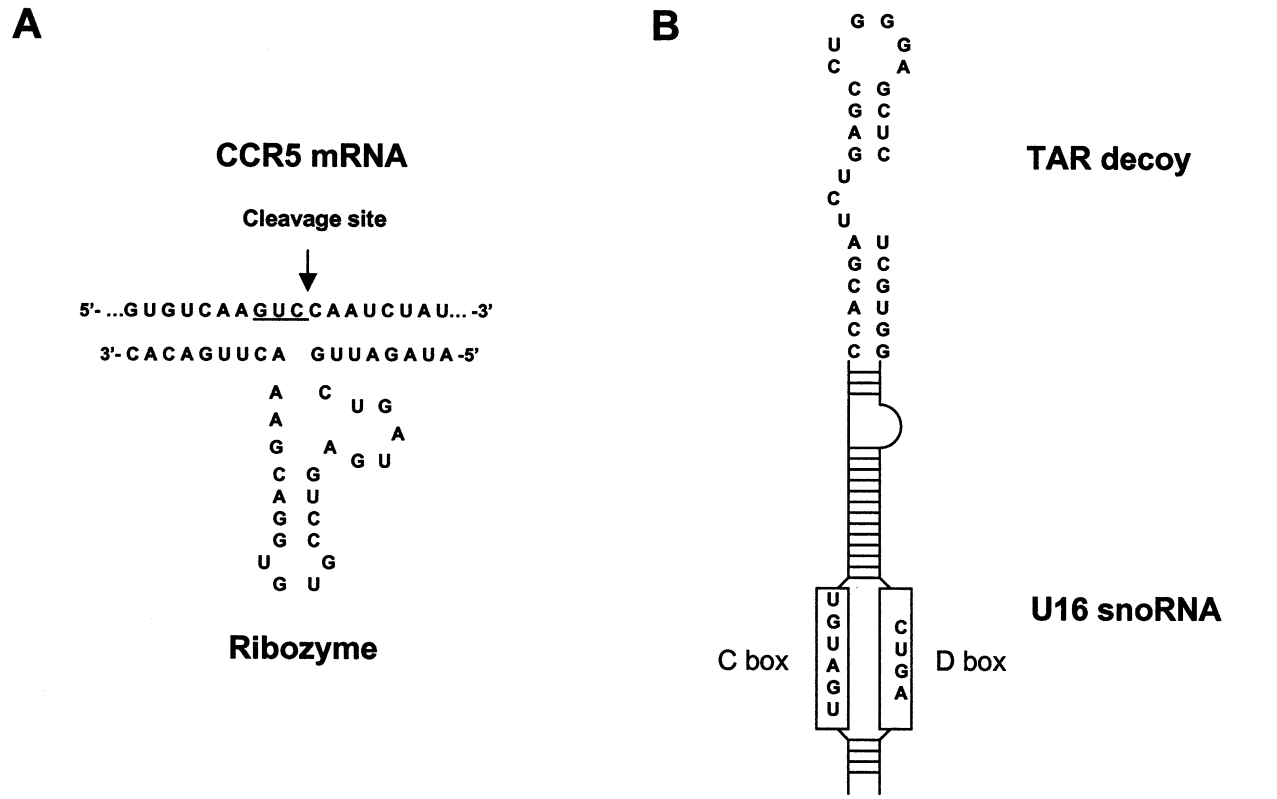

FIG. 1. Therapeutic genes delivered by the lentiviral vectors. (A) The sequence of the anti-CCR5 hammerhead ribozyme and the CCR5 sequence targeted by this ribozyme. The ribozyme binds to the substrate with its two arms and cleaves immediately 3′ of the GUC codon as indicated by the arrow. (B) Schematic representation of 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 U16 snoRNA sequence, allowing its nucleolar localization. C box and D box are the U16 nucleolar localization signals.

titers of 10^8–10^9 TU/ml were achieved. Based on the titer results, we chose only the forward orientation for each of the Pol III promoters (same direction as CMV-EGFP) for further analyses.

Transduction Efficiencies of the Various Lentiviral Vectors

We assessed the transduction efficiencies of the various lentiviral vector constructs using the cultured human cell lines 293, PM1, and CEM. Although inclusion of the Pol III-promoted transcripts resulted in somewhat reduced titers compared with the vector backbone, when we used the same multiplicity (multiplicity of infection (m.o.i.) of 8), the transduction efficiencies were equivalent for each of these vectors in 293 and PM1 cells. We evaluated the transduction efficiencies as the percentage of cells expressing EGFP. In 293 cells, the transduction efficiencies for pHIV-7-GFP, pHIV-VA1-CCR5RZ-GFP, and pHIV-U6-U16TAR-GFP were 96, 98, and 97%, respectively (Fig. 3). In PM1 cells the values were 79, 78, and 77%, respectively. We observed no substantial reductions in the percentages of transduced cells even after 3 months of culture without selection (data not shown). Similar results were obtained from transductions using the siRNA expression constructs (Fig. 3).

Expression of the Anti-CCR5 Ribozyme, U16TAR Decoy, and siRNAs in T Cells

To evaluate expression of the cloned therapeutic RNAs in target cells, we transduced lentiviral vectors bearing the CCR5RZ, U16TAR, or siRNA sequences into PM1 and/or CEM cell lines. We extracted total RNA from these cells and analyzed it by Northern blotting. As shown in Fig. 4, when cells were transduced with the CCR5RZ- or U16TAR-expressing vectors, regardless of whether the cassettes were on separate vectors or combined in a single vector, there were less than twofold differences in the relative levels of expression between the two different Pol III transcripts (Fig. 4B). We also probed for expression of the antisense component of the siRNAs targeting site I or site II (Fig. 4C). These results indicate that each of the VA1-CCR5RZ, U6-U16TAR, and U6-siRNA cassettes is actively expressed in our lentiviral vector contexts. Furthermore, our data demonstrate that two therapeutic genes in the same lentiviral vectors can be independently expressed from Pol III promoters without interfering with

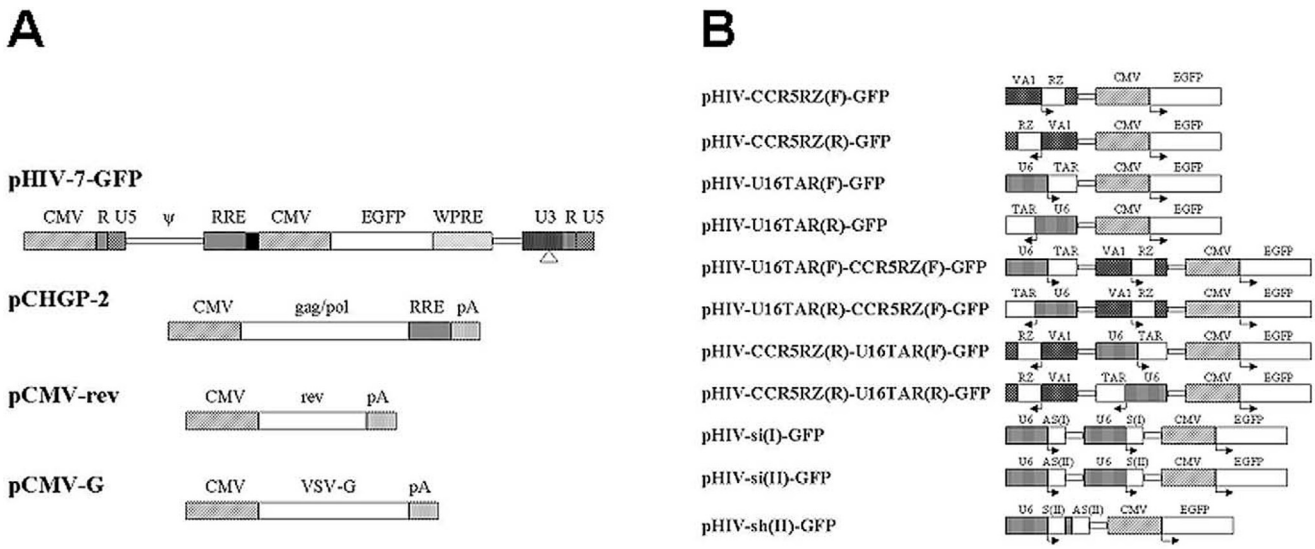

FIG. 2. The lentiviral vectors and the vector production system. (A) The packaging system. The lentiviral vector pHIV-7-GFP contains a hybrid 5′ LTR in which the U3 region is replaced with the CMV promoter, the packaging signal (Ψ), the RRE sequence, the EGFP gene driven by CMV promoter, and the 3′ LTR in which the *cis* regulatory sequences are completely removed from the U3 region. The genes of interest along with a Pol III promoter are inserted directly upstream of the CMV promoter of EGFP in the pHIV-7-GFP vector. The solid box between RRE and CMV-EGFP represents the FLAP sequence of HIV-1. pCHGP-2 contains the *gag* and *pol* genes and RRE sequence from HIV-1 under the control of the CMV promoter. pCMV-Rev contains the coding sequence of Rev driven by the CMV promoter. pCMV-G contains the VSV-G protein gene under the control of the CMV promoter. pA indicates the polyadenylation signal from the human β-globin gene. (B) Schematic representation of the pHIV-7-GFP-derived vectors. Only the inserted genes with their respective promoters and the CMV-EGFP region of the vectors are shown. VA1, VA1 promoter; RZ, anti-CCR5 ribozyme; U6, U6 promoter; TAR, U16TAR RNA decoy. S(I) and AS(I) are sense and antisense sequences of siRNA against a common *tat/rev* exon (site I), whereas S(II) and AS(II) are sense and antisense sequences for site II that are in the *rev* coding sequence [52]. Arrows indicate the orientation of transcription.

each other. Previously published results using the U6 Pol III promoter in a retroviral vector demonstrated higher expression levels of the U16TAR decoy in the forward orientation relative to the retroviral Pol II promoters [37]. Qin *et al.* [65] also chose the forward orientation of U6-promoted siRNAs relative to the UbiC-EGFP gene in a lentiviral vector. Aside from the present study, there is little information available on the effects of orientation of transcription units using multiple Pol III expression cassettes in a single retroviral or lentiviral backbone.

Down-regulation of CCR5 by the Anti-CCR5 Ribozyme

We transduced lentiviral vectors harboring chimeric VA1-CCR5 ribozyme constructs into HOS-CD4.CCR5 cells [26, 31]. We monitored down-regulation of the CCR5 mRNA by RT-PCR. When cells were transduced with the vector expressing the anti-CCR5 ribozyme alone, CCR5 mRNA levels were substantially reduced relative to controls, although a more modest reduction was achieved in cells transduced with TAR-CCR5RZ double construct (Fig. 5).

Inhibition of HIV Infection in Primary T Cells Transduced with Anti-HIV Genes

We next sought to evaluate the antiviral efficacy of the lentiviral vector-transduced anti-HIV constructs in pri-

TABLE 1: Transduction efficiency of lentiviral vectors

Construct[a]	Titer (TU/ml)[b]	Relative efficiency (%)
pHIV-7-GFP	8.2×10^6	100
pHIV-CCR5RZ(F)-GFP	4.5×10^6	55
pHIV-CCR5RZ(R)-GFP	3.0×10^5	4
pHIV-U16TAR(F)-GFP	3.5×10^6	43
pHIV-U16TAR(R)-GFP	2.6×10^6	32
pHIV-U16TAR(F)-CCR5RZ(F)-GFP	1.4×10^6	17
pHIV-U16TAR(R)-CCR5RZ(F)-GFP	3.0×10^5	4
pHIV-CCR5RZ(R)-U16TAR(F)-GFP	6.0×10^4	0.7
pHIV-CCR5RZ(R)-U16TAR(R)-GFP	1.4×10^5	1.7
pHIV-si(I)-GFP	1.0×10^6	12
pHIV-si(II)-GFP	1.1×10^6	13
pHIV-sh(II)-GFP	2.8×10^6	34

[a]CCR5RZ is driven by the VA1 promoter; U16TAR and siRNA are driven by the U6 promoter. (F) and (R) indicate forward orientation or reverse orientation of the Pol III promoters compared with the CMV promoter for GFP.
[b]HT1080 cells were transduced with the lentiviral vectors shown above as described under Materials and Methods. The titers were obtained with FACS analysis and expressed as the number of GFP-positive cells transduced with 1 ml viral supernatant. The values are averages from two independent experiments. The relative efficiency was calculated as the ratio of the titer in the construct indicated to that in the control vector, pHIV-7-GFP.

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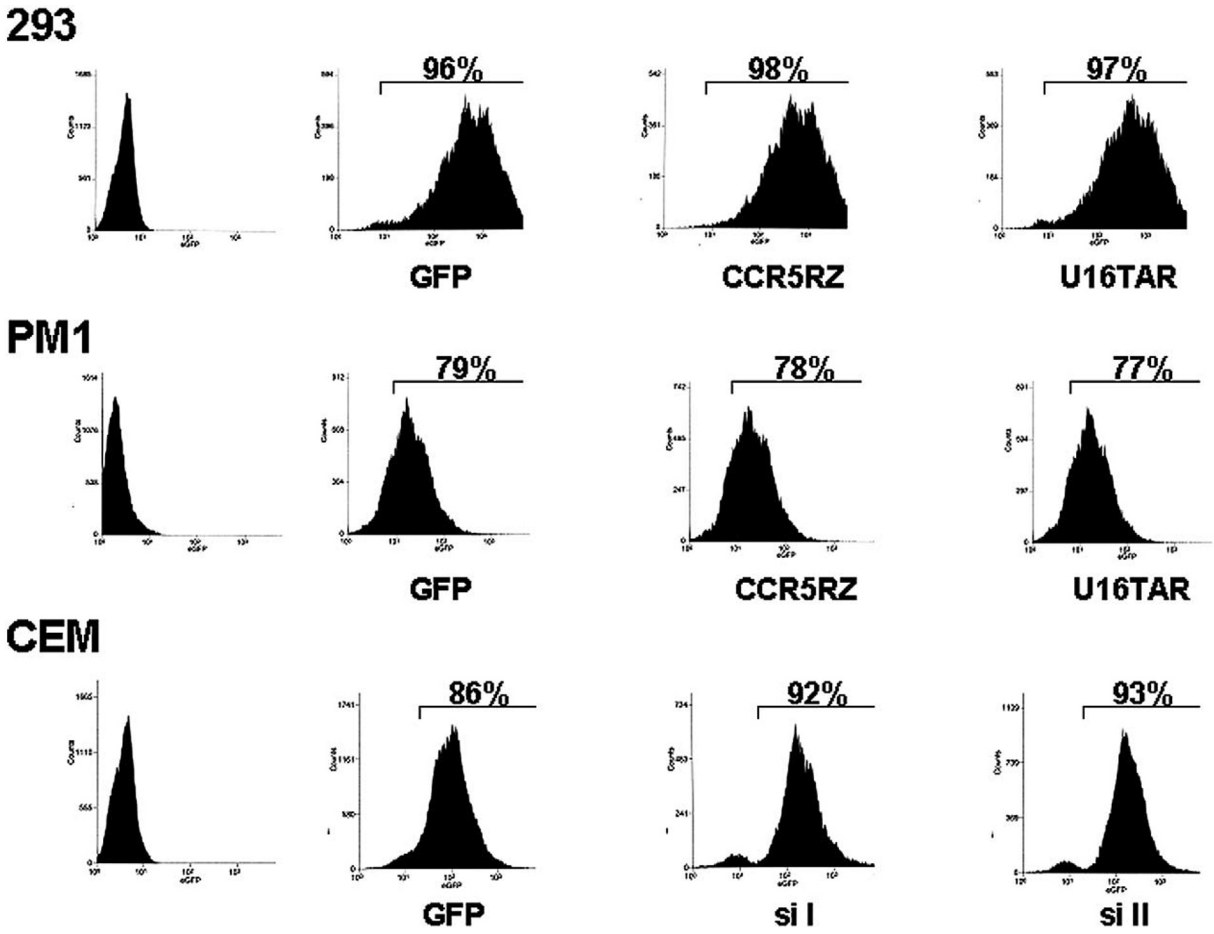

FIG. 3. EGFP expression in 293, CEM, and PM1 cells following transduction with the various lentiviral vectors. Cells were transduced with the lentiviral vectors at an m.o.i. of 8. FACS analyses were carried out 7 days posttransduction. The *y* axis indicates cell number. The *x* axis indicates the intensity of fluorescence.

mary cells. PBMCs were enriched for $CD4^+$ cells and activated prior to transduction. To evaluate the antiviral activities of the various constructs, we transduced PBMCs with the various vectors and monitored the initial transduction efficiencies for EFGP expression via FACS analyses. We cultured unsorted populations of cells in the absence or presence of M-tropic JR-FL viral challenge. To monitor selective survival of protected cells, we removed only 50% of the cells at each indicated time point and added an equivalent volume of fresh medium. We conducted FACS analyses at the indicated days to monitor the relative frequencies of EGFP cells in the population.

As shown in Fig. 6, after being challenged with the JR-FL strain of HIV-1, the EGFP-positive cell population increased over time in primary T cells transduced with U16TAR decoy. Although the anti-CCR5 ribozyme alone was not sufficient to maintain the survival advantage, cells expressing both the anti-CCR5 ribozyme and the U16TAR decoy showed greater protection against HIV-1 infection than those expressing only the U16TAR decoy. These results suggest a potential synergistic effect of combining RNA-based HIV-1 inhibitors in HIV-1 gene therapy.

$CD34^+$ hematopoietic stem cells can differentiate into a variety of hematopoietic cell lineages including CD^+ lymphocytes, monocytes, and macrophages. Stem cell-based gene therapy has the potential to provide protection for all these cell lineages against HIV-1 infection. Therefore, we felt it was important to evaluate the anti-HIV-1 efficacy of our therapeutic RNAs in $CD34^+$ stem cells. We isolated $CD34^+$ cells from umbilical cord blood using monoclonal antibody-conjugated magnetic beads. We transduced the resulting $CD34^+$ cells with lentiviral vectors expressing U16TAR, the anti-CCR5 ribozyme, and the U16TAR plus anti-CCR5 ribozyme. One week after transduction, the cells were infected with HIV-1 JR-FL. We monitored $EGFP^+$ cells up to 4 weeks by FACS analysis. Similar to the results in primary T cells, the combinatorial

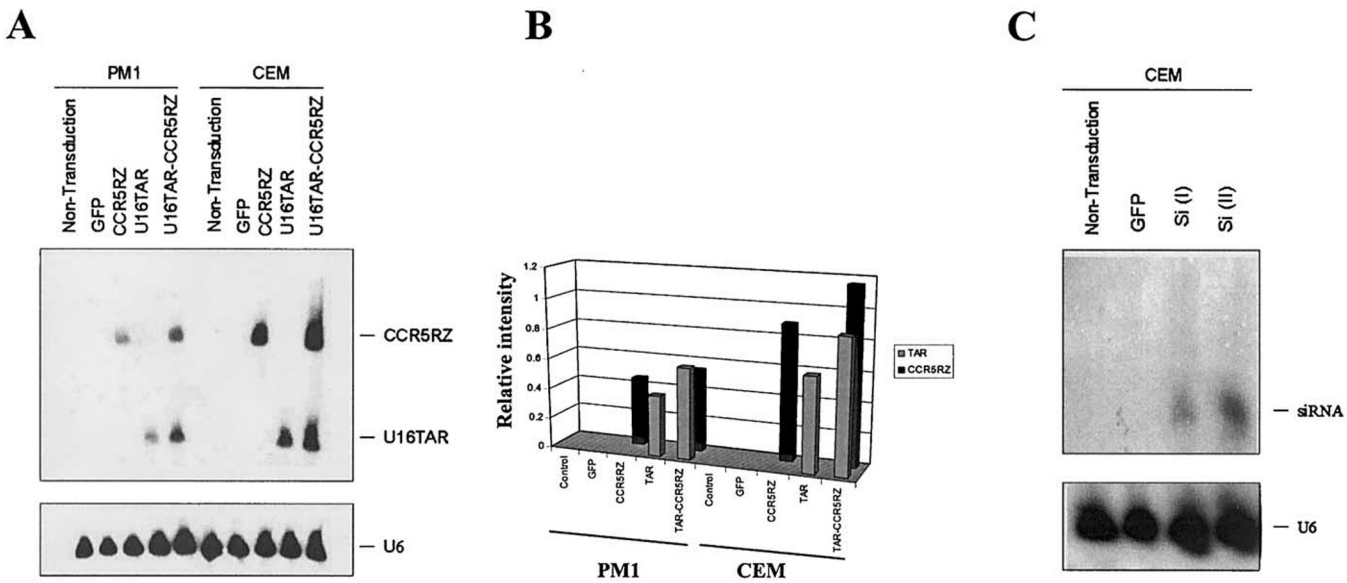

FIG. 4. Expression of the anti-CCR5 ribozyme, TAR decoy, and siRNAs in PM1 and CEM cells. (A) Cells were transduced with the lentiviral vectors harboring the VA1-CCR5 ribozyme, the U16TAR decoy, or a combination of the two. Total RNA (15 μg) was electrophoresed in a 6% polyacrylamide gel with 7 M urea, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled probes for the anti-CCR5 ribozyme and U16TAR decoy. A U6 snRNA complementary probe was used as an internal control. RNA extracts from untransduced cells or cells transduced with the EGFP parental vector alone were used as negative controls. (B) Quantitation of the results from (A) by PhosphorImager analyses. (C) Total RNA was extracted from cells transduced with lentiviral vectors carrying various siRNA expression cassettes and hybridized with probes complementary to the antisense sequences of the siRNAs. The experimental conditions were the same as in (A) except the gel concentration was 8%.

effects of the U16TAR and anti-CCR5 ribozyme provided a selective survival advantage to the monocytes derived from transduced CD34$^+$ cells (Fig. 7). HIV-1 p24 antigen assays also demonstrated reduction of this protein in cells transduced with the combination of the CCR5RZ and U16TAR (data not shown).

To evaluate further the functional expression of Pol III-expressed RNAs in the lentiviral vector backbone, we assessed the anti-HIV-1 efficacy of siRNAs targeted against *rev* and *tat* in PBMCs. We transduced PBMCs with pHIV-si(I)-GFP (targeting both *rev* and *tat*), pHIV-si(II)-GFP (targeting *rev*), or pHIV-sh(II)-GFP (targeting *rev*). Transduced cells expressing EGFP were FACS sorted and then challenged with either HIV-1 strain JR-FL or strain IIIB. As shown in Fig. 8, after infection of HIV-1 strain JR-FL, the siRNAs provided an approximate 3 log reduction of p24 antigen relative to the vector backbone control. When the same cell populations were challenged with the more robustly replicating T-tropic HIV-1 IIIB, the siRNAs exhibited various levels of anti-HIV-1 activity. Strikingly, the hairpin-formed siRNA maintained p24 antigen at a background level for up to 19 days postinfection. Taken together, these data demonstrate for the first time that lentiviral vector backbone-expressed siRNAs targeting HIV itself result in efficient inhibition of HIV-1 replication in primary cells.

DISCUSSION

Development of drug-resistant HIV-1 variants and the side effects of chemotherapy for the treatment of HIV-1 infection make it necessary to develop alternative therapeutic approaches for the treatment of HIV-1 infection. Murine-based retroviral vectors have long been favorite tools for gene delivery. Traditional retroviral vectors can deliver therapeutic genes into target cells; however, they are inefficient in transducing nondividing cells [15,61]. In contrast, lentiviral vectors have been shown to transduce nondividing cells efficiently, including human hematopoietic progenitor cells [62–64]. This property makes them attractive as vectors for hematopoietic stem cell gene therapy. In the present study we have investigated the lentiviral vector-mediated delivery of Pol III-driven anti-HIV constructs, including a VA1-driven anti-CCR5 ribozyme, a U6-promoted nucleolar-localizing TAR RNA decoy, and U6-driven siRNAs.

We demonstrate efficient delivery and expression of the lentiviral constructs in a variety of cultured and primary cells. Most importantly, we show that a combination of two different anti-HIV-1 RNAs can be functionally expressed from the same vector backbone, and significantly, the dual expression of a ribozyme targeting a cellular target (CCR5) and a TAR decoy that binds Tat provides a selective survival advantage in both PBMCs and

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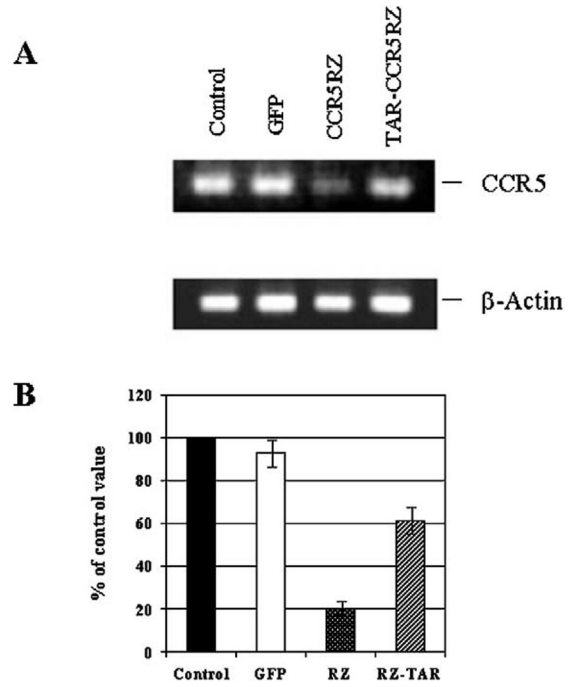

FIG. 5. Down-regulation of CCR5 transcripts by the anti-CCR5 ribozyme. HOS-CD4.CCR5 cells were transduced with the following lentiviral vectors: parental EGFP, VA1-CCR5RZ, or U16TAR/VA1-CCR5RZ. Total RNA was extracted, DNase I treated, and analyzed by RT-PCR. (A) Top: cDNA was amplified using primers complementary to CCR5. Bottom: β-Actin mRNA was amplified and serves as an internal control. (B) Quantitation of the RT-PCR results. The values are the averages ± SD from three independent experiments.

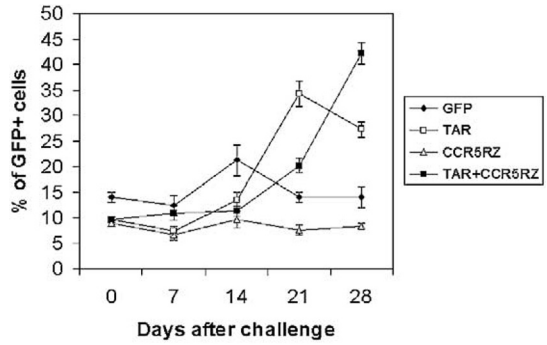

FIG. 6. Selective survival of primary T cells after challenge with HIV-1. Primary T cells were transduced with lentiviral vectors with pHIV-7-GFP, pHIV-CCR5RZ-GFP, pHIV-U16TAR-GFP, or pHIV-U16TAR-CCR5RZ-GFP. After 24 h, the cells were challenged with the HIV-1 strain JR-FL at an m.o.i. of 0.01. On the days indicated, one-half of the cells were removed and the cultures fed with fresh medium as described under Materials and Methods. This procedure allows free virus to continue infecting cells. Percentages of EGFP-positive cells in the total cell population were determined by FACS analyses at the indicated days after the initial HIV-1 infection. The values are averages of two independent experiments.

CD34$^+$-derived monocytes under conditions of HIV-1 challenge.

CCR5 is an ideal cellular target for ribozyme- [26] or siRNA-mediated down-regulation [65] since naturally occurring homozygous deletions do not appear to have a deleterious effect on hematopoiesis. There is of course a danger in utilizing only CCR5 as a target in an HIV-1 gene therapy setting since the switch to CXCR4 usage could negate any therapeutic effect. Therefore cloning the VA1-CCR5 ribozyme with one or more anti-HIV-1 inhibitory RNAs should provide further protection against CXCR4 viruses, as well as enhancing protection from M-tropic viruses. In the present study we find that the lentiviral vector-delivered VA1-CCR5 ribozyme can down-regulate CCR5 expression by as much as 70% in cultured cells, but this on its own does not provide complete protection against viral infection [26]. On the other hand, interaction of Tat with the TAR element is essential for efficient transcription of all HIV transcripts, both accessory and structural [66]. We have previously demonstrated that a nucleolar-localized TAR decoy can provide strong inhibition of HIV-1 infection when transduced into cultured T cells with a retroviral vector [27]. We now demonstrate effective expression of this nucleolar-localizing TAR decoy from the backbone of our lentiviral vector, and perhaps more importantly, when combined with the VA1-CCR5RZ, it provides both potent inhibition of HIV-1 infection and a marked survival advantage of primary PBMCs and monocytes under continuous challenge by HIV-1 (Figs. 6 and 7).

RNAi has recently been demonstrated to be a potent antiviral mechanism in a variety of studies targeting HIV-1 and other viruses [45,47,49,50,52,53,67–70]. Functional delivery of shRNA expression constructs using lentiviral vectors has recently been demonstrated for CCR5

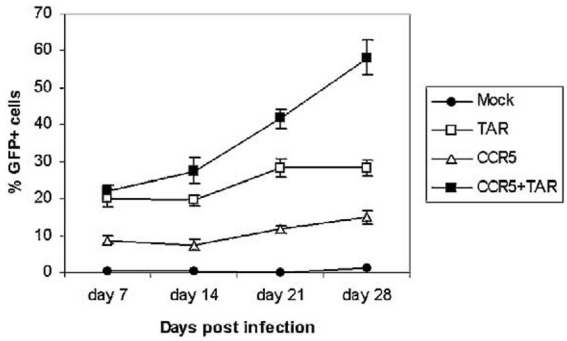

FIG. 7. Selective survival of CD34$^+$-derived monocytes challenged with HIV-1. CD34$^+$ cells were transduced with lentiviral vectors expressing the anti-CCR5 ribozyme, U16TAR decoy, or both, followed by challenge with the HIV-1 JR-FL at an m.o.i. of 0.01. On the days indicated, one-half of the cells were removed and the cultures fed with fresh medium as described under Materials and Methods. This procedure allows free virus to continue infecting the cells. Percentages of EGFP-positive cells in the total cell population were determined by FACS analyses at the indicated days after the initial HIV-1 infection. The values are averages of two independent experiments.

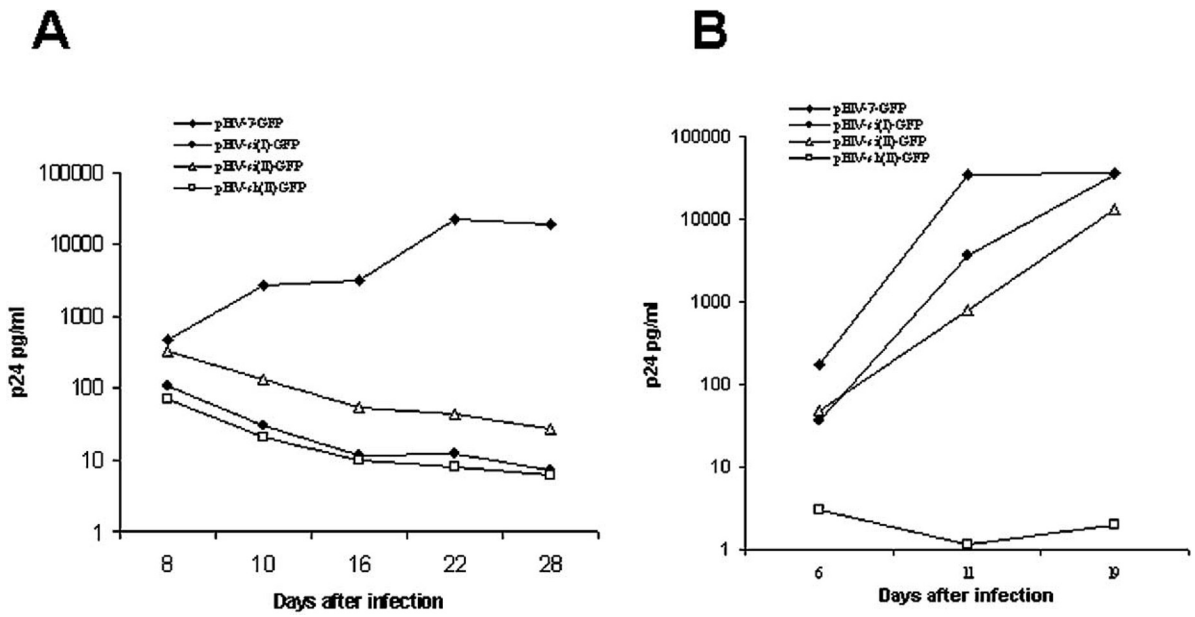

FIG. 8. Inhibition of HIV-1 replication in PBMCs expressing anti-*tat* and -*rev* siRNAs. (A) PBMCs were transduced with pHIV-7-GFP, pHIV-si(I)-GFP, pHIV-si(II)-GFP, or pHIV-sh(II)-GFP. Twenty-four hours later, the cells were infected with the HIV-1 strain JR-FL at an m.o.i. of 0.01. Culture supernatants were collected at the days indicated. The HIV-encoded p24 antigen was determined by ELISA. (B) PBMCs challenged with HIV-1 strain IIIB. The experiment procedures and the symbols are the same as in A.

[65]. In this study, we demonstrate functional expression and inhibition of HIV-1 strains JR-FL and IIIB using siRNAs targeting *tat/rev* and *rev* (Fig. 8). The shRNA appears to be more potent than the individually expressed sense and antisense in the HIV-1 IIIB challenge assay (Fig. 8B). Nevertheless, we have routinely been able to obtain good anti-HIV activity with the individually expressed siRNAs when the challenge virus is JRFL (Fig. 8A) and have also obtained good inhibition of HIV-1 IIIB when lower multiplicities of the challenge virus were used (data not presented). Nevertheless, the differences in effectiveness are interesting. Aside from the fact that the local concentration of sense and antisense siRNAs is higher when they are expressed as a hairpin relative to individual transcription units, other factors such as better nuclear to cytoplasmic transport and coupling with the microRNA pathway may also be involved. We are currently carrying out experiments to understand the bases for these differences in potency better.

A major concern about lentiviral vector gene therapy is the safety issue. These vectors have a propensity to integrate into active genes [71], although there has not been a direct link between lentiviral integration and host gene expression abnormalities. Nevertheless, this is a concern. It should be pointed out that the lentiviral vectors described in our study have inactive LTR promoters, and the only transcripts emanating from these vectors are from those that are inserted within the body of the vector. Obviously for gene therapy the CMV-EGFP gene would be eliminated. Other safety issues are centered around the possibility that replication-competent lentivirus might arise during the packaging process. The constructs used in our study lack all viral proteins required for viral replication, and the four-plasmid packaging system greatly reduces the probability that a replication-competent recombinant can arise during the packaging process. Since our constructs rely entirely on expression of Pol III transcripts, which efficiently terminate in a string of U's at the 3′ terminus of the transcripts, there is little likelihood that readthrough transcription into adjacent genes will take place following integration of these vectors. The safety issue is currently being addressed in a rigorous fashion using *in utero* gene transfer in fetal rhesus macaques [72,73] in collaboration with Dr. Alice Tarantal. To date three monkeys transduced with our lentiviral vectors expressing the U16TAR decoy and VA1-CCR5 ribozyme have come to term and there have been no adverse effects on hematopoiesis despite robust marking of progenitor cells (data not presented).

Finally, our demonstration that both PBMCs and $CD34^+$-derived monocytes transduced with the combination of the U16TAR/VA1-CCR5 ribozyme have selective survival advantages under conditions of HIV-1 challenge is critical in thinking about how to use gene therapy for the treatment of HIV-1 infection. Since the Pol III cassettes are relatively small, it should be possible to increase further the number of transcription units in a single vector. Our future goal is to attempt to combine the VA1-

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CCR5 ribozyme, U16 TAR decoy, and one or more shRNAs in the same lentiviral vector. Such a combination, if feasible, should provide marked resistance to HIV-1 and also hinder the generation of escape mutants. The constitutive expression of these two therapeutic RNAs has no apparent toxicities. It remains to be determined whether long-term expression of siRNAs will have toxicity. The constructs and vectors described in this study will now allow us to address this important question.

MATERIALS AND METHODS

Plasmid construction. The hammerhead ribozyme designed for cleaving CCR5 along with the adenoviral VA1 promoter was excised from pBSVACCR5RZ [26] with XhoI and SacI, rendered blunt ended with T4 DNA polymerase, and cloned into the lentiviral vector pHIV-7-GFP (Fig. 2A) at the BamHI site (DNA polymerase I, Klenow fragment filled in) immediately upstream of the CMV promoter–EGFP insert. This generated pHIV-CCR5RZ(F)-GFP and pHIV-CCR5RZ(R)-GFP in both forward and reverse orientations, respectively (Fig. 2B). The U16TAR cloned in pTz/U6+1 [27,54] under the U6 promoter (U6-U16TAR) was excised from pTz/U6+1/U16TAR with BamHI and XbaI and ligated to BamHI-linearized pHIV-7-GFP. This was followed by filling in the XbaI and the remaining vector BamHI site with DNA polymerase I (Klenow). The resultant products were religated, resulting in pHIV-U16TAR(F)-GFP and pHIV-U16TAR(R)-GFP in the forward and reverse orientations, respectively. To generate the lentiviral vectors expressing both the anti-CCR5 ribozyme and the U16TAR decoy, the U6-U16TAR cassette was excised from pHIV-U16TAR(F)-GFP by BamHI, filled in, and cloned into the NotI site of pHIV-CCR5RZ(F)-GFP and pHIV-CCR5RZ(R)-GFP, generating pHIV-U16TAR(F)-CCR5RZ(F)-GFP, pHIV-U16TAR(R)-CCR5RZ(F), pHIV-CCR5RZ(R)-U16TAR(F)-GFP, and pHIV-CCR5RZ(R)-U16TAR(R)-GFP as depicted in Fig. 2B.

The siRNA expression cassettes for target site I or site II of *rev* mRNA were excised from the pTZ U6+1-based vectors [52] at the *Eco*RI and *Hin*dIII restriction sites, blunt-end treated with Klenow fragment, and inserted into the blunt-ended *Bam*HI site in pHIV-7-GFP. Transcription orientations for antisense and sense sequences of the siRNAs were the same as for CMV-EGFP. A hairpin form of the siRNA expression cassette against site II of *rev* (designated sh(II)) was constructed by PCR with one primer complementary to the sequence upstream of the U6 promoter and another primer that covers the antisense, loop, sense sequences of the siRNA and downstream of the U6 promoter [55]. The PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, CA) and subsequently cloned into the *Bam*HI site of pHIV-7-GFP.

Cell lines and cell culture. HT1080 and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. The 293 cell line (American Type Culture Collection) was maintained in Eagle's minimum essential medium supplemented with 10% FBS. The human T cell lines PM1 [56] and CEM were maintained in RPMI 1640 medium supplemented with 10% FBS. The HOS-CD4.CCR5 cell line (obtained from the AIDS Research and Reference Reagent Program) was maintained in DMEM supplemented with 10% FBS and 1 μg/ml Puromycin.

Vector production. 293T cells were cultured until they reached 80% confluency in a 100-mm culture dish. Fifteen micrograms of lentiviral vector with the appropriate insert, 15 μg of pCHGP-2, 5 μg of pCMV-G, and 5 μg of pCMV-Rev were cotransfected into 293T cells using the calcium phosphate precipitation procedure [57]. The packaging system is shown in Fig. 2A [58]. Six hours after transfection, the culture medium was replaced. The culture supernatants were collected at 24 and 36 h after transfection. The supernatants were pooled together, passed through a 0.45-μm filter, concentrated by ultracentrifugation, and stored at −80°C until use. Vector titers were determined by transduction of HT1080 cells and assayed for EGFP expression using flow cytometry. The vectors were free of replication-competent lentivirus as determined by both RT-PCR and p24 antigen assays.

Transduction of target cells. To transduce 293 cells and HOS-CD4.CCR5 cells, 1×10^5 cells were seeded in a 6-well plate 16 h prior to transduction. Lentiviral vectors were added at an m.o.i. of 8 in the presence of Polybrene at 8 μg/ml. The culture medium was replaced 24 h after transduction. To transduce PM1 and CEM cells, 2×10^5 cells were placed in a 15-ml centrifuge tube with 1 ml culture medium in the presence of lentiviral vector at an m.o.i. of 8 and 8 μg/ml Polybrene. Following centrifugation at 2000 rpm for 1 h, the cells were transferred into a 24-well culture plate and after 24 h the culture medium was replaced. To transduce primary T lymphocytes, mononuclear cells (MNCs) were isolated by Ficoll–Paque gradient (Pharmacia, Piscataway, NJ). The recovered MNCs were cultured in RPMI 1640 medium supplemented with 10% FBS in the culture dish pretreated with antibodies against CD3 and CD28 (BD PharMingen, San Diego, CA). Two days after stimulation, the cells were transduced with lentiviral vectors at an m.o.i. of 10 and cultured in Retronectin- (Biowhittaker, Walkersville, MD) pretreated plates for 4 h. The transduced cells were washed and placed back into CD3/CD28 antibody-coated plates in the culture medium supplemented with 5 U/ml IL-2. For transduction of $CD34^+$ cells, $CD34^+$ stem cells were enriched from umbilical cord blood by anti-$CD34^+$ antibody-coupled magnetic beads (Miltenyi Biotech, Auburn, CA). The purity of $CD34^+$ cells was above 90% as determined by FACS analysis. After overnight culture in Iscove's modified Dulbecco's medium (IMDM) supplemented with 20% FBS, IL-3 (10 ng/ml), IL-6 (10 ng/ml), and SCF (10 ng/ml) in fibronectin-treated plates, the cells were transduced with lentiviral vectors at an m.o.i. of 20.

Northern blot analyses. RNA extracts were prepared using the RNA STAT-60 reagent (Tel-Test "B"; Friendswood, TX) according to the manufacturer's protocol. Fifteen micrograms of total RNA was separated by electrophoresis in a 6 or 8% polyacrylamide gel containing 7 M urea. The RNA was electroblotted onto a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and the membrane was hybridized with γ-^{32}P-labeled oligodeoxynucleotide probes complementary to the anti-CCR5 ribozyme, the U16TAR decoy, or the antisense siRNAs. A probe specific for U6 snRNA was used as an internal control.

RT-PCR assay. Total RNA was extracted from cells using the RNA STAT-60 reagent followed by digestion with RNase-free DNase I to remove residual DNA. cDNA synthesis was carried out with an oligo(dT) primer using MuLV reverse transcriptase (GIBCO BRL) at 42°C for 45 min. The PCR primers used for amplifying the CCR5 mRNA were 5′-TGTGTTTGCTTTA-AAAGCC-3′ (sense) and 5′-TAAGCCTCACAGCCCTGTG-3′ (antisense) [59]. PCRs were initiated at 95°C for 4 min, followed by 28 cycles of 95°C for 40 s, 56°C for 40 s, and 72°C for 1 min. The β-actin mRNA was also amplified as an internal standard.

HIV-1 challenge. Twenty-four hours after transduction, 1×10^6 primary T cells were infected with HIV-1 strain JR-FL or IIIB at an m.o.i. of 0.01. After overnight incubation, the cells were washed three times with Hanks' balanced salts solution (HBSS) and cultured in medium with 50% R10 (RPMI 1640 + 10% FBS) and 50% Acticyte (BIO-E, St. Paul, MN). On a weekly basis, 50% of the cells were removed for survival advantage experiments and the remaining cells were refed with Acticyte. Cell culture supernatant was also collected at various days for HIV-1 p24 antigen analysis. The p24 values were determined with the Coulter HIV-1 p24 antigen assay (Beckman Coulter, Brea, CA) according to the manufacturer's instructions. For challenges of $CD34^+$ cells, lentiviral vector-transduced $CD34^+$ cells were propagated in IMDM containing 20% FBS, IL-3 (10 ng/ml), IL-6 (10 ng/ml), and SCF (10 ng/ml) on irradiated human stroma cells for 1 week. $CD34^+$ cells (1×10^6) were exposed to the JR-FL strain of HIV-1 at an m.o.i. of 0.01 overnight. The infected cells were washed four times with HBSS and the cultures were continued in the medium containing the cytokines. Selective survival assays of transduced $CD34^+$ cells were carried out as described for PBMCs above.

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