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Optimization and Functional Effects of Stable Short Hairpin RNA Expression in Primary Human Lymphocytes via Lentiviral Vectors

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Specific, potent, and sustained short hairpin RNA (shRNA)-mediated gene silencing is crucial for the successful application of RNA interference technology to therapeutic interventions. We examined the effects of shRNA expression in primary human lymphocytes (PBLs) using lentiviral vectors bearing different RNA polymerase III promoters. We found that the U6 promoter is more efficient than the H1 promoter for shRNA expression and for reducing expression of CCR5 in PBLs. However, shRNA expression from the U6 promoter resulted in a gradual decline of the transduced cell populations. With one CCR5 shRNA this decline could be attributed to elevated apoptosis but another CCR5 shRNA that caused cytotoxicity did not show evidence of apoptosis, suggesting sequence-specific mechanisms for cytotoxicity. In contrast to the U6 promoter, PBLs transduced by vectors expressing shRNAs from the H1 promoter could be maintained without major cytotoxic effects. Since a lower level of shRNA expression appears to be advantageous to maintaining the shRNA-transduced population, lentiviral vectors bearing the H1 promoter are more suitable for stable transduction and expression of shRNA in primary human T lymphocytes. Our results suggest that functional shRNA screens should include tests for both potency and adverse metabolic effects upon primary cells.

Key Words: lentiviral vector, shRNA, RNAi, CCR5, primary T lymphocytes, H1 RNA polymerase III promoter, U6 RNA polymerase promoter

INTRODUCTION

RNA interference (RNAi) using short interfering RNAs (siRNAs) to inhibit specific gene expression is a powerful and promising technology for both basic research and therapeutic intervention. Using a highly conserved cellular machinery, siRNAs recognize cognate mRNAs and induce sequence-specific mRNA degradation [1]. Initially, synthetic double-stranded siRNAs, approximately 21 nucleotides long, were transiently transfected into mammalian cells to mediate specific RNAi silencing while avoiding nonspecific global inhibition of gene expression by the interferon response [2]. Subsequently, short hairpin RNAs (shRNAs) were devised to be transcribed by RNA polymerase III promoters in mammalian cells [3–7]. shRNAs are processed into siRNA duplexes by a cellular enzyme called Dicer [8]. The processed siRNA duplexes are subsequently incorporated into RNA-induced silencing complexes to guide target mRNA degradation.

A number of vector systems have been reported to mediate stable transduction and expression of shRNAs in mammalian cells [9]. Among them, lentiviral vectors have the ability to transduce nondividing cells stably through integration of the vector DNA into the genome [10]. RNA polymerase III promoters, most commonly the H1 and U6 promoters, have been incorporated into the lentiviral vectors for stable expression of shRNAs [11–15]. We and others have utilized such vectors in various

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experimental systems to analyze gene function and to inhibit expression of viral and cellular genes [11–17].

Several recent publications have shown some adverse effects of expressing shRNAs in mammalian cells. First, Fish *et al*. observed significant nonspecific cytotoxic effects and up-regulation of an interferon-responsive gene, oligoadenylate synthase-1 (*OAS1*), in shRNA-transduced cells [18]. They also reported that target gene silencing could not be maintained in extended cell culture despite persistent marker gene expression from the lentiviral vector in their shRNA-transduced cells. Later, several reports described type I interferon responses against si/shRNAs in mammalian cells, even though the length of the si/shRNA duplexes was less than 30 bp [19,20]. Moreover, some si/shRNA constructs induce down-regulation of noncognate target genes with less than 100% sequence homology, resulting in global alterations in gene expression profiles termed off-target gene effects [21–23]. These and possibly other adverse outcomes may diminish the therapeutic potential of the lentiviral vector-mediated shRNA expression system. Thus, it is important to investigate the functional consequences of shRNA expression from lentiviral vectors in mammalian cells and identify optimal conditions to achieve stable silencing of specific genes without unintended effects.

In this study, we systematically examined the effectiveness of gene silencing by shRNAs expressed from two commonly used H1 and U6 RNA polymerase III promoters. Further, we studied the long-term effects of lentiviral vector-mediated shRNA expression in human primary peripheral blood-derived T lymphocytes (PBLs) cultured *ex vivo* and in a NOD/SCID-hu PBL mouse model *in vivo*. We obtained a marked reduction of chemokine (C-C motif) receptor 5 (CCR5) expression in the T lymphocytes using the U6 promoter *ex vivo*; however, this was accompanied by a gradual decline in the positively transduced cell population and elevated levels of apoptosis. We also injected transduced PBLs into NOD/SCID mice to examine long-term maintenance of RNAi silencing *in vivo*. Consistent with the *ex vivo* observations, the U6 promoter vector-transduced population diminished in the mice. However, these adverse effects on cell persistence could be prevented by using H1, a transcriptionally weaker RNA polymerase III promoter [24], thereby reducing the shRNA expression level.

Results and Discussion

U6 Promoter-Driven shRNA Expression Is More Potent Than That of the H1 Promoter for Reducing CCR5

To determine which RNA polymerase III promoter is optimal for driving shRNA expression from a lentiviral vector, we compared directly the effectiveness of the U6 and H1 promoters in reducing CCR5 expression in primary human T lymphocytes. We designed H1- or U6-driven shRNA expression cassettes in such a way that identical shRNA transcripts were produced from the integrated lentiviral vectors (see Materials and Methods). We used our previously reported shRNA targeting nucleotides 186–205 of the CCR5 coding sequence [designated CCR5-shRNA (186)] [12] and a new shRNA targeting nucleotides 13–32 [designated CCR5-shRNA (13)]. As controls, we included an shRNA against firefly luciferase or a null expression cassette that did not produce a functional shRNA. As previously reported, the FG12 vector also expresses the enhanced green fluorescent protein (EGFP) marker under the human UbiC internal promoter for tracking transduced cells [12].

We isolated human PBLs from leukopacks, stimulated them with phytohemagglutinin (PHA) for 2 days, and transduced them with various lentiviral constructs at an m.o.i. of 5. Subsequently we cultured transduced PBLs in the presence of human interleukin-2 (IL-2) and harvested them at day 7 posttransduction for FACS analysis of CCR5 expression. Because the percentage of the EGFP-positive (transduced) cell population in each culture was slightly different, we measured CCR5 expression as the percentage of CCR5-positive cells within the $EGFP^+$ population (Fig. 1). The U6 promoter-driven CCR5-shRNA (186) and CCR5-shRNA (13) reduced the fraction of CCR5-expressing cells 10- and 25-fold, respectively, compared to the Luc shRNA controls (Figs. 1a, 1c, and 1e). In contrast, the H1 promoter-driven shRNA-CCR5 (186) and CCR5 (13) reduced CCR5 expression 3- and 6.5-fold, respectively (Figs. 1b, 1d, and 1f). Similarly, the mean fluorescence intensity as an estimate of CCR5 expression was decreased approximately 5-fold in cells expressing CCR5 shRNA (186) and (13) by the U6 promoter versus 2- and 4-fold by CCR5 shRNA (186) and (13), respectively by the H1 promoter (these represent minimum estimates since the majority of cells are at a background level of fluorescence). Thus, the U6 promoter is more potent than the H1 promoter for driving shRNA-mediated silencing of CCR5 in primary PBLs.

The U6 Promoter Expresses Higher Levels of shRNA Than the H1 Promoter

To examine whether CCR5 silencing correlates with the level of shRNA expression, we measured the level of shRNA transcripts in CCR5-shRNA (186)- and CCR5-shRNA (13)-transduced human CEM.NKR-CCR5 cells by Northern blot analysis. We isolated total cellular RNA from the CCR5-shRNA-transduced cells at 14 days posttransduction and detected the antisense strand of processed CCR5-shRNAs by specific radiolabeled oligonucleotide probes. We found that levels of shRNA transcripts were at least sixfold higher in the U6 promoter vector-transduced cells than in the H1 promoter vector-transduced cells based on Phosphorimager quantitation (Fig. 2). The higher level of shRNA transcripts from the U6 promoter correlates with a greater reduction in CCR5 expression (Supplementary Fig. 1).

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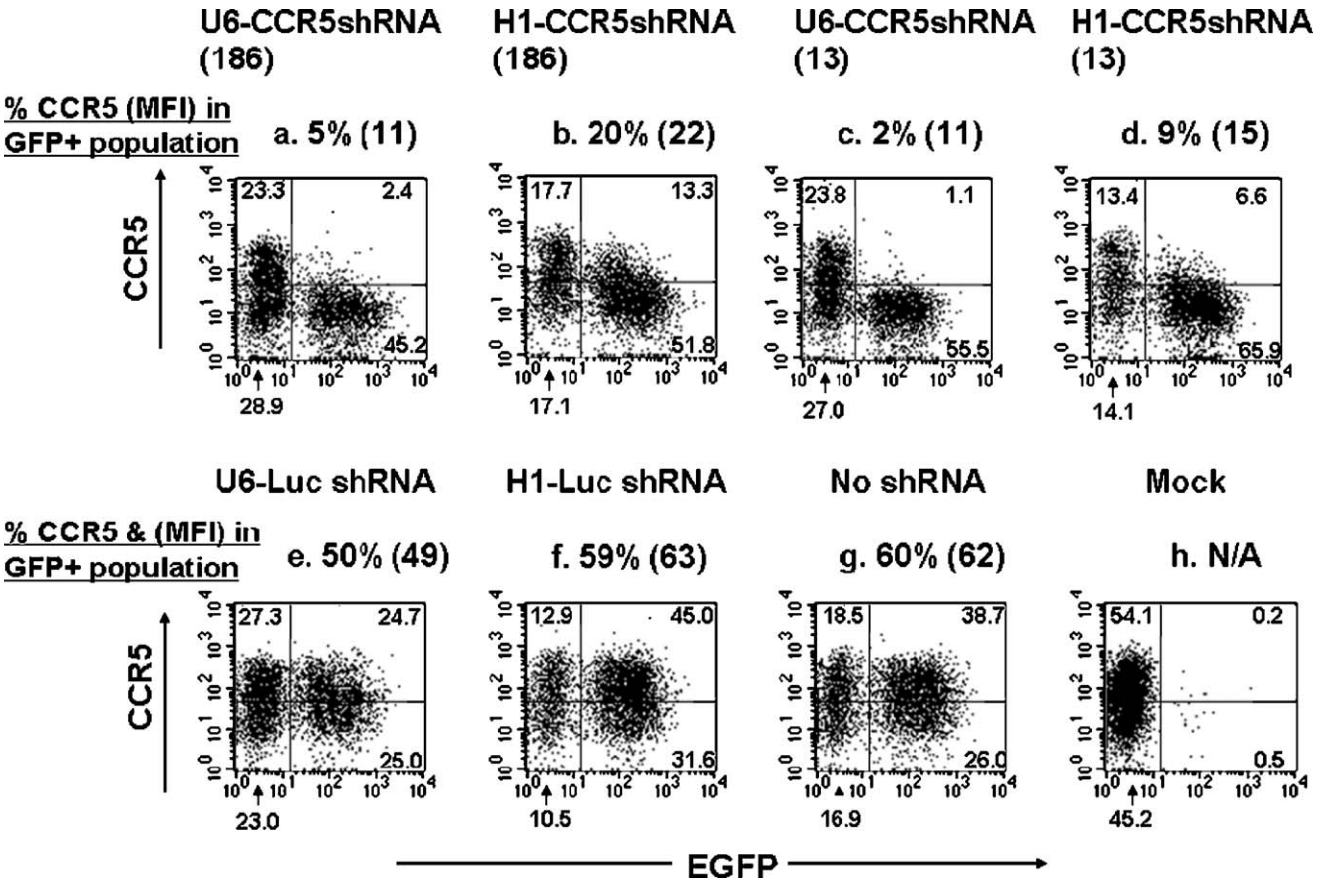


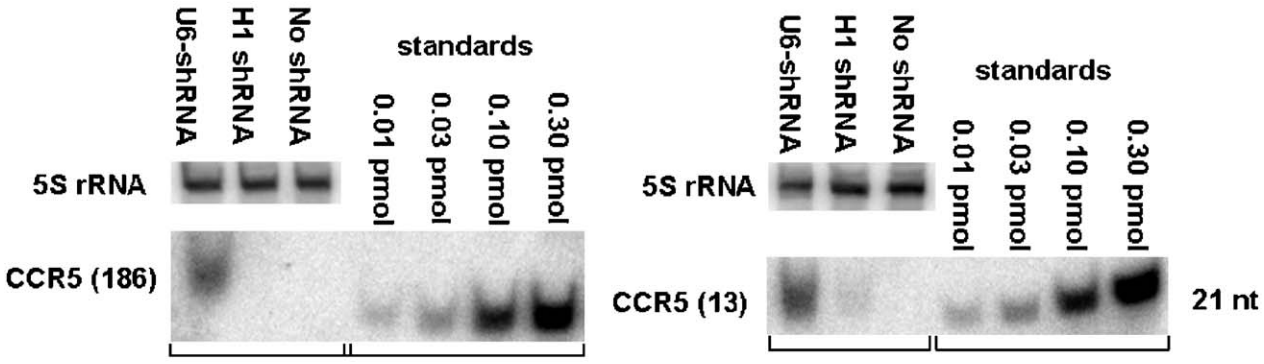
FIG. 1. The U6 promoter is more potent than the H1 promoter in driving the expression of shRNAs in primary human PBLs. PHA/IL-2-stimulated PBLs were transduced at a m.o.i. of 5 with lentiviral vectors expressing various shRNAs under the control of a U6 or an H1 promoter as indicated. The transduced cells were further cultured in IL-2-containing medium for 7 days before flow-cytometric analysis for CCR5 and EGFP expression. The *x* axis indicates GFP expression; the *y* axis indicates CCR5 expression of the cell populations in the live cell gate. Each graph shows the CCR5/EGFP plot from the U6- and the H1-shRNA vector-transduced PBLs for each shRNA against CCR5 (186) (a, b), CCR5 (13) (c, d), or firefly luciferase (e, f). (g) Cells transduced with vector without shRNA expression unit (no shRNA) and (h) mock-transduced PBLs are also shown. The quadrant lines were defined by mock transduction (h) and isotype-control stain. Percentage CCR5 positivity and mean fluorescence intensity (MFI) of CCR5 in the EGFP-positive population are shown at the top. The percentage of cells in each quadrant is also indicated. The data are representative of four independent experiments. The U6-shRNA vector-transduced PBLs had a greater CCR5 reduction than H1-shRNA vector-transduced PBLs in the four experiments ($P = 0.004$) by the Wilcoxon rank sum test.

This result, combined with the CCR5 reduction data in primary PBL (Fig. 1), suggests that a higher level of shRNA expression induces a more effective reduction in CCR5 expression. Passage of transduced CEM.NKR-CCR5 cells over 4 weeks showed stable EGFP expression and no apparent cytotoxicities (data not shown), in contrast to what we observed in primary PBL (see below).

Stable Maintenance of Transduced PBLs Using H1 Promoter Driven shRNA Vectors

To investigate the persistence of shRNA expression by lentiviral vector and the long-term effects of shRNA expression on cell growth, we examined the kinetics of total cell numbers in the U6- and H1-promoter shRNA-transduced PBLs at three time points during culture (Fig. 3A). We noted a significant decline in total cell numbers in all U6-shRNA-transduced cell cultures. The presence or absence of target mRNA in shRNA-transduced cells did not affect the decline of cell number, since it occurred for shRNAs against CCR5 (with target) and firefly luciferase and LacZ (without target). Next, we measured the percentage of $EGFP^+$ cells in each vector-transduced PBL culture (Fig. 3B). Consistently, the percentage $EGFP^+$ population in all U6-shRNA-transduced PBLs declined more rapidly over time. In contrast, the $EGFP^+$ population persisted in the H1-shRNA-transduced PBLs although a slight decline was observed at the later time points.

To examine further whether the loss of $EGFP^+$ cells was a consequence of a loss of vector-transduced cells, we



	CCR5 (186)		CCR5 (13)	
	U6 promoter	H1 promoter	U6 promoter	H1 promoter
Absolute quantity of shRNA (pmol)	0.058	<0.01	0.059	<0.01
Fold over H1 promoter driven shRNA	>5.8		>5.9	

FIG. 2. The U6 promoter expresses higher levels of shRNA than the H1 promoter. CEM.NKR-CCR5 cells were transduced at an m.o.i. of 1 with lentiviral vectors bearing either the U6 or the H1 promoter. As CEM.NKR-CCR5 cells are fivefold more susceptible to lentiviral vector transduction than primary PBLs, we used an m.o.i. of 1 to achieve a similar percentage EGFP expression between CEM.NKR-CCR5 and primary PBLs. The transduced cells were cultured for 12 days and total RNA was isolated from the cells. The total RNA was blotted with probes against the CCR5-shRNA (186) or (13). Known amounts of synthetic CCR5-siRNAs (186) or (13) were blotted in parallel as standards for the quantitation. The data are representative of two independent experiments.

measured the average copy number of vector DNA in transduced cells at the three time points. We isolated total cellular DNA from the transduced PBLs and subjected it to real-time quantitative DNA PCR analysis using a primer pair specific to the vector sequence. We used human β-globin quantitative PCR to normalize the amount of cellular DNA in each sample. Table 1 shows normalized vector copy numbers per cell at each time point. Corresponding to the loss of total cell counts and the $EGFP^+$ population, the amount of vector DNA decreased more rapidly in the U6-shRNA-transduced PBLs relative to the H1-shRNA or no-shRNA vector-transduced PBLs. The average copy number of the U6-shRNA vector DNA in transduced PBLs decreased approximately 10-fold between day 4 and day 12, much greater than the drop in total cell counts (Fig. 3A) and the percentage of $EGFP^+$ cells (Fig. 3B). This might reflect a preferential loss of cells with multiple copies of vector integration in which higher levels of shRNA expression might result in a greater impact on cell growth.

IFN-Responsive Genes Were Not Triggered in shRNA Transduced PBLs

We examined the type I interferon (IFN) response in the shRNA-transduced PBLs as a possible cause of the decline in U6-shRNA-transduced PBL populations. Activation of interferon responses has been reported to cause attenuated cell growth and apoptosis [25]. Although the stem sequences of our shRNAs are designed to yield a 19-bp RNA duplex after Dicer processing, several recent reports suggested that some shRNAs with less than 30-bp stems can activate IFN-responsive genes [19,20,26]. To analyze whether IFN-responsive genes were induced in the U6-shRNA- and H1-shRNA-transduced PBLs, we measured the mRNA levels for *OAS1* and interferon stimulated gene-15 kDa (*ISG15*), two common IFN-responsive genes. We isolated total cellular RNA from transduced PBLs 4 days posttransduction and subjected it to Northern blot analysis using probes for *OAS1* and *ISG15* sequences (Fig. 4). Although poly(I:C) electroporation induced both *OAS1* and *ISG15* gene expression up to 9.5-fold, we could

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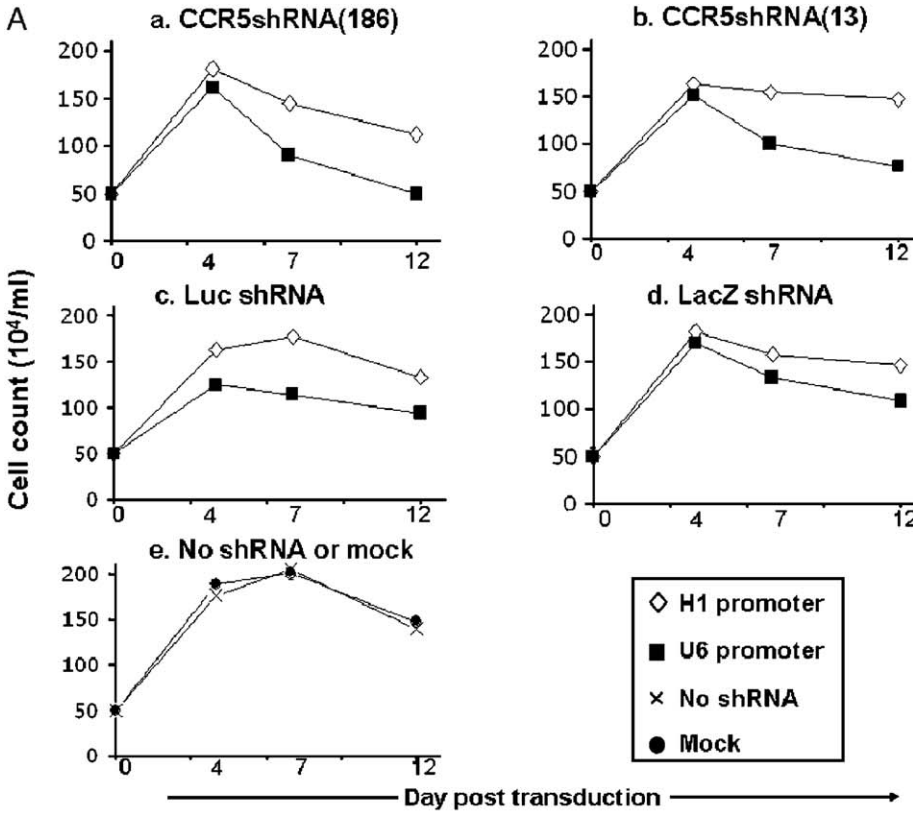

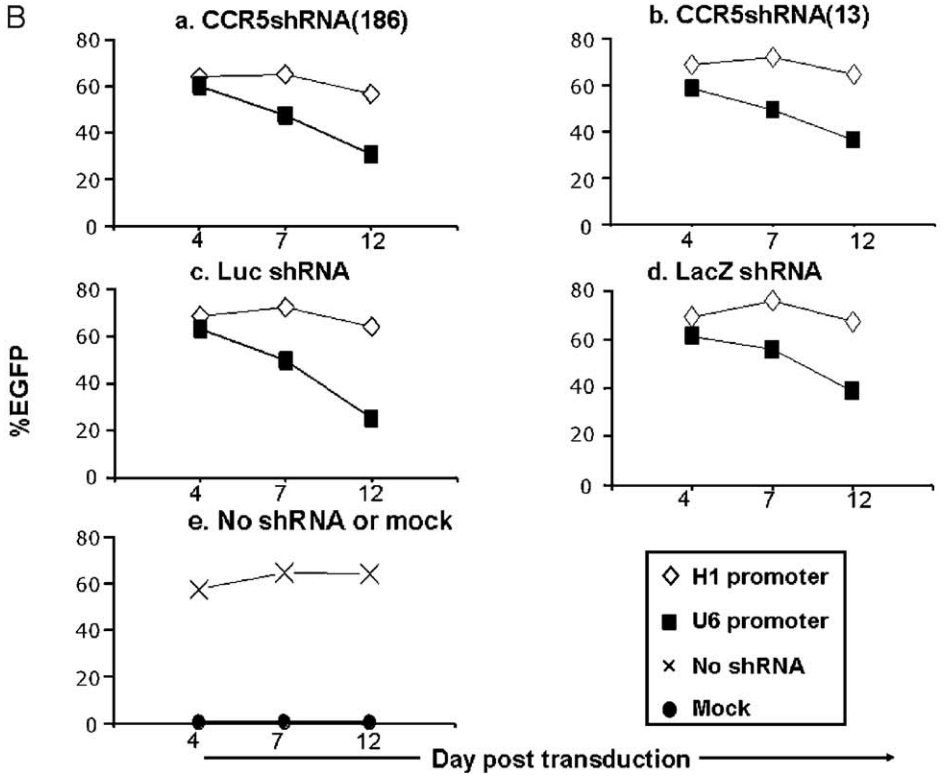

TABLE 1: Loss of vector DNA copy number in U6-promoter-driven shRNA-expressing primary PBLs

Vector	Vector DNA copies per cell		
	Day 4	Day 7	Day 12
H1-CCR5-shRNA (186)	3.8	2.5	2.6
U6-CCR5-shRNA (186)	4.8	2.3	0.3
H1-CCR5-shRNA (13)	2.5	2.4	1.2
U6-CCR5-shRNA (13)	3.3	1.6	0.4
H1-luc-shRNA	3.2	1.7	1.0
U6-luc-shRNA	3.5	1.0	0.4
H1-lacZ-shRNA	3.7	3.0	1.9
U6-lacZ-shRNA	3.8	2.1	0.4
No shRNA	1.5	0.8	0.6
Mock	<0.01	<0.01	<0.01

Total DNA was isolated from various lentiviral vector-transduced PBLs at days 4, 7, and 12 posttransduction and subjected to real-time DNA PCR analysis for lentiviral vector DNA sequence and human β-globin. The vector DNA copy number was normalized to human β-globin copy number in each sample and expressed as vector DNA copies per cell.

not detect significant up-regulation of *OAS1* or *ISG15* expression in the shRNA-transduced cells. Based on these observations, we conclude that the loss of U6-shRNA-transduced PBLs was unlikely to be related to a general IFN-mediated response.

U6 Promoter-Driven shRNA Expression Can Cause Elevated Apoptosis in Primary PBLs

We next examined whether the cells underwent accelerated apoptosis. We harvested transduced PBLs at 7 and 12 days posttransduction and stained them with Annexin V to measure apoptosis. We calculated the percentage of $EGFP^+$ cells that were also Annexin V positive as measured by FACS analysis and compared the Annexin V positivity of U6- and H1-shRNA-transduced cells (Table 2). We observed two- to fourfold higher percentages of Annexin V^+ cells in populations transduced by U6-shRNA vectors against CCR5 shRNA (186), firefly luciferase, and LacZ in several independent experiments.

We examined the loss of EGFP-positive cells and apoptotic effects in more detail by infection at different multiplicities of viral vector and analysis at more time points. Consistent with the results of Fig. 3B, we observed declines in the percentage of $EGFP^+$ cells when CCR5 shRNA was driven by the U6 promoter, but not when CCR5 shRNA was driven by the H1 promoter (Supplementary Fig. 2A). At the higher m.o.i. both CCR5 shRNAs showed slightly greater cytotoxic effects. In replicate samples over time we observed significant apoptosis only with CCR5-shRNA (186) and not with CCR5-shRNA (13), despite reductions in percentage $EGFP^+$ cells over time with both shRNAs (Supplementary Fig. 2B). We observed slightly more apoptosis over time in cells transduced at the higher m.o.i. We observed no significant apoptosis for either shRNA when expressed using the H1 promoter. These results suggest that high level expression of shRNA from the U6 promoter may lead to elevated apoptosis in human PBLs, providing one explanation for the decline in the U6-shRNA-transduced cells. However, since CCR5-shRNA (13) expression from the U6 promoter did lead to a decline in $EGFP^+$ cells, but was not associated with elevated apoptosis, apoptosis appears not to be the only underlying mechanism for cytotoxicity.

IFN-γ Production Was Not Altered by shRNA Expression

Although we observed increased apoptosis in the U6-shRNA-transduced PBLs, the majority of transduced PBLs still remained Annexin V negative and maintained reduced CCR5 expression levels, indicating that the GFP^+/Annexin V^- cell population might still be functionally intact. To test this hypothesis, we examined whether the transduced PBLs were capable of responding to a T cell stimulus and producing the effector cytokine IFN-γ. Seven and 12 days posttransduction, we stimulated the transduced PBLs with phorbol 12-myristate 13-acetate (PMA)/ionomycin for 4 h in the presence of brefeldin A. We permeabilized the cells and stained them for intracellular IFN-γ. We calculated the percentage of EGFP-positive cells that were also IFN-γ positive (Supplementary Table1). We observed high levels of IFN-γ production with H1- and U6-shRNA and control vector-transduced cells, and there was no significant difference in the frequency of the IFN-γ^+ population among them. Thus, this study shows that at least one key T cell functional response was intact in the shRNA-transduced PBLs.

H1 Promoter-Driven shRNA Reduced CCR5 in a NOD/SCID-hu PBL Model

The life span of PHA/IL-2-activated PBLs restricts *in vivo* experiments to a rather short time period (12 days). To investigate further the long-term effects of shRNA expression in human primary PBLs, we utilized a NOD/SCID-hu

FIG. 3. (A) Slower cell growth of PBLs expressing U6-promoter-driven shRNA. Vector-transduced PBLs were plated at 50×10^4/ml and counted at 4, 7, and 12 days posttransduction. After each cell count, cells were divided 1:3 and fresh medium was added. Each graph shows the cell count ($\times 10^4$/ml) from the H1- and the U6-shRNA vector-transduced PBLs for shRNA against CCR5 (186) (a), CCR5 (13) (b), firefly luciferase (c), or LacZ (d). No shRNA indicates cells transduced with vector that did not express shRNA. (B) Decline in EGFP-positive population in primary PBLs expressing U6-promoter-driven shRNA. EGFP populations were monitored by FACS analysis in the shRNA-transduced PBLs at days 4, 7, and 12 post-vector transduction. Each graph shows percentage EGFP positivity from the H1- and the U6-shRNA vector-transduced PBLs for shRNA against CCR5 (186) (a), CCR5 (13) (b), firefly luciferase (c), or LacZ (d). No shRNA indicates cells transduced with vector that did not express shRNA. The data are representative of four independent experiments. The U6-shRNA vector-transduced PBL had an overall greater decrease (greater negative slope) than H1-shRNA vector-transduced PBL in the four experiments ($P = 0.0001$) by the Wilcoxon rank sum test.

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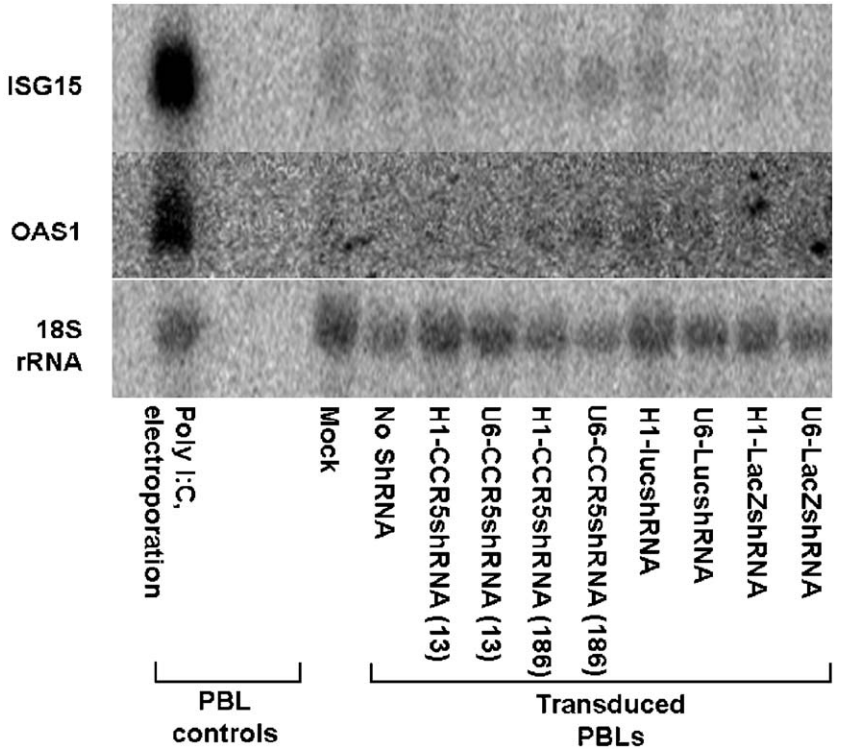

FIG. 4. IFN-induced genes *OAS1* and *ISG15* are not up-regulated in shRNA-transduced PBLs. RNA was isolated from transduced PBLs 4 days posttransduction and subjected to Northern blot analysis using specific probes for human *OAS1* and *ISG15*. As a control, poly(I:C) was transfected into PBLs by electroporation. The data are representative of two independent experiments.

PBL model [27]. The absence of a functional immune system in NOD/SCID mice permits long-term engraftment (30 days) of human PBLs in the mouse blood circulation. In this setting, the engrafted human PBLs are also highly stimulated by mouse antigens, resulting in a high level of CCR5 expression and enabling us to examine shRNA-mediated CCR5 silencing. We injected H1- or U6-CCR5-shRNA (13)-transduced PBLs into the mice through an intraperitoneal route and analyzed peripheral blood at 30 days postinjection for CCR5 and EGFP expression within the human CD45-positive lymphocyte population. Representative FACS data are shown in Fig. 5; quantitative analysis of the FACS results from all animals is summarized in Table 3. In four of four animals reconstituted with PBLs transduced with a no-shRNA vector control, 38–56% of human CD45-positive cells expressed EGFP (Table 3). Likewise, 27 and 30% of human CD45-positive cells were $EGFP^+$ in two of four animals injected with the human PBLs transduced with H1-CCR5-shRNA (13). The other two animals of this group did not have human CD45-positive cells due to the lack of human cell engraftment. In contrast, four of four animals injected with the U6-CCR5-shRNA (13)-transduced human PBLs had less than 1.5% $EGFP^+$ cells in the engrafted human CD45-positive cells. Thus, in agreement with the *ex vivo* experiments, U6-driven shRNA expression resulted in a remarkably low percentage of EGFP-expressing cells, presumably due to a cell growth disadvantage.

To examine whether CCR5 expression was reduced in the H1-CCR5-shRNA (13)-transduced PBLs, we measured the level of CCR5 reduction in the $EGFP^+$ population.

TABLE 2: U6-promoter-driven shRNA expression causes elevated apoptosis in primary PBLs

	% Annexin V^+ cells in the $EGFP^+$ population		
	Experiment 1	Experiment 2	
Vector	Day 7	Day 7	Day 12
H1-CCR5-shRNA (186)	3.8	4.2	6.7
U6-CCR5-shRNA (186)	10.2	13.0	24.1
H1-CCR5-shRNA (13)	1.6	3.5	5.5
U6-CCR5-shRNA (13)	3.2	3.9	5.5
H1-luc-shRNA	3.0	7.3	7.2
U6-luc-shRNA	7.0	11.3	11.8
H1-lacZ-shRNA	4.4	9.4	7.6
U6-lacZ-shRNA	10.0	9.3	13.9
No shRNA	3.5	6.6	6.9

Cells were transduced with lentiviral vectors expressing shRNAs under the control of the U6 or H1 promoter as described for Fig. 1 and under Materials and Methods. At the indicated times posttransduction, cells were analyzed by FACS for apoptotic cells (Annexin V positive) in the EGFP-expressing population. The numbers indicate the percentage of cells that are both Annexin V and EGFP positive. Different donor PBLs were used for experiments 1 and 2.

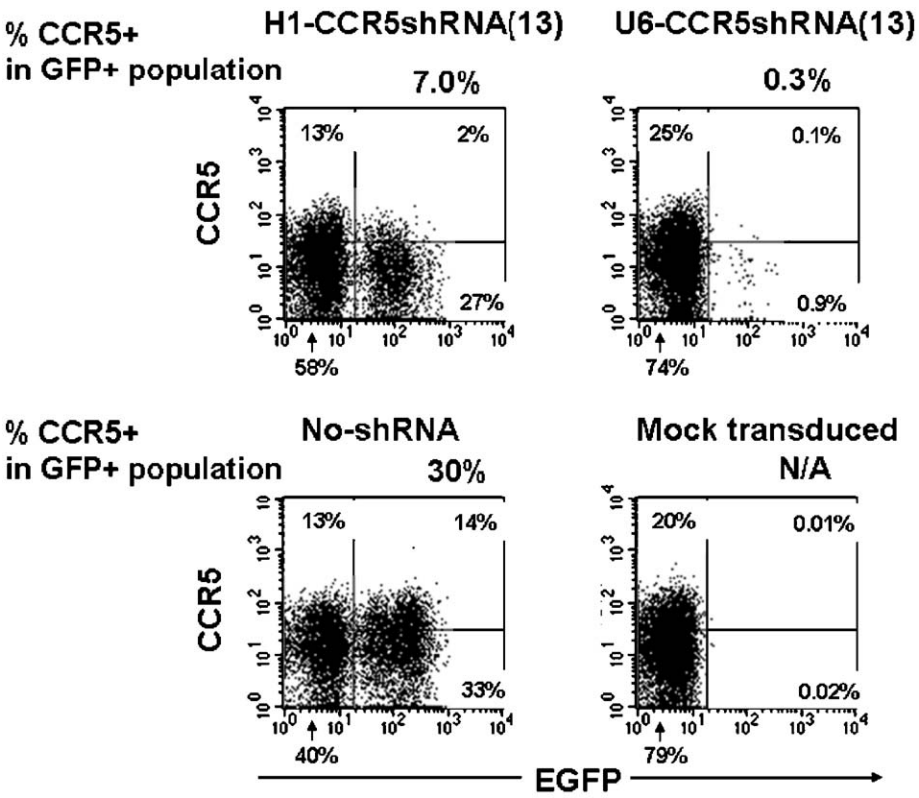

FIG. 5. Reduction of CCR5 in NOD SCID-Hu PBL model. PBLs (1×10^7) transduced with the lentiviral vector expressing shRNA as indicated were injected into NOD SCID mice. Peripheral blood was obtained from the mice at 30 days postinjection and treated with red blood cell lysis buffer and the remaining cells were analyzed by FACS for human CD45 and CCR5 expression. The human CD45-positive population was gated and analyzed for EGFP and CCR5 expression. The percentage CCR5 positivity in the EGFP-positive population was calculated and is shown at the top. The percentage of cells in each quadrant is also shown. The data shown are a representative flow-cytometric analysis data set. N/A, not applicable.

CCR5 expression was reduced two- to fourfold in the H1 promoter-driven shRNA-transduced PBLs relative to the no-shRNA vector-transduced PBLs (compare animals 1 and 2 with animals 9, 10, 11, and 12 in Table 3 and the representative data shown in Fig. 5). Taken together, we conclude that the H1 promoter is more suitable than the U6 promoter for stable shRNA expression against CCR5 in human lymphocytes *in vivo*.

In summary, we systematically examined the functional consequences of shRNA-lentiviral vector transduction and the expression of shRNA in human primary peripheral blood lymphocytes in *in vivo* culture and in a NOD/SCID-hu PBL mouse model. Although U6-promoter-driven shRNA inhibited CCR5 to a greater extent than H1-promoter-driven shRNA, we observed a gradual decline in the $EGFP^+$ population and total cell numbers in the U6-shRNA-expressing PBLs, accompanied by a decline in the copy number of U6-shRNA vector DNA. For one of the CCR5 shRNAs these effects could be at least partly attributed to elevated apoptotic cell death.

With H1-promoter-driven shRNA, the cell number and $EGFP^+$ population were steadily maintained with only minor decreases in levels of transduced cells, and no increase in apoptosis was detected. These results suggest that a high level of continuous shRNA expression might have adverse effects on cell growth in human primary PBLs, partly due to increased apoptosis. Based on these observations, we conclude that it is important to control the level of shRNA expression to maintain stable gene silencing. We thus propose that the H1 promoter is a better choice for lentiviral-shRNA expression in human primary PBLs. However, the lower level of shRNA expression by the H1 promoter resulted in inefficient reduction of CCR5 gene expression; to achieve efficient reduction of target gene expression, highly potent shRNAs must be identified. Based on our results and other published results, the potency of an shRNA is largely dependent on its primary sequence [12,28]. Several rational design and library screening methods have been proposed for selecting efficient shRNA sequence for gene silencing [28–30], but it remains important to screen a large number of potential shRNAs. We anticipate further systematic screening will yield even more potent shRNAs against CCR5 but potential cyto-

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TABLE 3: Survival of shRNA-transduced PBLs in NOD/SCID-hu PBL mice

Vector	% GFP$^+$ before injection	Mouse	% human CD45$^+$	% GFP$^+$ in human CD45$^+$	% CCR5$^+$ and GFP$^+$ in human CD45$^+$	% CCR5$^-$ and GFP$^+$ in human CD45$^+$	% CCR5$^+$ in GFP$^+$
H1-CCR5-shRNA (13)	73.5	1	84.8	27.7	1.9	25.8	7.0
		2	73.6	22.1	2.0	20.1	9.0
		3	0.02	<0.01			
		4	0.5	<0.01			
U6-CCR5-shRNA (13)	62.3	5	88.3	0.4	0.1	0.3	
		6	63.9	0.8	0.1	0.7	
		7	40.5	0.6	0.2	0.3	
		8	38.2	1.5	0.3	1.2	
No shRNA	73.4	9	84.2	45.9	7.9	38.0	17.0
		10	32.9	56.3	12.1	44.2	22.0
		11	90.8	38.6	11.7	27.0	30.0
		12	81.7	45.5	10.1	35.5	22.0
Mock	0.2	13	75.4	<0.01			
		14	81.0	<0.01			
		15	9.6	<0.01			
		16	1.2	<0.01			
	Mock injection	17	0.7	<0.01			

Lentiviral vector-transduced PBLs (1×10^7) were injected into NOD/SCID mice. Peripheral blood was obtained from the mice at 30 days postinjection and treated with red blood cell lysis buffer and the remaining cells were analyzed by FACS for human CD45 and CCR5 expression. Mouse 17 did not receive human PBLs (mock injection).

toxic effects must be evaluated empirically even when a weaker promoter such as H1 is utilized.

RNAi technology has been widely applied for reducing specific target gene expression and may be used as a therapeutic intervention in the future. For silencing CCR5 as a therapy for AIDS, stable shRNA expression is critical in preventing HIV-1 entry. Although lentiviral vectors are well suited to stable expression of shRNA in mammalian cells, high-level shRNA expression from the human U6 promoter caused a progressive loss of the transduced human PBLs. Such undesirable effects will impede the advancement of this technology into clinical applications. In accordance with our observations, several groups reported cytotoxicity associated with shRNA expression in mammalian cells [18,31,32]. In contrast, other reports did not report any obvious toxicities from U6-promoter-driven shRNAs [24,33,34]. Given the variability of data in different cell types and experimental settings, we propose that the best approach to identifying suitable shRNAs for any given target gene is to select multiple shRNA sequences having the desired functional effect and test them empirically in the context of different expression cassettes for adverse metabolic effects upon cells. Furthermore, given that cytotoxicity was observed only in primary PBLs and not in a T cell line (CEM.NKR-CCR5), it is important to design assays using primary cells.

It is noteworthy that two shRNAs directed against CCR5 show distinct phenotypes. Both CCR5-shRNA (186) and CCR5-shRNA (13) are potent at suppressing CCR5 expression. Both shRNAs induce cytotoxic effects as evidenced by loss of EGFP$^+$ transduced cells over time. Yet, only CCR5-shRNA (186) shows obvious proapoptotic effects. These results indicate that there are likely to be multiple mechanisms for cytotoxicity induced by shRNAs and that these effects are likely to be dependent on the primary sequence of the shRNA.

At present, we do not fully understand the mechanisms behind elevated cytotoxicity in the U6-shRNA-transduced PBLs. Induction of the IFN response has been reported to cause slower cell growth [25], and several reports have shown induction of type I interferon response by introducing si/shRNA into mammalian cells [19,20,26]. On the other hand, other reports suggest no induction of type I interferon responses by shRNAs [2]. In our study, we did not detect significant induction of IFN-responsive genes *OAS1* and *ISG15*. Bridge *et al*. initially reported that U6- and H1-promoter-driven shRNA expression from a lentiviral vector caused over 500-fold induction of *OAS1* gene expression in human lung fibroblasts when two vectors were used concomitantly [19]. However, in a subsequent publication, they showed the IFN response was caused by a flawed vector design; removing additional AA dinucleotides and preserving the transcriptional start site with a consensus C/G sequence at position −1/+1 prevented the *OAS1* induction [35]. Our shRNA design is a 19-nt stem coupled with the intact cognate C/G at the U6 promoter transcriptional start site. This could explain why we did not observe activation of IFN-responsive genes.

Another possible mechanism underlying the adverse effects caused by the overexpression of shRNA from a

strong promoter is that it may interfere with endogenous microRNA (miRNA) pathways. Mammalian cells express more than 200 endogenous miRNAs, which are structurally similar to shRNA [36]. Some miRNAs appear to play important roles in regulating differentiation and development processes at the posttranscriptional level [37,38]. The same cellular machinery is utilized for shRNA processing and maturation [39,40]. We postulate that overexpression of shRNA from strong promoters might compete with endogenous miRNAs for limiting cellular factors, thus interfering with normal miRNA function. Consistent with this notion, Yi *et al*. recently reported that both miRNAs and shRNAs were stabilized and exported to the cytoplasm by karyopherin exportin 5. Exportin 5 expression was shown to be at very low abundance and appeared be one of the limiting factors for RNAi process in human cells [41].

Another possible mechanism is off-target gene silencing by shRNAs. Microarray analysis data revealed that si/shRNA expression may cause a global change in gene expression profiles [21–23]. In addition, several reports showed that si/shRNA causes down-regulation of non-cognate genes with some degree of sequence homology. The off-target effects were siRNA sequence specific, in both concentration-dependent and concentration-independent manners [21–23]. Although this study does not provide any direct evidence, it is possible that global changes in gene expression may cause adverse effects on cell growth.

While the RNAi technology holds great promise for potential therapeutic applications such as AIDS therapy, careful optimization of shRNA expression in target cells is necessary. We still do not fully understand the mechanisms underpinning the loss of transduced cells expressing high levels of shRNAs. Nevertheless, our results demonstrate that such an adverse effect can be avoided, and further studies should provide us with more information and bring us closer to applying RNAi technology in human clinical settings.

Materials and Methods

Vector construction. Human H1-RNA polymerase III promoter DNA (−241 to −9) was amplified from HEK-293 genomic DNA with primers 5′-CCATGGAATTCGAACGCTGACGTC-3′ and 5′-GCAAGCTTTTG-GATCCGTGGTCTCATACAGAACTTATAAGATTCCC-3′, containing a novel 3′ *Bam*HI site for the insertion of shRNA sequences at the +1 position of the H1 transcript. The PCR fragment was cloned into the pBS-SKII plasmid (Stratagene) between the *Xba*I and *Xho*I sites (designated pBS-H1-3). The cloning of a human U6-RNA pol III promoter, the sequence of the CCR5-shRNA (186), and the shRNA against lacZ were previously described [12]. The new CCR5-shRNA (13) was designed to target nucleotide position 13–31 of the human CCR5 coding sequence (GenBank Accession No. U57840). The firefly luciferase shRNA was designed to target nucleotide position 1915–1933 of the firefly luciferase gene. Each shRNA was inserted downstream of a human U6 promoter in the FG12 lentiviral vector as previously described [12]. To construct H1-promoter-driven shRNA expression cassettes, two complementary DNA oligos were synthesized, annealed, and inserted between the *Bam*HI and *Xho*I sites of pBS-hH1-3, immediately downstream of an H1 promoter: 5′-GATCCCC$(N)_{19}$TTCAAGAGA$(N)_{19}$TTTTTC-3′, 3′-GGG$(N)_{19}$AAGTTCTCT$(N)_{19}$AAAAAGAGCT-5′. The 19-nt sense and reverse complementary targeting sequences are highlighted in bold. The shRNA cassette also features a TTCAAGAGA loop situated between the sense and the reverse complementary targeting sequences and a TTTTT terminator at the 3′ end. To construct the shRNA-expressing lentiviral vectors, the shRNA expression cassette was subcloned into the FG12 plasmid between the *Xba*I and *Xho*I sites. The resulting plasmids were confirmed by restriction enzyme digestion and DNA sequencing.

Lentiviral vector production. All vesicular stomatitis virus G-protein-pseudotyped lentiviral vector stocks were produced by calcium phosphate-mediated transient transfection of HEK-293T cells, as previously described [12,42].

Cell culture. CEM.NKR-CCR5 [43] (AIDS Research and Reference Reagent Program of the National Institutes of Health) were maintained in Iscove's MEM, 10% FCS. Human primary PBLs were isolated from leukopacks by Ficoll and cultured in RPMI 1640 medium, 20% FCS, with 2.5 μg of PHA, 100 units of penicillin, 100 μg/ml streptomycin for 2 days. After 2 days of PHA stimulation, PBLs were cultured in RPMI 1640 medium containing 20 units/ml IL-2.

Vector transduction. Human PBLs were isolated from leukopacks by Histopaque (Sigma) and cultured in RPMI 1640 medium, 20% FCS, with 2.5 μg of PHA (Murex Biotech, Dartford, UK)/100 units of penicillin/100 μg/ml streptomycin for 2 days. After 2 days of PHA stimulation, PBLs were used for lentiviral vector transduction. Briefly, 50×10^4 cells were incubated with various lentiviral vectors at an m.o.i. of 5 for 2 h in the presence of 8 μg/ml Polybrene. After the incubation, virus supernatants were removed and replaced with 1.5 ml of fresh RPMI 1640 medium, 20% FCS containing 20 units/ml IL-2 (Roche Molecular Biochemicals). The transduced cells were harvested 4, 7, and 12 days later and stained with APC-labeled mouse anti-human CCR5 mAb (2D7; PharMingen) or a mouse IgG2a/k isotype control (OX-35; PharMingen), according to the manufacturer's instructions. GFP and CCR5 expression was analyzed by FACS at multiple time points after transduction.

Real-time DNA PCR. DNA was isolated from lentiviral vector-transduced PBL by urea lysis, as previously described [44]. Quantitative, real-time DNA PCR was performed as previously described [45] using a primer/probe pair that amplifies cellular β-globin sequences and one that amplifies HIV-1 DNA (R-U5 of the LTR). All amplifications were performed on the ABI Prism 7700 (Applied Biosystems).

Northern blot analysis. Total RNA was isolated using Tri Reagent (Invitrogen). For small RNA blotting analysis, 50 μg of the RNA samples was resolved on a 10% denaturing polyacrylamide gel containing 50% urea. Subsequently the gel was transferred to a Hybond Plus membrane (Amersham) using TransBlotter (Bio-Rad). Hybridization was performed using ^{32}P-labeled oligonucleotide probe against the antisense strand of CCR5 (186) or (13). The membranes were also hybridized with a probe against 5S rRNA for loading control. To generate the standard curves for quantitation, known amounts of oligonucleotide of the corresponding antisense strand were loaded on the same gel. Radioactive signal was measured by PhosphoImager (Storm; Amersham). For regular Northern blot analysis, RNA samples were resolved on a 1% agarose gel and then transferred to a Genescreen membrane (NEN). Hybridization was performed using ^{32}P-labeled cDNA probes against human *OSA1* and *ISG15* in a sequential manner after the previous probe was stripped. The membrane was also hybridized with a oligonucleotide probe against 18S rRNA for loading control.

Annexin V staining. Lentiviral vector-transduced cells (1×10^5) were harvested from cell cultures and incubated with PE-conjugated anti-Annexin V monoclonal antibodies for 30 min according to the manufacturer's manual (BD Biosciences). The stained cells were analyzed immediately by FACS analysis using FACSCalibur (Becton–Dickinson).

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IFN-γ production and intracellular IFN-γ staining. Lentiviral vector-transduced cells were restimulated with 5 ng/ml PMA (Sigma–Aldrich) and 0.5 μg/ml ionomycin (Sigma–Aldrich), in the presence of 10 μg/ml brefeldin A (Sigma) for 4 h. After the incubation, the cells were fixed with 2% paraformaldehyde, permeabilized by saponin, and subjected to intracellular staining with PE-conjugated anti-human IFN-γ antibody.

NOD/SCID-hu PBL mouse model. NOD/LtSz-Prkdc scid/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in the BSL3 SCID mouse facility at UCLA. Mice were treated with 1 mg of TMβ-1 anti-mouse interleukin 2 receptor β chain monoclonal antibodies to deplete mouse NK cells 1 day before human PBL injection. Lentiviral vector-transduced PBLs (1×10^7 cells in 0.5 ml of RPMI 1640 containing 20% fetal calf serum per mouse) were injected into the peritoneal cavity. Mouse peripheral blood was obtained from the retro-orbital plexus 30 days post-cell injection. The blood was treated with red blood cell lysis buffer and remaining cells were subjected to monoclonal antibody staining for human CD45, CCR5.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ymthe.2006.05.015.

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