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ORIGINAL ARTICLE

Complete knockdown of CCR5 by lentiviral vector-expressed siRNAs and protection of transgenic macrophages against HIV-1 infection

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The CCR5 co-receptor is necessary for cellular entry by R5 tropic viral strains involved in primary HIV infection, but is dispensable for normal human physiology. Owing to its crucial role in HIV-1 infection, the CCR5 co-receptor has been the subject of many therapeutic approaches, including gene therapy. siRNA targeting was shown to be effective in downregulating CCR5 expression and conferring significant protection against HIV-1 in susceptible cells. However, complete knockdown of CCR5 expression has not been achieved and thus remains an elusive goal. In these studies, we identified new CCR5 siRNAs capable of achieving complete knockdown of the co-receptor expression. Our transfection studies have shown that longer 28-mer short hairpin siRNAs are very effective in gene downregulation as assessed by fluorescence-activated cell sorting and transcript quantitation by quantitative real-time polymerase chain reaction. These siRNAs conferred strong antiviral protection during viral challenge. To obtain stable expression, highly potent siRNA expression cassettes were introduced into lentiviral vectors. Similar high levels of CCR5 downregulation were observed in stably transduced cells with concomitant viral protection in cultured cell lines. To translate these results to a stem cell gene therapy setting, CD34 hematopoietic progenitor cells were transduced with lentiviral vectors to derive transgenic macrophages. The transgenic cells also exhibited high levels of CCR5 downregulation and viral resistance. With regard to Pol-III promoter-mediated siRNA expression, higher efficacies were obtained with U6-driven CCR5 siRNAs. However, in contrast to previous reports, no apparent cytotoxicities were observed in transgenic cells containing U6-driven siRNA constructs. Thus the above anti-CCR5 siRNAs are among the most effective demonstrated to date and are very promising candidates for clinical applications.
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Introduction

HIV-1 as the causative agent of AIDS continues to be a major public health threat as global infections continue to rise and current therapies do not completely cure the disease.[1,2] Gene therapy for HIV-1 infection is a potentially rewarding approach for long-term control of this chronic and fatal disease. So far, a number of promising intracellular immunization strategies have been employed using different molecules that act by a variety of mechanisms such as ribozymes, antisense constructs and siRNAs.[3–8] Of these, siRNA-based constructs have received considerable attention due to their target specificity and improved potency. siRNAs use the endogenous RNAi pathway for target recognition and gene silencing.[9–11] Numerous laboratories have tested a number of anti-HIV siRNAs targeted to both viral as well as essential cellular genes.[12–32] A number of siRNAs targeted to different viral genes have shown impressive gene downregulation and consequent viral inhibition. However, since siRNAs function with high target specificity, there remains the possibility for viral escape mutants to arise during prolonged treatment. In fact, recent studies have documented the generation of viral escape mutants against specific siRNAs.[33,34] In this regard, targeting essential cellular molecules that aid in viral replication is likely to yield more sustainable therapies. Many cellular molecules have been shown to be involved in HIV-1 infection and replication and offer potential roadblocks that can be exploited for inhibiting the viral life cycle at various stages. Inhibiting cellular molecules that aid in the viral infection is likely to afford broad protection against many strains of HIV-1 and decrease the chance of generating viral escape mutants.

Macrophages and helper T cells are the predominant host cells for HIV-1 replication.[35] Viral entry into these cells is mediated by the presence of essential cellular receptors.[36] The initial infection is established by macrophage tropic viruses (R5) that use the chemokine receptors CCR5 and CD4 for cellular entry. During the later stages of disease, T-cell tropic viruses (X4) use CXCR4 as co-receptor predominates.[36] Since HIV-1 co-receptors play a key role during early viral–cell

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interactions, they are attractive targets for many antiviral approaches. A segment of the human population containing a 32 bp deletion in the CCR5 gene was found to be resistant to infection by R5 tropic HIV-1.[37–39] Since these individuals lacking a functional CCR5 gene are apparently normal, CCR5 is an excellent candidate for gene knockdown to inhibit HIV-1 infection. Based on this rationale, this gene has been targeted in many studies to confer viral resistance.

Earlier studies used ribozymes targeting CCR5 to prevent HIV-1 entry both *in vitro* and *in vivo* in a SCID-hu mouse model, whereas more recent studies used siRNAs to downregulate CCR5 expression.[4–7,20,22–27,29] Both synthetic siRNAs delivered via transfection and endogenously expressed siRNAs via viral vectors have been employed. Although marked downregulation of CCR5 expression has been observed, complete silencing of co-receptor expression has yet to be achieved. Complete knockdown of CCR5 expression is critical as to not leave any residual co-receptor present for viral attachment. Recent developments and improvements in siRNA design have allowed derivation of improved siRNA constructs.[40–42]

In our present studies, we designed and evaluated a new generation of anti-CCR5 siRNAs to achieve complete knockdown of CCR5 expression. For long-term gene therapy to be successful, constitutive expression of anti-HIV transgenes needs to be achieved. Lentiviral vectors offer numerous advantages over other vector systems for gene delivery and expression. These include their ability to transduce both nondividing and dividing cells as well as being less prone to transgene silencing.[43] Lentiviral vectors are also capable of transducing CD34 hematopoietic stem cells (HSCs) which can give rise to all blood cell lineages, including T cells, macrophages and dendritic cells which are the main target cells of HIV-1 infection.[17,30,31,44–47] Accordingly, we have introduced a set of new and highly effective CCR5 siRNAs into lentiviral vectors for stable gene transfer. Here, we demonstrate that these siRNAs are highly potent in CCR5 downregulation and show high antiviral efficacy in transgenic macrophages derived from lentiviral vector-transduced CD34 HSCs.

Results

Downregulation of CCR5 expression in siRNA-transfected cells

Five 19-mer duplex synthetic siRNAs were initially tested by transfection of Ghost R5/X4/R3 cells at high concentrations (640 nM) (Figure 1). Previous experiments have shown that longer siRNAs derived by extending their sequence downstream are more potent effectors of RNAi than their shorter counterparts.[48] Therefore, testing of these initial 19-mer siRNAs was followed by evaluation of their longer short hairpin (sh) versions (Figure 1). Cells were analyzed by flow cytometry at 72 h post-transfection to determine the level of downregulation of CCR5 surface expression. As shown in Figure 2, the 19 bp duplex siRNAs showed various levels of CCR5 downregulation with #4, #5, #6, #7 and #B transfected cells showing expression levels of 71, 10, 9, 46 and 7%, respectively, translating into corresponding knockdown levels of 29, 90, 91, 54 and 93% (Figure 2). These values were determined in comparison to nontransfected con-

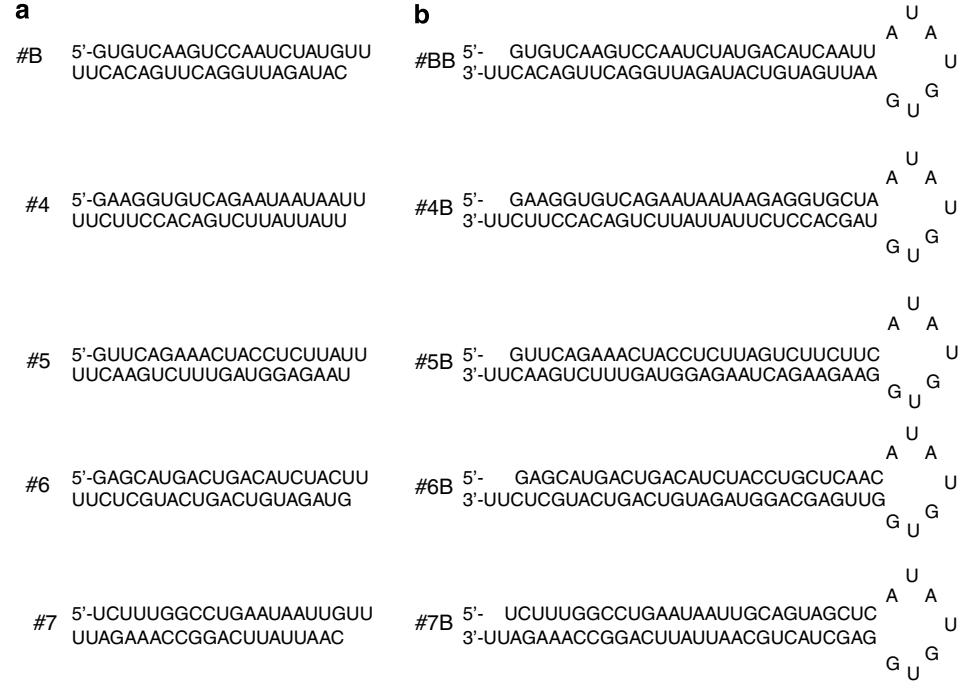


Figure 1 Sequences of CCR5 siRNA constructs: siRNAs targeted to CCR5 were designed by rational target sequence identification criteria. (**a**) siRNA sense and antisense strands containing two nucleotide uracil 3′ overhangs were annealed together to form 19-mer duplexes. (**b**) siRNA 28-mer sequences with the sense and antisense strands connected by the loop sequence AUAUGUG with a two-nucleotide uracil 3′ overhang. The 28mer siRNAs target the same region of the CCR5 mRNA as their 19-mer counterparts but were extended nine nucleotides downstream.

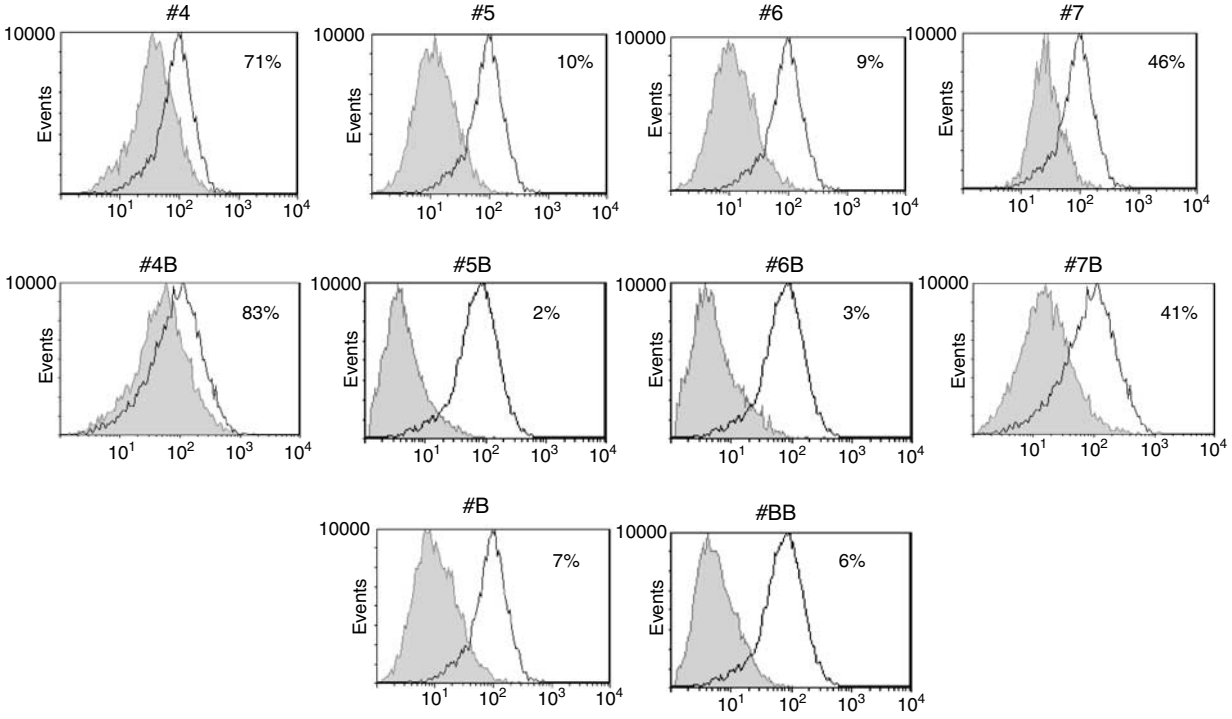


Figure 2 Downregulation of CCR5 co-receptor in siRNA-transfected Ghost R5/X4/R3 cells: cells were transfected with 640 nM of the respective siRNA. Seventy-two hours post-transfection, cells were analyzed for CCR5 cell surface expression by FACS. The percent CCR5 positive cells are indicated in comparison with control cells (unshaded areas). These results are representative of triplicate experiments.

trol cells (unshaded areas). As shown, #5, #6 and #B were more effective compared to #4 and #7. When the longer 28 bp hairpin versions were similarly tested, greater knockdown of CCR5 expression was achieved with the constructs #5B, #6B and #BB, showing respective knockdown levels of 98, 97 and 94%. The constructs #4B and #7B showed inefficient knockdown of CCR5 expression. Since the efficacies of CCR5 siRNAs #4, #7, #4B and #7B were relatively modest, they were omitted from further analysis and characterization.

SiRNAs at higher doses of transfection may be toxic and are prone to exhibit off-target effects.[49,50] Therefore, it is important to identify siRNAs that work efficiently at lower concentrations. We next proceeded to evaluate if the effective siRNAs #B, #5 and #6 and their longer sh versions retained their efficacy at lower concentrations. Tenfold decreasing concentrations of 64 and 6.4 nM were then evaluated for efficacy. Results showed that at 64 nM siRNA concentration, the efficacy was largely retained with the siRNAs #5, #6, #B, #5B, #6B and #BB showing $>90\%$ knockdown of CCR5 surface expression (Figure 3a). Even at 100-fold lower concentrations (6.4 nM), these siRNAs still significantly downregulated CCR5 surface expression (Figure 3b). Since the relative half-life of cell surface receptors varies considerably, fluorescence-activated cell sorting (FACS)-derived data may not truly reflect the actual gene knockdown conferred by siRNAs. Therefore, to more accurately quantitate CCR5 downregulation, quantitative real-time polymerase chain reaction (QRT-PCR) was performed to evaluate the levels of CCR5 transcripts. As shown in Figure 4a, complete knockdown ($>99\%$) of CCR5 transcript levels was achieved using constructs #5, #B, #5B, #6B and #BB at a concentration of 64 nM. Again, at relatively low levels of siRNA (6.4 nM), significant knockdown of CCR5 expression was observed (Figure 4b). Control, glyceraldehyde-6-phosphate (G-6-P) and transcript levels were similar for all samples tested (Figure 4c).

HIV-1 resistance of CCR5 siRNA-transfected cells

To determine if the observed downregulation of the essential co-receptor CCR5 conferred viral resistance, transfected Ghost R5/X4/R3 cells were challenged with R5-tropic HIV-1 BaL-1 at a multiplicity of infection (MOI) of 0.01. Viral p24 antigen levels at different days post-challenge were determined by enzyme-linked immunosorbent assay to quantify levels of HIV-1 resistance. Over a ninefold reduction in viral antigen levels was seen with #B, #5, #6, #BB, #5B and #6B transfected Ghost cells as compared to control nontransfected, unrelated transfected, #4 and #7 transfected cells (Figure 5).

Cytotoxicity of transfected siRNAs

Some siRNAs have been shown to be directly cytotoxic.[49,50] To determine if the above CCR5 siRNAs were toxic to cells upon transfection, cells were stained for the extracellular expression of phosphatidyl-serine suggesting apoptosis. CCR5 siRNA-transfected cells showed no dramatic increase in cell death, as analyzed by Annexin V staining. The cytotoxicity values (16.0–27.6%) were similar to that of mock-transfected control cells with lipofectamine alone (17.6%) and lower than siTOX-transfected cells (56.9%) (Figure 6). Cells transfected with the siRNA constructs #B and #BB showed the greatest level of cell death among the CCR5 constructs as indicated by 27.6 and 22.3% of cells staining with

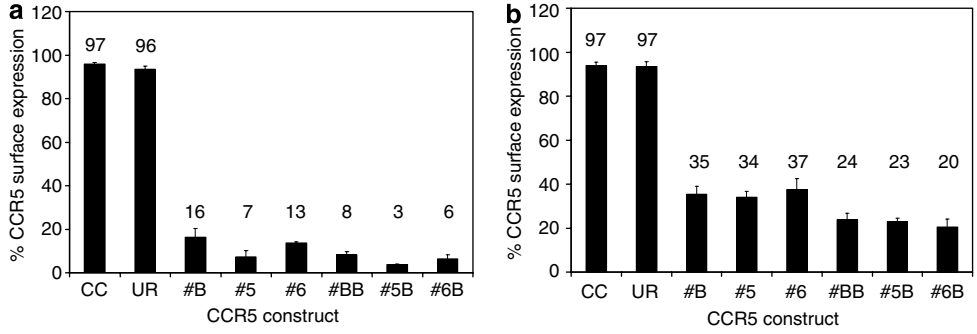
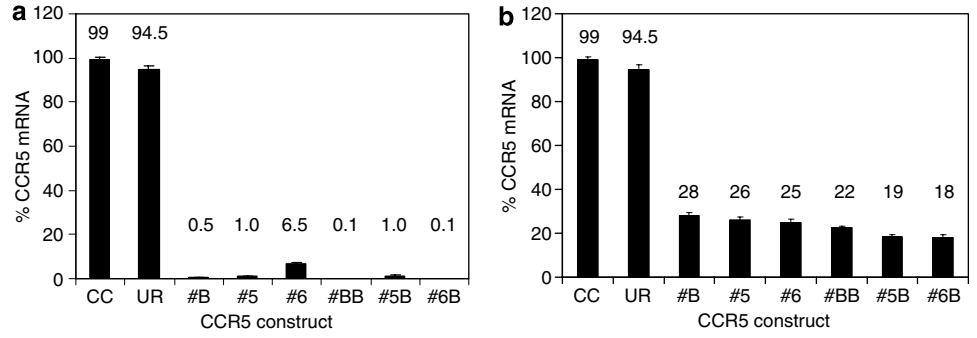


Figure 3 CCR5 co-receptor downregulation in siRNA-transfected Ghost R5/X4/R3 cells: cells were transfected with lower amounts of siRNAs than in Figure 1. (**a**) 64 nM or (**b**) 6.4 nM of respective siRNAs. Seventy-two hours post-transfection, cells were analyzed for CCR5 cell surface expression by FACS and the percent positive cells are indicated for each of the siRNA-treated cells. CC, control unmanipulated cells. UR, cells transfected with an unrelated siRNA. Other siRNAs are indicated below each bar. Data were obtained in triplicate.



c

shRNA	CC	UR	#B	#5	#6	#BB	#5B	#6B
C_T	19.9	18.5	19.6	19.5	19.8	19.6	19.7	19.3

Figure 4 QRT-PCR of CCR5 transcripts downregulated by chemically synthesized siRNAs. Cells were transfected with (**a**) 64 nm or (**b**) 6.4 nM of the respective siRNAs. Seventy-two hours post-transfection, intracellular CCR5 transcript levels were quantified by QRT-PCR to determine the level of CCR5 downregulation. Percent levels of CCR5 transcripts are indicated above the bar graphs. (**c**) The levels of G-6-P transcripts were used as an internal control by comparing threshold cycles (C_T) between samples. Data were obtained in triplicate.

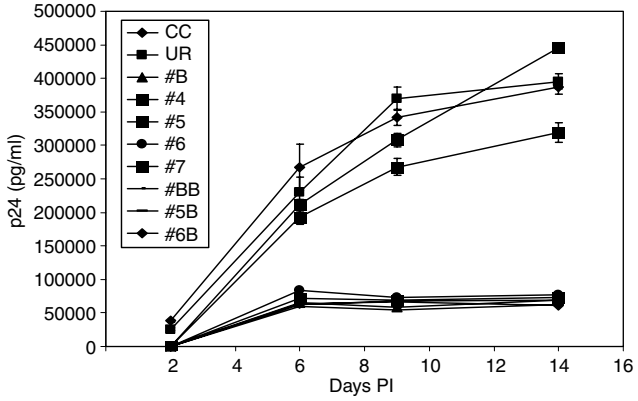


Figure 5 HIV-1 challenge of CCR5 siRNA-transfected Ghost R5/X4/R3 cells. Cells were transfected with 64 nM of the respective CCR5 siRNAs. Seventy-two hours post-transfection, cells were challenged with an R5-tropic BaL-1 strain of HIV-1 at an MOI of 0.01. On various days post-infection, cell culture supernatants were collected and analyzed for p24 antigen levels by ELISA. Data were obtained in triplicate.

Annexin V, respectively. These results demonstrated that these specific siRNAs did not have any cytotoxic effects detrimental to cell survival.

Downregulation of CCR5 expression in siRNA vector-transduced cells

Since the effect of transfected siRNAs is transient due to eventual degradation and dilution of siRNAs during cell division, constitutive expression using such methods as lentiviral transduction is necessary for long-term siRNA gene therapy. Thus, a major goal in these studies is to introduce highly effective CCR5 siRNAs into lentiviral constructs to achieve stable constitutive expression in transduced cells. Highly potent siRNA constructs, namely #B, #5, #6, #BB, #5B and #6B were therefore cloned individually into a third-generation lentiviral vector HIV7-GFP in which expression of the siRNA was driven by a Pol-III H1 promoter (Figure 7a). The sense and antisense synthetic duplex constructs, #B, #5 and #6, were converted into stem loop-structured shRNAs by connecting the two strands with the loop sequence UUCAAGAGA. The 28-mer constructs #BB, #5B and #6B retained their original design (Figure 1b) with the loop sequence AUAUGUG. Located downstream to the siRNA cassette is the reporter gene enhanced green fluorescent protein (EGFP), which is driven by a cytomegalovirus (CMV) promoter (Figure 7). The control vector, HIV7-GFP, contains only the reporter gene.

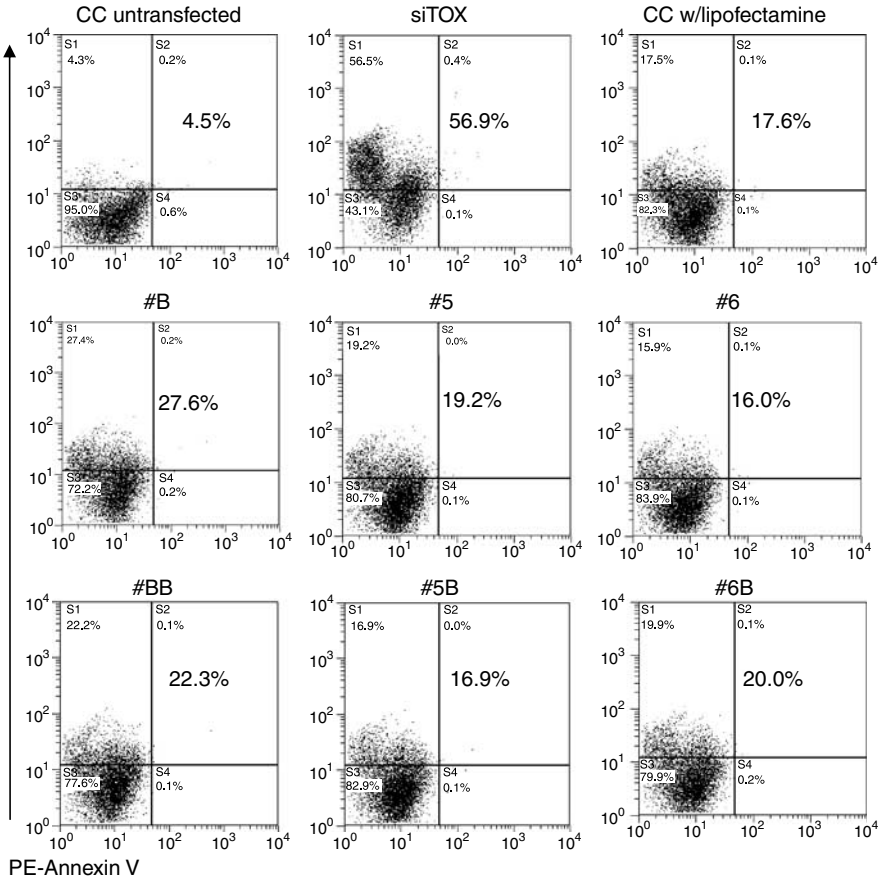


Figure 6 Cytotoxicity and IFN induction by CCR5 siRNAs Ghost R5/X4/R3 cells were transfected with the respective CCR5 siRNAs (640 nm). Forty-eight hours post-transfection, transfected cells were stained with a PE-conjugated Annexin V antibody and analyzed by FACS. These data were representative of triplicate experiments. Percentage cell death is shown in each panel.

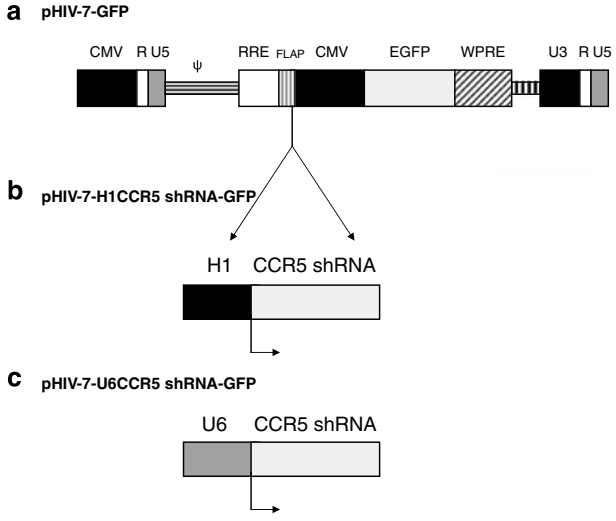


Figure 7 Lentiviral vector constructs: a third-generation lentiviral vector containing an EGFP reporter gene was used to generate the CCR5 siRNA constructs. (**a**) HIV7-GFP control vector. CCR5 shRNA constructs, #B, #5, #6, #BB, #5B and #6B were cloned individually upstream of the EGFP reporter gene under the control of the human Pol-III (**b**) H1 and (**c**) U6 promoters. CMV (cytomegalovirus immediate early promoter), RRE (Rev responsive element), EGFP (enhanced green fluorescent protein), WPRE (Woodchuck post-transcriptional regulatory element).

Ghost R5/X4/R3 cells constitutively expressing CCR5 on the cell surface were transduced with the individual lentiviral vectors containing different CCR5 siRNA constructs. Transduced cells were sorted by FACS based on EGFP expression to enrich for vector-containing cells. The enriched populations were then analyzed for CCR5 downregulation by FACS. Marked co-receptor downregulation was seen in all CCR5 siRNA vector-transduced cell populations as compared to nontransduced and HIV7-GFP vector-transduced cells (Figure 8a). The levels of downregulation ranged from 94 to 98% with the isotype control staining 2%. To confirm the data observed by FACS analysis, QRT-PCR was performed on CCR5 siRNA-transduced Ghost R5/X4/R3 cells. As seen in Figure 8c, substantial gene knockdown was observed for all CCR5 siRNA-transduced cells as compared to control cells. The levels of transcript knockdown observed ranged from 94–99%. The CCR5 construct #BB was the most efficient in downregulating CCR5 expression with transcript levels decreasing $>99\%$.

The above results showed the efficacy of CCR5 siRNAs in cultured cell lines. However, for stem cell-based gene therapy strategies to succeed in the long range, self-renewing HSCs are the ideal targets for lentiviral transduction. The resulting transgenic stem cells are expected to generate resistant progeny that will

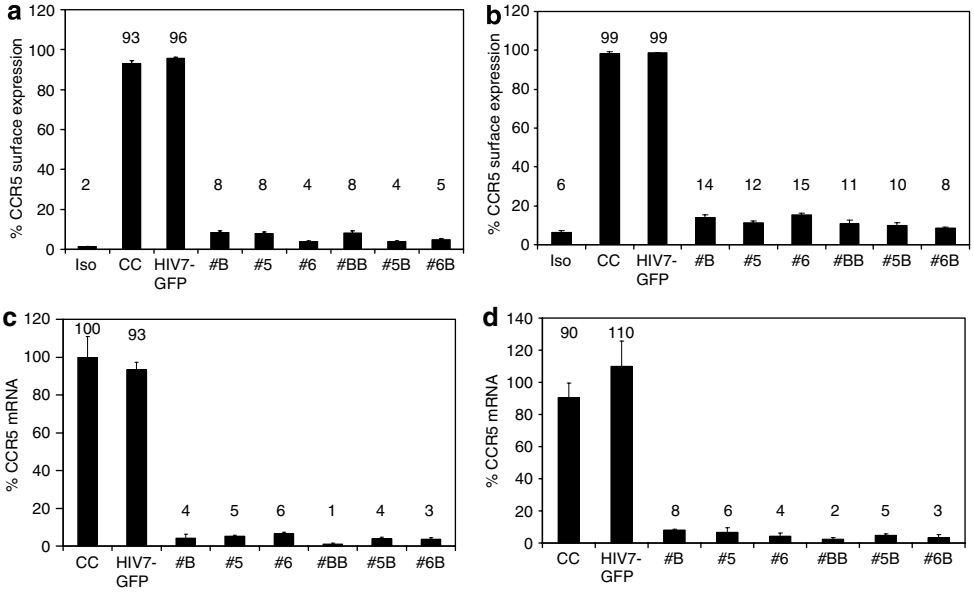


Figure 8 Downregulation of CCR5 mRNA and protein levels in siRNA lentiviral vector-transduced cells: Ghost R5/X4/R3 cells were transduced with the various CCR5 siRNA lentiviral vectors. Cells were subsequently analyzed to detect CCR5 downregulation by (**a**) FACS and (**c**) QRT-PCR. CD34 HSCs were transduced with the various CCR5 siRNA vectors and differentiated into mature macrophages. Transgenic macrophages were analyzed to detect CCR5 downregulation by (**b**) FACS and (**d**) QRT-PCR. Data were obtained in triplicate.

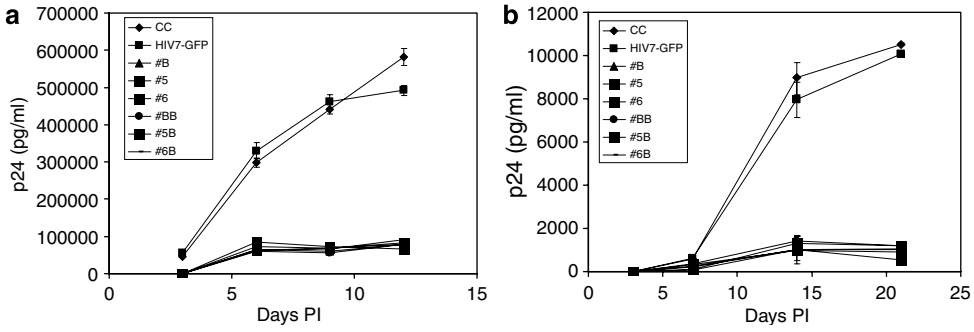


Figure 9 HIV-1 challenge of CCR5 siRNA lentiviral vector-transduced cells: transgenic (**a**) Ghost R5/X4/R3 and (**b**) CD34 cell-derived macrophages were challenged with an R5-tropic BaL-1 strain of HIV-1 at an MOI of 0.01. Cell culture supernatants were collected on various days post-infection and analyzed for viral p24 antigen levels by ELISA. Data were obtained in triplicate.

replenish the population of immune system cells depleted during the course of HIV infection. Accordingly, CD34 hematopoietic progenitor stem cells were subjected to transduction with various CCR5 siRNA lentiviral constructs and cultured in cytokine media to derive siRNA transgenic macrophages. The resulting transgenic macrophages were then evaluated to assess CCR5 downregulation both by FACS and QRT-PCR. As seen in Figure 8b, CD34-derived CCR5 siRNA transgenic macrophages displayed a marked reduction in CCR5 surface expression as compared to control cells. The levels of downregulation ranged from 92–98% with the isotype control staining 6% positive. To confirm these results, QRT-PCR was performed to determine knockdown of CCR5 RNA transcripts in transgenic macrophages. CCR5 transcript downregulation ranged from 92–98% (Figure 8d). Of the various constructs tested, #BB was found to be the most efficient in CCR5 downregulation with transcript levels decreasing 98% from those seen in control cells.

HIV-1 resistance in siRNA-transduced cells

To determine if the observed downregulation of CCR5 surface expression translated into viral resistance, Ghost R5/X4/R3 cells and transgenic macrophages were challenged with R5-tropic HIV-1 BaL-1 at an MOI of 0.01. Viral p24 antigen levels at different days post-challenge were determined by enzyme-linked immunosorbent assay (ELISA) to quantify levels of HIV-1 resistance. Over a tenfold inhibition was seen in CCR5 vector-transduced cells compared to nontransduced or HIV7-GFP control-transduced cells (Figure 9). These data together established that all of the above CCR5 siRNAs transduced via lentiviral vectors were also effective in inhibiting HIV-1 infection in both cultured cells and CD34 cell-derived transgenic macrophages.

Lack of interferon induction

To determine if the above CCR5 siRNAs induced an interferon (IFN) response in transgenic CD34-derived

macrophages, the levels of the IFN-related mRNAs, *MX1*, *OAS1* and *IFNβ* were measured by QRT-PCR. IFN-related transcripts were measured in relative levels to that of internal control albumin mRNA levels. No marked changes in the levels of their expression was observed in CCR5 siRNA transgenic macrophages when compared to nontransduced or HIV7-GFP control vector-transduced cells (Figure 10). However, a significant increase in *MX1* and *OAS1* mRNA levels was observed in cells transfected with poly I:C long double-stranded RNA, a known IFN inducer (Figure 10).

U6 promoter expression of CCR5 siRNAs

Recent evidence has shown that Pol-III U6 promoter-expressed siRNAs are more efficient in targeted gene silencing compared to that of Pol-III H1 promoter-driven expression attributed to a higher level of expression.[51] Therefore, CCR5 siRNAs were cloned individually under the control of an U6 promoter to evaluate if an increased level of CCR5 downregulation could be achieved (Figure 7c). Transgenic Ghost R5/X4/R3 cells were analyzed by FACS and QRT-PCR to determine the levels of co-receptor knockdown. Complete downregulation of CCR5 expression was observed in siRNA-transduced cells as measured by cell surface expression and intracellular transcript levels (>99%) (Figure 11a and b).

To rule out any IFN induction and related toxicity, the levels of the IFN-specific mRNAs, *MX1*, *OAS1* and *IFNβ* were measured by QRT-PCR. CCR5 siRNA-transduced Ghost R5/X4/R3 cells did not show any marked increase in the levels of IFN gene expression similar to control cells (Figure 11c). However, as expected, a significant increase in *MX1* and *OAS1* gene expression was observed in poly I:C transfected cells, which were used as a positive control (Figure 11c). The above results showed that a more robust and complete downregulation of CCR5 expression could be achieved with Pol-III U6 promoter-driven siRNA expression and that higher levels were not toxic to the transduced cells.

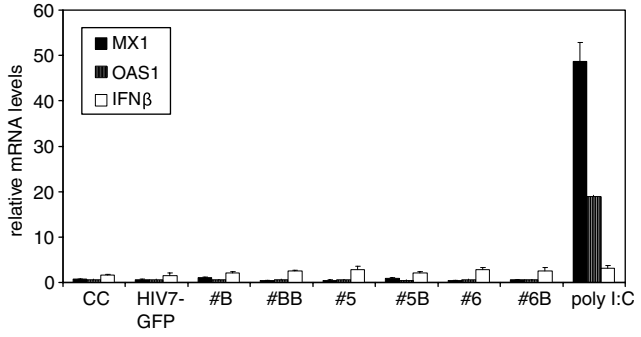


Figure 10 IFN-related gene expression: total RNA was extracted from CCR5 siRNA transgenic CD34 cell-derived macrophages. The expression levels of the IFN-specific genes *MX1*, *OAS1* and *IFNβ* were analyzed by QRT-PCR. Poly I:C transfected cells were used as a positive control. Measured transcript levels are shown relative to that of internal control albumin mRNA levels.

Discussion

Due to its critical role during primary HIV-1 infection as an essential co-receptor, CCR5 is an ideal therapeutic target. Therefore, derivation of fully efficacious anti-CCR5 siRNAs has been a long-standing goal in many laboratories. Although various levels of downregulation with marked viral inhibition have been reported recently, complete knockdown is yet to be achieved.[22–27,30] In our present study, we derived several highly potent siRNAs capable of complete suppression of CCR5 expression. Both synthetic transfected as well as endogenously expressed siRNAs via lentiviral vectors were found to be highly effective. Moreover, we have shown that these potent siRNAs retained their efficacy in stably transduced HSC-derived macrophages. Since genetic polymorphism and allelic variation exists in the CCR5 gene, availability of multiple effective siRNAs targeted to

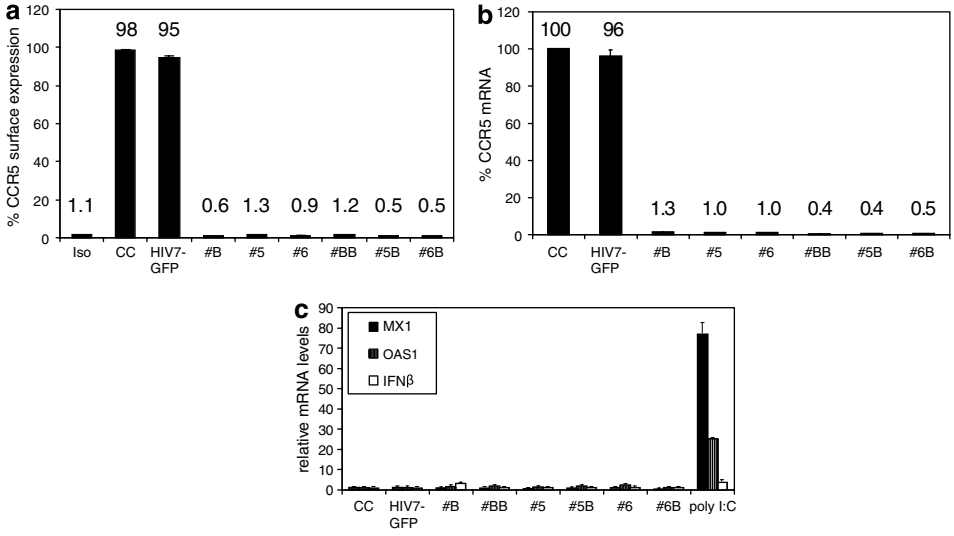


Figure 11 Efficacy of U6 promoter-driven CCR5 siRNAs: Ghost R5/X4/R3 cells were transduced with the various U6 promoter-driven CCR5 siRNA containing lentiviral vectors. Cells were subsequently analyzed to detect CCR5 downregulation by (**a**) FACS and (**b**) QRT-PCR. (**c**) IFN-specific gene expression was analyzed by QRT-PCR. Poly I:C transfected cells were used as positive controls. Measured transcript levels are shown relative to that of internal control albumin mRNA levels.

different regions, as we described here, will offer additional options for clinical applications.

To identify highly potent CCR5 siRNAs, the SMART-selection design algorithm (Dharmacon Inc., Lafayette, CO, USA) was employed. First, we began with 19-mer duplexes with dinucleotide 3′ uracil overhangs, followed by testing longer constructs with an sh design. The sh design permitted the use of Pol-III promoters to drive the expression of a single transcript containing both the sense and antisense strands of the siRNA duplex for introduction into a lentiviral vector. Of the initial five 19-mer constructs tested in transient transfection experiments, three showed >90% efficacy in CCR5 knockdown. The longer hairpin versions of these constructs displayed an increased potency with all three constructs downregulating expression >94%. Two of the constructs, namely 5B and 6B showed 98 and 97% reduction in cell surface expression of CCR5. Initial proof-of-concept and later reports with anti-CCR5 siRNAs described gene knockdown levels ranging between 48–90%.[23–26] The present gene silencing levels are higher than that of any previously published CCR5 siRNAs to date including ones tested previously in our laboratory.[22,30]

To determine if the above siRNAs were also effective at lower concentrations, tenfold and 100-fold lower concentrations were tested in transfection experiments. Our results have shown that the six effective siRNAs retained their efficacy at tenfold lower levels (64 nm), and still showed moderate efficacy at 100-fold lower concentration (6.4 nm). Since the sole measurement of cell surface expression of CCR5 may not accurately reflect the true knockdown of gene expression due to differences between half-lives of the expressed protein versus the parent transcript, the intracellular levels of CCR5 mRNA were assayed. On the basis of QRT-PCR, the reduction in CCR5 transcript levels was found to be almost complete with all six of the effective siRNAs, namely #5, #6, #B, #5B, #6B and #BB. These data confirm that the above siRNAs are highly potent in CCR5 gene silencing.

Certain siRNAs have been shown to exhibit overt toxic effects in transfected as well as transgenic cells. Therefore, we evaluated if any apparent toxicities exist with the above siRNAs. With the exception of the constructs #B and #BB, cytotoxic effects seen in transfected cells with high concentrations of siRNAs (640 nm) were similar to that seen with the transfection reagent alone. It is known that cytotoxicities seen with some siRNAs can be prevented by designing siRNAs that act at very low concentrations. Since the six effective siRNAs retained their full efficacy even at tenfold lower concentrations, overt cellular toxicity/cell death may not be an issue with these constructs.

To determine if the observed knockdown of CCR5 in transfected cells conferred HIV-1 resistance, cells were challenged with an R5-tropic strain of HIV-1. Co-receptor knockdown coincided with viral resistance to HIV infection with each of the highly potent CCR5 siRNA constructs. We next introduced these siRNAs into lentiviral vectors for stable expression since gene silencing is only transient in siRNA-transfected cells. A Pol-III H1 promoter was initially used to drive expression of the respective siRNA constructs. Ghost R5/X4/R3 cells that constitutively express CCR5 were used to verify siRNA efficacy. Our results showed effective downregulation of both cell surface expression of CCR5 and its intracellular transcripts. When stably transduced cells were challenged with R5 tropic HIV-1, marked resistance to viral infection was observed.

As mentioned above, to obtain long-range protection *in vivo*, it is necessary to introduce effective siRNAs into HSCs such that constitutive expression can be obtained in end-stage virus susceptible cells such as macrophages and helper T cells. Therefore, we transduced CD34 cells with the respective siRNA constructs to derive transgenic macrophages. These experiments were designed to reveal if the transduced progenitor cells were capable of differentiation into end-stage cells, and if these differentiated cells were resistant to HIV-1 infection. Normal differentiation would suggest no apparent siRNA-mediated toxicities during cell differentiation, whereas co-receptor downregulation and viral resistance would confirm transgene expression levels sufficient for potent knockdown and prevention of viral entry. Our analysis of CCR5 siRNA transgenic macrophages by FACS and QRT-PCR showed expected downregulation of cell surface CCR5 expression and reduction in transcript levels. HIV-1 challenge experiments also showed remarkable resistance to infection of transgenic macrophages indicating the effectiveness of the above siRNAs in a gene therapy setting.

Constitutive CCR5 siRNA expression was not found to be toxic or detrimental to cell viability as CD34 HSCs underwent normal differentiation into mature macrophages. The morphology and yield of both myeloid colonies and end-stage macrophages were similar for control as well as all siRNA transgenic cells (data not shown). Upon analysis of IFN-specific gene expression, namely that of *MX1*, *OAS1* and *IFNβ*, no significant increases in their transcript levels could be seen in transgenic macrophages, thus indicating no IFN induction by the above siRNAs. Further testing of these constructs *in vivo* in systems such as SCID-hu mice is likely to reveal their potential for use in a clinical setting.

Recent data suggested that siRNAs expressed under the control of U6 promoters are more efficacious than those under H1 promoter due to higher expression levels.[51] Increased levels of U6-driven siRNA expression was also found to be cytotoxic in the above recent report. To evaluate further these findings, we tested the above siRNAs under the control of a U6 promoter. As observed by both FACS and QRT-PCR, U6 promoter-driven CCR5 siRNA expression resulted in a more complete CCR5 knockdown in transduced Ghost R5/X4/R3 cells confirming higher efficacy. However, in contrast to the above studies,[51] no apparent toxicities could be detected for these highly efficacious CCR5 siRNAs when driven by the U6 promoter. Therefore, cellular toxicities caused by siRNA expression are more likely due to the specific siRNA sequences rather than an increased level of U6 promoter-driven expression.

The use of CCR5 siRNAs alone would not be an adequate strategy for effective HIV gene therapy as they will not protect against X4 or dual tropic strains of HIV-1. Furthermore, the use of CCR5 siRNAs alone may promote rapid emergence of X4 tropic viruses *in vivo*. Therefore, it is necessary to combine the present CCR5 siRNAs with other effective anti-HIV genes in a single lentiviral construct to protect against divergent viral

strains. We have previously shown that such a strategy would be successful.[30,31] For improved long-range efficacy, an ideal combinatorial vector for HIV gene therapy should incorporate anti-HIV genes targeted to both viral and cellular targets to minimize the development of escape mutants. Such strategies are currently under development.

Materials and methods

CCR5 siRNA constructs

siRNAs targeting CCR5 were identified with the help of Dharmacon using the SMARTselection design algorithm. The siRNA duplexes (#4, #5, #6 and #7) are 19-mers consisting of sense and antisense strands each with two 3′ uracil overhangs. The siRNA #B was used as a reference standard as it is the most potent CCR5 siRNA published to date.[26] The target sequences are as follows: #B-5′-GUGUCAAGUCCAAUCUAUG-3′, #4-5′-GAAG GUGUCAGAAUAAUAA-3′, #5-5′-GUUCAGAAACUA CCUCUUA-3′, #6-5′-GAGCAUGACUGACAUCUAC-3′, #7-5′-UCUUUGGCCUGAAUAAUUG-3′. The second set of siRNAs includes shRNAs which are 28-mers of sense-loop-antisense design with the loop sequence AUAU GUG (#BB, #4B, #5B, #6B, #7B). These shRNAs target the same regions of CCR5 as described above but were extended nine nucleotides downstream of the 3′ end, converting the 19-mer siRNAs into 28-mer shRNAs.

siRNA transfection

Ghost R5/X4/R3 cells that constitutively express the HIV-1 co-receptor CCR5 were obtained from the AIDS Reference and Reagent Program. They were maintained in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% heat inactivated fetal bovine serum (FBS), 0.2 mg/ml G418, 0.1 mg/ml hygromycin B and 1.0 μg/ml puromycin. Cells were plated in 2.5 cm wells at 5×10^5 cells/well 24 h before transfection. Lipid–RNA complexes were prepared by incubating various amounts (640, 64 and 6.4 nM) of the appropriate siRNA with 4 μl lipofectamine (Invitrogen, Carlsbad, CA, USA) at room temperature for 25 min. Complexes were then added to the cells in a final volume of 700 μl. Cells were incubated with transfection mixtures at 37°C for 6 h followed by the addition of 2 ml DMEM containing 10% heat inactivated FBS. Transfections were repeated on day 2. Seventy-two hours post-transfection, cells were analyzed by flow cytometry and QRT-PCR analyses to determine the downregulation of CCR5 surface expression and transcript levels, respectively.

Plasmid and lentiviral vector construction

A third-generation lentiviral vector backbone, pHIV-7-GFP, was used to derive the CCR5 constructs.[46] shRNA expression cassettes targeting CCR5 under the control of the polymerase-III H1 (Figure 7b) or U6 (Figure 7c) promoters were PCR-amplified as described previously.[52] In generating shRNA expression cassettes for the sense and antisense duplex constructs, #B, #5 and #6, these siRNAs were converted into stem loop-structured shRNAs by connecting the two strands with the loop sequence UUCAAGAGA. These six shRNA expression cassettes, #B, #5, #6, #BB, #5B and #6B, were then cloned individually into the pHIV-7-GFP transfer vector in a *Bam*HI site immediately upstream of the CMV immediate early promoter-enhanced green fluorescent protein (CMV-EGFP) reporter gene. Sequencing and confirmation of candidate clones was performed by Laragen Inc. (Los Angeles, CA, USA).

293 T cells, used for vector production, were maintained in DMEM supplemented with 10% FBS. To generate lentiviral vectors, 15 μg of transfer vector (either HIV7-GFP or one of the CCR5 constructs) was transfected along with 15 μg pCHGP-2, 5 μg pCMV-Rev and 5 μg pCMV-VSVG into 293 T cells at 80% confluency in 100 mm culture dishes using a calcium phosphate transfection kit (Sigma-Aldrich, St Louis, MO, USA). Six hours after transfection, fresh medium was exchanged. Cell culture supernatants containing the vector were collected at 24, 36, 48 and 60 h post-transfection and pooled. Vector supernatants were concentrated by ultracentrifugation and later titrated on 293 T cells using FACS analysis for EGFP expression.

Lentiviral vector transduction and derivation of macrophages

Ghost R5/X4/R3 cells were seeded in 2.5 cm wells 24 h before transduction, 5×10^5 cells per well. Cells were transduced with lentiviral vectors at an MOI of 20 in the presence of 4 μg/ml polybrene for 2 h. Seventy-two hours post-transduction, cells were sorted based on EGFP expression.

CD34 hematopoietic progenitor cells were purified from human fetal liver by selection with monoclonal antibody-conjugated immunomagnetic beads (Miltenyi Biotech, Auburn, CA, USA). The purity of CD34 cells was determined by FACS using a phycoerythrin (PE)-conjugated CD34 antibody. The purity of cells was routinely $>95\%$ (data not shown). CD34 cells were maintained in Iscove's modified Dulbecco's growth medium containing IL-3, IL-6 and stem cell factor each at 10 ng/ml (R&D Systems, Minneapolis, MN, USA) supplemented with 10% FBS. Lentiviral vector transductions were performed on two consecutive days at an MOI of 20 in the presence of polybrene (4 μg/ml). Transduced cells were then sorted by FACS for EGFP expression and subsequently placed in semisolid methylcellulose Methocult media (Stem Cell Technologies, Vancouver, BC, Canada) for 10–12 days to derive myeloid colonies. Total myeloid colonies were then pooled and cultured *in vitro* in DMEM supplemented with the cytokines M-CSF (25 ng/ml) and GM-CSF (25 ng/ml) (R&D Systems, Minneapolis, MN, USA) for 4 days to derive mature macrophages.

Flow cytometry

To determine the effect of transfected and lentiviral vector-expressed siRNAs on the cell surface expression of CCR5, FACS analysis was performed. Seventy-two hours post-transfection or transduction of Ghost R5/X4/R3 cells, cells were washed twice with 0.5% bovine serum albumin/phosphate-buffered saline (PBS). PE-conjugated mouse anti-human CCR5 and matching isotype (Pharmingen, San Jose, CA) were used for staining. Data were obtained and analyzed on the Coulter EPICSXL-MCL with EXPO32 ADC software (Coulter Corporation, Miami, FL, USA). After allowing for differentiation and maturation of transgenic

CD34-derived macrophages, FACS analysis of transgenic macrophages was performed as described above.

QRT-PCR

To confirm the results of CCR5 downregulation as seen by FACS analysis, QRT-PCR was performed on siRNA-transfected and lentiviral vector-transduced cells to determine the intracellular levels of CCR5 transcripts. Total RNA was isolated from cells using RNA-STAT-60 as described by the manufacturer (Tel-Test Inc., Friendswood, TX). Using the Superscript-III First Strand Synthesis kit (Invitrogen, Carlsbad, CA), 5 μg cDNA was generated from cellular RNA using oligo dT primers. QRT-PCR was then performed on 10 ng cDNA using the SYBR Green kit (Invitrogen). QRT-PCR was performed and analyzed on the BioRad iCycler. G-6-P was used as an internal control. Primer sequences for each siRNA set are as follows: #B and #BB (forward) 5′-GGATTATCA AGTGTCAAGTCCAA-3′ and (reverse) 5′-TTGCCCACA AAACCAAAGAT-3′, #5 and #5B (forward) 5′-CAGGAA TTCTTTGGCCTGAA-3′ and (reverse) 5′-CTGAACTTCT CCCCGACAAA-3′, #6 and #6B (forward) 5′-ACTGCAA AAGGCTGAAGAGC-3′ and (reverse) 5′-AGCATAGTG AGCCCAGAAGG-3′.

To determine if siRNA expression induced an IFN response in transduced CD34-derived macrophages, the mRNA levels of *MX1*, *OAS1* and *IFNβ* were evaluated. Total RNA was isolated and QRT-PCR was performed as described above with specific primer sets for these genes. The primers used are as follows: *MX1* (forward), 5′-CT GGTGCTGAAACTGAAGAAAC-3′ and (reverse), 5′-AT CTCAATCTCGTAGTCCTGGTA-3′; *OAS1* (forward), 5-CGAGGGAGCATGAAAACACATTT-3 and (reverse), 5′-GCAGAGTTGCTGGTAGTTTATGAC-3′; *IFNβ* (forward), 5′-AGACTTACAGGTTACCTCCGAA-3′ and (reverse), 5′-CAGTACATTCGCCATCAGTCA-3′.

Long double-stranded poly I:C RNA was used as a positive control to induce IFN-specific gene expression. Poly I:C RNA (500 ng) was transfected into target cells as described above on two consecutive days. Total RNA was isolated and the mRNA levels of *MX1*, *OAS1* and *IFNβ* were determined.

HIV-1 challenge of siRNA-transfected and -transduced cells

Ghost R5/X4/R3 cells are susceptible to infection with R5-tropic HIV-1 BaL-1, as they express the co-receptor, CCR5. To determine viral resistance conferred by the downregulation of CCR5, siRNA-transfected or -transduced Ghost cells were subjected to viral challenge. Seventy-two hours post-transfection/transduction, culture media was removed and virus was added to the cells (MOI 0.01) in the presence of polybrene (4 μg/ml). Virus was allowed to adsorb for 2 h at 37°C. Cells were then washed twice with PBS and 2 ml of complete DMEM was added. Transgenic macrophages were also challenged similarly. Culture supernatants collected at different days post-challenge were assayed for p24 antigen by ELISA (Beckman-Coulter, Fullerton, CA, USA).

Cytotoxicity of siRNA-transfected cells

To determine if these specific CCR5 siRNAs were cytotoxic, transfected cells were stained for the expression of phosphatidyl-serine. Transfections were performed as described above. When cells undergo apoptosis, membrane-associated phosphatidyl-serine molecules become exposed on the extracellular surface for detection by phagocytes. Phosphatidyl-serine can be detected by staining with Annexin V. Seventy-two hours post-transfection, cells were stained with a PE-conjugated Annexin V antibody and analyzed by FACS. A reagent that causes cell death, si*CONTROL* TOX transfection control reagent (Dharmacon), was used as a positive control.

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