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DISSERTATION

GENE THERAPY FOR HIV/AIDS: HARNESSING RNA INTERFERENCE AND THE RESTRICTION FACTOR TRIM5alpha TO INHIBIT HIV-1 INFECTION

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

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COLORADO STATE UNIVERSITY

November 1, 2005

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSEPH STERLING ANDERSON ENTITLED GENE THERAPY FOR HIV/AIDS: HARNESSING RNA INTERFERENCE AND THE RESTRICTION FACTOR, TRIM5alpha, TO INHIBIT HIV-1 INFECTIONS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

GENE THERAPY FOR HIV/AIDS: HARNESSING RNA INTERFERENCE AND THE RESTRICTION FACTOR TRIM5alpha TO INHIBIT HIV-1 INFECTION

HIV/AIDS continues to be a major public health problem worldwide with millions of people currently infected and new infections continuing to rise. As no effective vaccines are currently available for prevention, new and innovative therapies need to be developed. Current therapies such as highly active antiretroviral therapies (HAART) which includes protease and reverse transcriptase inhibitors have shown to be efficacious but also possess certain drawbacks. Intracellular immunization against HIV through gene therapy approaches offers a promising alternative approach to current therapies. Based on this concept, a number of strategies that showed varied efficacies *in vitro* have been tested. These include antisense RNA, RNA decoys, ribozymes, transdominant proteins, and bacterial toxins. The recently discovered phenomenon of RNA interference (RNAi) offers another novel approach, which appears to be even more potent in targeted gene silencing and therefore can be potentially harnessed for HIV gene therapy. With the recent advances in knowledge of HIV evolution and species specific restriction, the intracellular protein, TRIM5α, has been shown to be responsible for restricting retroviral infections between species. By harnessing this evolutionarily selected mechanism, its potential for inhibiting HIV infections can be exploited in gene therapy applications. Here I describe the use of siRNAs and the rhesus macaque isoform of TRIM5α for inhibiting HIV-1 infections in a gene therapy setting. The siRNAs used target the critical coreceptors CXCR4 and CCR5 required for HIV attachment and entry. A monospecific short hairpin form CXCR4 siRNA was initially designed to down regulate CXCR4 alone and confer resistance to T cell tropic NL4-3 HIV-1. Transfecting this shRNA into HIV-1 susceptible cells resulted in significant cell surface down regulation with a concomitant resistance to HIV-1 infection. To extend these findings to inhibit both T cell and macrophage tropic strains of HIV-1, a bispecific synthetic shRNA was designed targeting both CXCR4 and CCR5. Again, transfecting this bispecific construct into cultured cells

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resulted in down regulation of both coreceptors which conferred resistance to HIV-1. For constitutive expression of these shRNAs, Pol-III expression cassettes were designed and inserted into a third generation lentiviral vector (XHR) for transduction of target cells. Upon transduction and subsequent analysis, stable down regulation of both CXCR4 and CCR5 was achieved. This down regulation of both coreceptors conferred resistance to both X4 and R5-tropic strains of HIV-1 in both cultured cell lines and CD34+ derived macrophages. As the expression of shRNAs and the down regulation of normal cell surface markers may have detrimental effects on normal cell physiology, phenotypic and functional assays were performed on XHR transgenic macrophages. Normal levels of the cell surface markers, CD14, CD4, and MHCII were observed. Upon LPS stimulation, similar levels of B7.1 upregulation and IL-1 and TNF-α secretion were also found. The ability to phagocytose foreign material was also observed at normal levels. Engineered expression of TRIM5α_{rh} by lentiviral vector transductions also restricted productive infections of both tropisms of HIV-1 in both cultured cell lines and CD34+ cell derived macrophages. Constitutive expression of TRIM5α_{rh} did not alter normal macrophage phenotypes or functionality. Targeting multiple stages of the viral life cycle is critical to avoid generating escape variants as HIV-1 is prone to a high mutation rate. Accordingly, a Triple lentiviral vector containing a rev/tat shRNA, a TAR decoy, and a CCR5 ribozyme was used to generate transgenic thymocytes in a SCID-hu mouse model. Transgenic thymocytes were shown to be phenotypically normal displaying all cell subsets (CD4+, CD8+, CD4+/CD8+) and expressing the normal T cell markers CD45RA, CCR7, CXCR4, and CD28. Triple transgenic thymocytes were also resistant to T cell tropic HIV-1 infection. Together, these results show the potential of these constructs to inhibit HIV-1 infection in a stem cell gene therapy setting.

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DEDICATION

I would like to dedicate my work to my family who has always been there for me, especially my mom and big Kevin who, without them, I would not have been fortunate enough to experience everything that has brought me to where I am today.

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CHAPTER 1

GEHE THERAPY STRATEGIES FOR INHIBITING HIV/AIDS INFECTIONS

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The global HIV epidemic continues to spread at an alarming rate with high mortality in developing countries. As of the end of 2004, 39.4 million people were infected with HIV worldwide with 4.9 million new infections and 3.1 million deaths. HIV mainly infects and destroys CD4+ T lymphocytes and macrophages of the immune system rendering the body defenseless against other opportunistic infections. Once infection occurs, HIV integrates into the host's chromosome and can remain latent for 2-20 years until activation. Upon activation, there is a dramatic rise in viremia with a subsequent decrease in CD4+ cell counts. When CD4+ levels fall below 200 cells/ul, AIDS-related symptoms appear from recurring opportunistic infections that are normally cleared by the immune system such as tuberculosis, candidiasis, salmonellosis, cryptococcal meningitis, hepatitis, herpes, and Kaposis's sarcoma.[1]

The lack of effective vaccines, in conjunction with the paucity of drug-based therapy in certain parts of the world points to the need to develop alternative treatment strategies. Although combinatorial therapies such as HAART have proven to be effective in prolonging life, they do not afford a complete cure. HAART therapy includes protease inhibitors and nucleoside and non-nucleoside reverse transcriptase inhibitors.[2] Constraints with HAART therapy include the development of drug resistant viral mutants, toxicity after prolonged therapy, and cost.[2] Drug resistant HIV has even been found in patients receiving HAART therapy with undetectable levels of virus in the blood.[3] With continual viral replication in the presence of HAART therapy, accumulation of drug resistant mutants increases viral load and eventually leads to disease progression. These problems are encountered due to the characteristics of HIV

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which include a high mutation rate, stable integration in the host genome, and viral latency.

The principle of intracellular immunization against HIV using gene therapy offers a promising approach for therapeutic applications. For gene therapy to be successful, a number of issues need to be considered. First, relevant viral and/or cellular genes critical for viral replication need to be targeted. Second, the antiviral genes need to be delivered into hematopoietic stem cells that can differentiate into HIV target cells to confer resistance. The introduced transgenes also need to be expressed at therapeutic levels to confer viral resistance. For long term gene therapy to be effective, the anti-HIV transgene must also be expressed in a sustained manner from transduced progenitor cells to continuously generate resistant progeny.[4] Gene therapy can also be successful by introducing the transgene directly to HIV susceptible cells or discrete organ target sites.

Based on this concept, a number of strategies that showed varied efficacies *in vitro* have been tested. These include antisense RNA, RNA decoys, ribozymes, transdominant proteins, and bacterial toxins.[4-17] The recently discovered phenomenon of RNA interference (RNAi) offers another novel approach, which appears to be even more potent in targeted gene silencing and therefore can be potentially harnessed for HIV gene therapy.

The RNAi phenomenon, originally discovered in *Caenorhabditis elegans*, is a conserved biological response present in many eukaryotes.[18-19] More than a decade ago Rich Jorgensen and colleagues observed a phenomenon in petunias when trying to introduce a pigment producing gene under the control of a strong promoter.[20] Instead of seeing this pigment, they saw a decrease in pigment. They termed this observation

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"cosuppression" due to the silencing of both the introduced gene and the homologous endogenous gene.[20-21] The first evidence that dsRNA could lead to gene silencing occurred when Fire and Mello injected dsRNA into *C. elegans* and observed complete silencing of the homologous gene.[18] This phenomenon was also observed in Drosophila by microinjecting dsRNA into embryos.[22] RNAi is a mechanism of post-transcriptional gene silencing (PTGS) mediated by double-stranded small-interfering RNAs (siRNAs), ranging in size from 19-24 nucleotides in length, which can be targeted to any gene of interest.[23-25] The potency and specificity of RNAi as well as being a naturally occurring pathway holds considerable promise for being exploited in gene therapuetic applications.

In nature, RNAi is initiated when the cell encounters long dsRNA molecules transcribed from viral infections, degenerate cellular transcripts, or endogenously transcribed micro RNAs (miRNAs) which are involved in developmental and regulatory processes. When recognized, these dsRNA molecules induce a cascade of events that involve the RNase-III like enzyme Dicer and the multi-protein complex, RISC (RNA-induced silencing complex).[23-25] Dicer cleaves the long dsRNA molecules into smaller 19-24bp dsRNA molecules which are then handed off to the RISC complex. The RISC complex, using its ATP-dependent helicase activity, unwinds the short dsRNA molecules and uses the antisense strand as a guide. The guide strand allows the RISC complex and its endonuclease activity to recognize homologous mRNA transcripts for targeted degradation (Fig 1).

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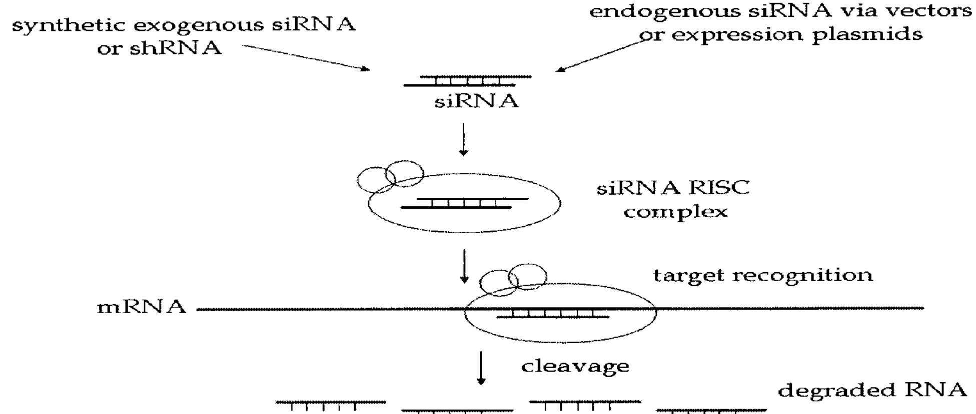

Fig 1. Mechanism of RNA interference. Either exogenously introduced or endogenously expressed si/shRNAs are introduced into a cell. These small 19-24bp dsRNA molecules are incorporated into the RISC complex where the antisense strand is used as a guide for homologous mRNA target degradation.

The RNAi phenomenon is a conserved biological response present in almost every eukaryote studied thus far.[26] RNAi was commonly used for genetic studies in plants, *C. elegans*, and *Drosophila melanogaster* and was attempted in mammalian cells using long dsRNAs. This approach was met with limited success due to the induction of the interferon response which results in total shutdown of cellular protein synthesis eventually leading to cell death.[27-28] However, several groups demonstrated that small 19-24bp dsRNAs could be introduced into mammalian cells and induce sequence-specific inhibition of target mRNA.[23,29] These small synthetic RNAs also avoided activating the interferon response. In mammalian cells, RNAi works independently from the IFN induced pathways.[30] Using standard transfection methods such as lipofection or calcium phophate precipitate mediated transfection, synthetic siRNAs can now be delivered into a broad spectrum of cell types including mammalian cells to achieve targeted gene

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silencing. *In vitro* synthesized siRNAs have the characteristic siRNA design being 21 nucleotide long RNA duplexes with 5' phosphates and 2-base 3' overhangs that are typically two uracils. Other lengths of siRNAs have also been used ranging from 18-28nt with varying results. In mammalian cells, increasing the length of a minimal effective 19mer siRNA increases its efficiency. However, doing the same to a highly effective siRNA does not increase its silencing ability.[31] To preserve the intracellular life of siRNAs, certain modifications have been developed. To protect siRNAs from nucleases, 2'-OH modifications have been added.[30] These modifications could be potentially exploited for increasing the half-life of siRNAs for *in vivo* delivery. The potency and spcificity of RNAi as well as being a naturally occurring pathway holds considerable promise for being exploited in gene therapeutic applications. siRNA-mediated silencing, therefore, could potentially be a powerful tool for inhibiting HIV infection/replication.

HIV can be targeted at various points in its replication cycle using siRNAs, whether introducing siRNAs targeted to the viral genome upon entry into cells or post-integration targeting viral transcripts or full length genomic RNA transcribed from proviral DNA (Fig 2).[4-5,32] siRNAs targeting essential cell surface receptors or cellular cofactors required for HIV replication can also be used to inhibit HIV infection/replication.[4-5]

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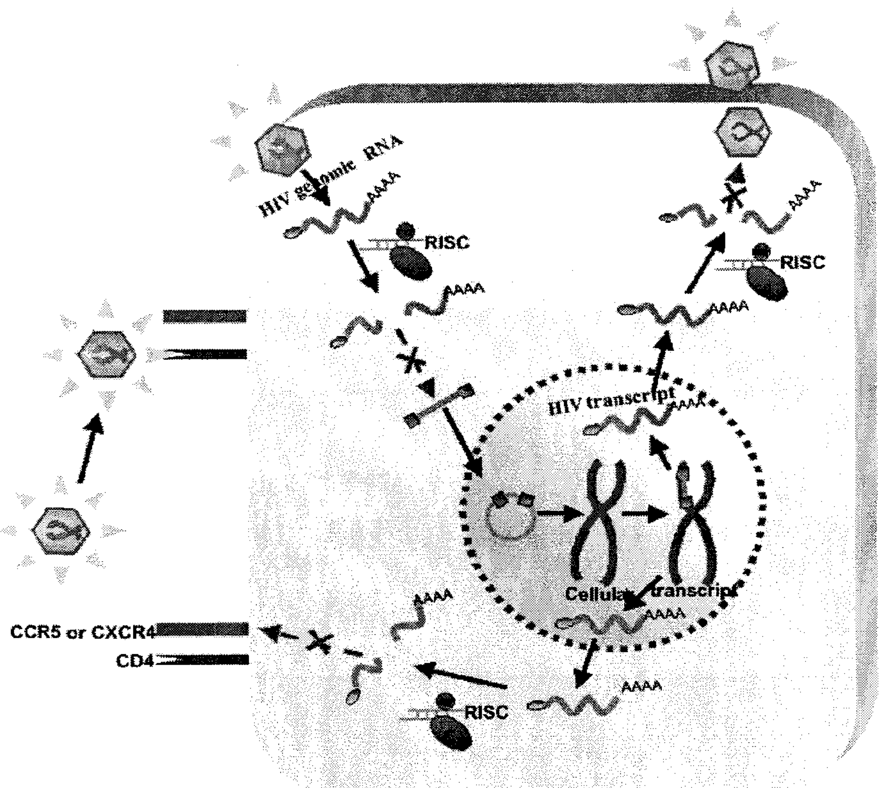

Fig. 2. HIV-1 life cycle and potential targets for siRNAs. The CD4 receptor and the coreceptors CCR5 and CXCR4 are critical for HIV entry. Each of these receptors is a potential target for RNAi which would block viral entry. Incoming HIV genomic RNA can also be targeted by siRNAs. Cellular cofactors essential for viral integration, transcription, and replication as well as viral transcripts are other areas for siRNA targeting.

The first cellular molecules HIV encounters when infecting a cell are the main receptor CD4 and the two critical coreceptors CXCR4 and CCR5. Infection of a susceptible cell by HIV-1 requires viral envelope interactions with the primary cell surface receptor, CD4, and a co-receptor, either CCR5, CXCR4, or other minor receptors.[33] Primary infection is established by R5 macrophage tropic HIV-1 that uses CCR5 as a coreceptor whereas X4 T-cell tropic viruses that predominate later in infection use CXCR4 as a coreceptor.[33] In the early stages of infection, HIV is mainly non-syncytia inducing and R5-tropic, predominantly infecting macrophages. The majority of infections of macrophages requires the use of the main cellular receptor CD4 and an

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essential coreceptor CCR5. These infections have minimal cytolysis and help to establish the base line viremia present throughout clinical latency. As disease progresses, there is a constant balance between production of new virions by infected cells and clearing of progeny virions by the immune system. During this period, there is a shift from R5 to X4-tropic strains of HIV. X4-tropic syncytia-inducing strains mainly infect CD4+ T lymphocytes and cause more destruction through cytolysis.[33]

An attractive strategy for HIV gene therapy is to target these essential surface receptors to prevent viral entry. However, disruption of CD4 surface molecules will have serious physiological consequences since CD4 is indispensable for normal immunological functions. To circumvent this drawback, an alternative strategy is to target the cellular co-receptors CCR5 and CXCR4, the disruption of which may not have undesireable effects. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of this coreceptor thus conferring significant resistance to HIV infection.[34-36] Homozygous or heterozygous individuals for this mutation remain physiologically normal. With regard to the CXCR4 coreceptor, it was found to be dispensable for T cell development and maturation in murine studies.[37] These findings suggest that CCR5 and CXCR4 are promising targets for HIV therapies.

Early transcripts such as nef, rev, and tat which are critical for further proviral gene expression are targets that have shown considerable promise in suppressing viral replication by siRNA mediated gene silencing.[38-42] Other HIV gene targets such as gag, pol, env, and vif have also been used in siRNA targeting and have been shown to inhibit HIV replication.[4] As the mechanism of RNAi is highly sequence specific, viral escape mutants have been isolated that contain single point mutations in the siRNA targeted

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region and are resistant to inhibition by cells expressing a single siRNA construct.[43-44] Therefore, when targeting viral genomes, care should be taken when evaluating potential targets. siRNAs would be best designed if directed against highly conserved HIV sequences. Combinations of siRNAs targeting multiple regions of the same gene or a variety of HIV genes should also be considered. As cellular genes mutate far less frequently than viral genes, cellular cofactors critical for HIV infection/replication may also be targeted by siRNAs.

HIV relies on numerous cellular molecules to complete a full replication cycle. Some of these cellular proteins include β-importin, high mobility group A1 protein (HMG), and LEDGF/p75 for nuclear transport and chromatin targeting of the pre-integration complex, NF-κB, Cyclin T, and CDK9 for Tat interaction and proviral transcription, and CRM-1, a cellular transport protein critical for Rev-dependent nuclear to cytoplasm transport of unspliced RNAs. These molecules have recently been targeted by siRNA mediated degradation and have conferred viral resistance.[4] Targeting these cellular proteins, however, may have detrimental effects on the cell and should be carefully evaluated for use in gene knockout experiments.

A number of recent reports have demonstrated the utility of siRNAs in inhibiting HIV-1 replication. Using direct transfection of siRNAs into mammalian cells, marked viral inhibition was achieved.[4] Cell types used included transformed cell lines susceptible to HIV infection, PBLs, blood monocytes, and CD34+ derived macrophages. Antiviral effects of transfection methods, however, are only transient due to eventual degradation and dilution of siRNAs during cell division. With the advent of expression cassettes driving transcription of siRNAs under the control of eukaryotic promoters,

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siRNA targeting experiments could then be performed using expression plasmids and/or vectors. The most common eukaryotic promoters used are the small RNA Pol-III promoters, the U6 small-nuclear RNA and the H1 RNA promoters.[39] The importance of transcribing siRNAs intracellularly is for long term constitutive expression and knockdown of target RNAs to be achieved. siRNAs against tat and tat-rev have been introduced into lentiviral vectors and their efficacy was demonstrated both in cell lines and primary T cells and macrophages.[39-42] Promising data was also obtained in experiments showing that anti-rev siRNAs against HIV were functional in conferring viral resistance in differentiated T cells and macrophages derived from transduced CD34+ hematopoietic progenitor cells.[41]

As the replication cycle of HIV is better understood with recent advances, it has become increasingly clear that host specified proteins play an important role in aiding and regulating viral expression. Of the many therapeutic approaches, restricting viral replication at the stage of viral entry is of particular advantage since the cell is protected from the toxicity of virally expressed proteins and prevents proviral formation and eventual latency in a sub-population of cells. It has long been known that species-specific restriction factors exist that confer resistance to retroviral and lentiviral infections.[45] Therefore the restriction factors that block viral infection could also be exploited for HIV-1 gene therapy. Certain nonhuman primates are naturally resistant to HIV-1 infection due to restricted species tropism. HIV-1 is unable to infect rhesus macaque cells because of a restriction at the post-entry step.[45-48] Retroviral restriction was shown to be due to the action of factors Ref1 and Lv1.[45,49-59] Through a genetic screen of rhesus monkey lung fibroblasts it was recently discovered that the MLV

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restriction factor Ref1 and the lentiviral restriction factor Lv1 are encoded by the same gene, TRIM5α.[60] The rhesus macaque isoform of TRIM5α ($TRIM5\alpha_{rh}$) was shown to confer strong resistance to HIV-1 when expressed in cells otherwise susceptible to infection.[61-63] It was also shown that $TRIM5\alpha_{rh}$ which restricts HIV-1 infection in monkey cells shares an 87% amino acid homology with that of the human TRIM5α isoform ($TRIM5\alpha_{hu}$).[60]

A component of cell cytoplasmic bodies, TRIM5α is a member of the tripartite motif (TRIM) family of proteins that contain RING domains, B boxes, and coiled coils.[64-66] TRIM5α also contains a carboxy terminal B30.2 SPRY domain that was shown, along with the RING domain, to be critical for HIV-1 restriction.[60-63] HIV-1 restriction mediated by $TRIM5\alpha_{rh}$ at the level of post-entry and pre-integration, is believed to be due to its destabilizing and ubiquitinating action on the viral capsid.[67-69] It is presumed to occur through ubiquitination as one TRIM isoform was shown to have ubiquitin ligase activity that is common to RING containing proteins.[70] The antiviral activity of $TRIM5\alpha_{rh}$ has recently been narrowed down to a 13 amino acid patch of positively selected amino acids in the SPRY domain.[62] Further analysis of this domain revealed a single amino acid change in the human isoform of $TRIM5\alpha_{hu}$ that conferred resistance to HIV-1[61,63]. Restriction differs from conventional innate immune responses in that restriction is present constitutively and is not activated by viral infection. Based on these observations, $TRIM5\alpha_{rh}$ could be exploited for conferring genetic resistance to HIV-1 in otherwise susceptible cells.

For HIV gene therapy strategies to succeed long range, it is necessary that anti-HIV coding transgenes be maintained and expressed long term in a virus susceptible

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target cell. In this regard, retroviral vectors have proven to be highly effective for high efficiency gene transduction and sustained gene expression.[71-72] The first retroviral vectors used in a gene therapy setting were murine leukemia virus derived vectors.[4] One drawback of these vectors is their preferential choice of integrating near transcriptional start sites.[73] Another major disadvantage of these vectors is their ability to traverse the nuclear membrane only in dividing cells.[74] Quiescent cells are extremely difficult to transduce with retroviral vectors. Lentiviral vectors such as the HIV based vectors, however, are effective means of delivering anti-HIV transgenes into mammalian cells because of their ability to transduce both dividing and non-dividing cells.[75] They also provide long term expression of the introduced genes.[75] Along with the favorable attributes of lentiviral vectors, there are also safety concerns to consider. Two of the major concerns surrounding the use of these are insertional mutagenesis and the generation of recombinants. Lentiviral vectors tend to integrate in chromosomal regions where there is a concentration of actively transcribed genes.[73] Also, recombination between human lentiviral vectors and endogenous retroviral sequences could possibly generate new viral strains. They do, however, offer safety in terms of oncogenesis as lentiviruses are not associated with tumor development.[4] To overcome the potential problems associated with lentiviral vectors, numerous alterations were made to the first generation lentiviral vectors.

Modifications to first generation lentiviral vectors involved deleting the accessory/regulatory genes Vif, Vpr, Vpu, and Nef from the packaging plasmid.[76] These deletions decreased the possibility of recombination and helped in avoiding to invoke an immune response. The vectors were further improved by replacing the 5' LTR U3 region

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with the cytomegalovirus immediate early promoter.[77] This allowed the vectors to be Tat independent. The deletion of Tat further reduced the ability of generating replication competent HIV. Enhancer and promoter sequences found in the 3' LTR U3 region were also removed. This modification rendered the lentiviral vector self-inactivating (SIN).[77] The property of self-inactivation is due to both LTRs being transcriptionally inactive. Transgene expression only occurs from promoters located in the individual expression cassettes found between the LTRs. The inactivation of both LTRs also makes recombination of replication-competent HIV highly unlikely. Recent modifications to the transfer vector have also improved the efficiency and expression of the introduced transgenes. The addition of the central DNA flap consisting of a polypurine tract sequence and a central termination sequence allow for more efficient nuclear import of the viral preintegration complex.[78] A second element, the WPRE (woodchuck post-transcriptional regulatory element), increases transgene expression (Fig 3).[78] To detect transduction of target cells, the reporter gene, EGFP (enhanced green fluorescent protein) under the control of the CMV IE promoter, was also inserted into the vector backbone.

pHIV-7-GFP

Fig 3. Third generation lentiviral vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. The vector backbone also contains the central flap and WPRE element as described above.

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Only three other HIV genes are needed to generate infectious vector, Gag, Pol, and Rev. Lentiviral vectors can be pseudotyped with the vesicular stomatitis virus-G protein which is used as the vector envelope. As VSV-G cellular receptors are found on nearly every cell of the body, pseudotyping lentiviral vectors with this envelope renders them pantropic with the capability to transduce many different cell types. Infectious viral vectors capable of delivering anti-HIV transgenes can then be produced using a four-plasmid transfection system (Fig 4).

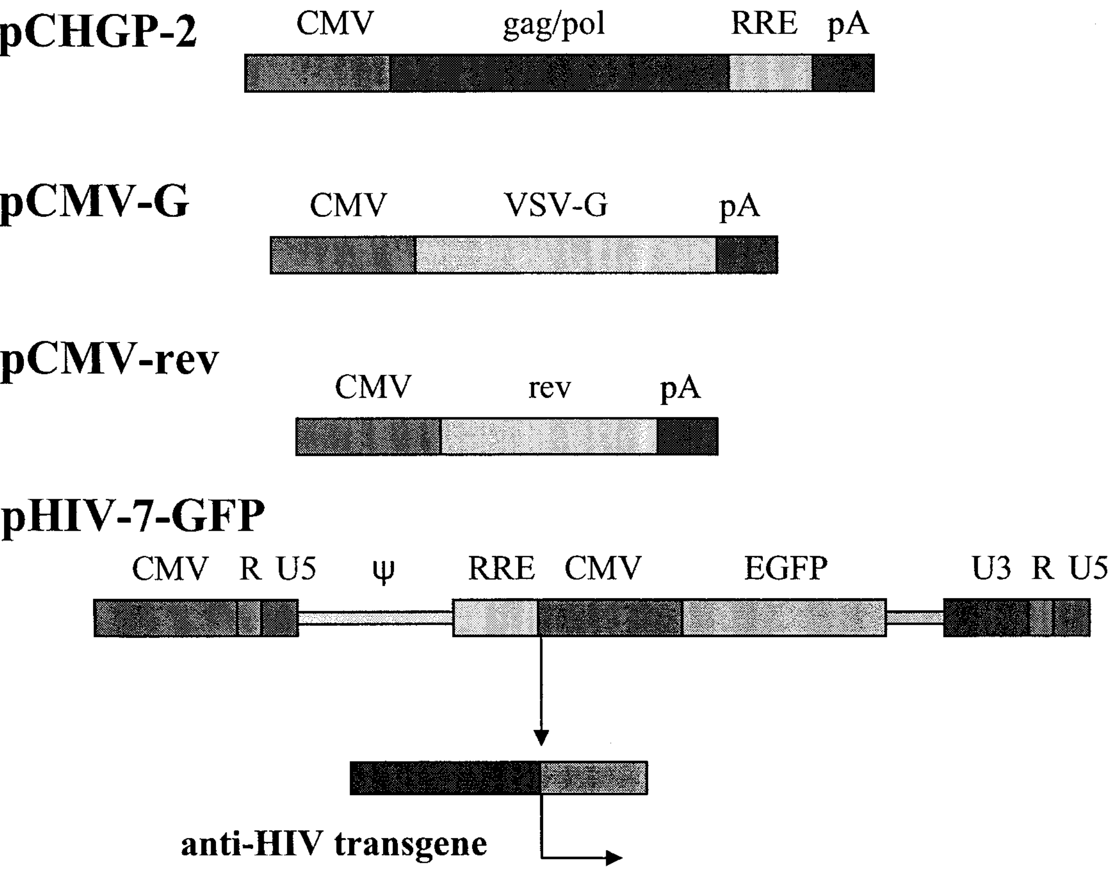

Fig 4. Lentiviral vector packaging system. A four plasmid transfection system is used to generate infectious viral vectors. pCHGP-2 contains the packaging genes Gag and Pol. pCMV-G encodes the envelope VSV-G used for pseudotyping. pCMV-rev encodes the Rev gene. pHIV-7-GFP contains the anti-HIV transgene along with EGFP reporter gene. Anti-HIV transgene expression cassettes can be inserted upstream of the CMV-EGFP gene.

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The advantage of using lentiviral vectors is their ability to transduce nondividing as well as dividing cells. Lentiviral vectors have also been shown to be highly efficient in transducing hematopoietic stem cells.[71] For stem cell gene therapy to succeed long term, efficient transduction and expression of the anti-HIV transgenes needs to occur. Transduction and infusion of long-lived progenitor cells will thereby generate HIV resistant progeny.

Macrophages along with T cells are major cell targets of HIV infections. Programming these cells to express anti-HIV siRNAs and eventually repopulating the immune system of immunodeficient HIV patients offers great promise for HIV stem cell gene therapy. To generate a continuous supply of resistant cells, hematopoietic progenitor cells need to be engineered to contain anti-HIV transgenes. CD34+ hematopoietic progenitor stem cells can differentiate into a variety of hematopoietic cell lineages including CD4+ lymphocytes, monocytes, macrophages, and dendritic cells. As these cells are the major targets of HIV infections, stem cell based therapy has the possibility of generating HIV resistant populations for all of these cell types. By using VSV-G envelope pseudotyped lentiviral vectors, efficient transduction of these stem cells can be achieved.[71] By combining these methods, effective gene transfer can be successfully achieved to inhibit HIV infections.

To better evaluate potential anti-HIV genes for use in a gene therapy setting, relevant animal models are needed to test vectors before they can be used in the clinic. The SCID-hu mouse model which harbors an intact human thymus provides an ideal environment for thymopoiesis and evaluation of potential constructs. The SCID-hu thy/liv model is generated by implanting fetal liver and thymus tissue under the kidney

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capsule of the mouse. The fetal liver tissue implanted along with the thymus tissue provides stem cells needed for thymus development. The fetal thymus tissue provides the environment needed for proper thymopoiesis. Within months, implanted tissue resembles an intact human thymus that has developed through vascularization with the kidney.[79] T cells in the thymus are phenotypically normal and can be infected by HIV.[9,41,79-80] CD34+ hematopoietic progenitor cells can be injected directly into the thymic grafts and develop normally into T lymphocytes. So far, the SCID-hu mouse model is the only *in vivo* system to evaluate the thymopoietic potential and HIV resistance of transgenic HSC derived thymocytes.

HIV gene therapy has the potential for long term treatment in inhibiting further infections. With the recent discovery of RNAi and its potent mechanism of target inhibition, therapeutic applications for inhibiting HIV infections have improved significantly. Many recent reports have demonstrated viral resistance by targeting HIV genes.[4] With the propensity to mutate at a rapid rate, HIV has been shown to circumvent siRNAs targeting its viral transcripts.[43-44] Also, as HIV is able to integrate into the host genome and remain latent and undetectable, targeting pre-integration steps of HIV's life cycle hold more promise in controlling infections. By inhibiting HIV infections pre-integration, viral latency, transcription, and the generation of escape mutants can be stopped. By targeting the critical coreceptors CXCR4 and CCR5 with siRNAs, subsequent decreases in cell surface expression have been shown to inhibit HIV-1 infections.[81-84] This method of inhibiting infections would thereby prevent any entry into susceptible cells. Also, by harnessing the species specific restriction factor, TRIM5α, in a gene therapy setting, viral integrations can be blocked.[85] Based on these concepts, the

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following work accentuates the potency and strong viral inhibition translated from the exploitation of the innate mechanism of RNAi. Being an evolutionarily selected restriction factor, TRIM5α also shows great potential for use in a gene therapy setting. By taking advantage of the recent advances in research in HIV's life cycle, a greater number of potent therapeutics have been discovered for use in HIV gene therapy.

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CHAPTER 2

POTENT SUPPRESSION OF HIV-1 INFECTION BY A STEM-LOOP STRUCTURED ANTI-CXCR4 siRNA

Anderson J, Banerjea A, Planelles V, Akkina R: Potent suppression of HIV type 1 infection by a short hairpin anti-CXCR4 siRNA. AIDS Res and Hum Retroviruses 2003, 19:699-706.

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ABSTRACT

The phenomenon of RNA interference (RNAi) sparked a new surge in the area of post-transcriptional gene silencing methodologies and their potential application for HIV-1 gene therapy. A potentially promising strategy is to exploit siRNAs to prevent viral entry at the cell surface by down regulating essential cell surface HIV-1 co-receptors. In the present studies we targeted the CXCR4 co-receptor for disruption with siRNA to inhibit HIV-1 entry as a first step towards the ultimate goal of translating this to gene therapy for AIDS. A stem-loop hairpin structured anti-CXCR4 siRNA was designed and synthesized *in vitro* by transcription with T7 polymerase. Down regulation of the co-receptor was assayed in U373-Magi-CXCR4 cells. FACS analysis showed marked-down regulation of CXCR4 on the cell surface and western blot analysis confirmed the reduced levels of intracellular synthesis. When challenged with X4-tropic HIV-1, NL4-3, the siRNA transfected cells exhibited marked viral resistance. Consistent with these results, siRNA transfected primary lymphocytes also exhibited significant resistance to HIV-1 entry. These proof-of-concept studies demonstrated the efficacy of a siRNA targeted to an essential cellular co-receptor CXCR4 in protecting from HIV-1 infection. Delivery of this siRNA into hematopoietic stem cells via lentiviral vectors may have potential gene therapeutic applications.

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INTRODUCTION

The recently discovered phenomenon of RNA interference (RNAi) offers a novel approach to be harnessed for HIV gene therapy. RNAi is a mechanism of post-transcriptional gene silencing mediated by double-stranded small-interfering RNAs (siRNAs), ranging in size from 19-24 nucleotides in length,[1-3] which can be targeted to any gene of interest. Silencing is carried out by an endogenous RNase III-like endonuclease, dicer, that uses specific siRNAs as triggers for cleaving the target homologous mRNA. The RNAi phenomenon, originally discovered in *Caenorhabditis elegans*,[4] is a conserved biological response present in many eukaryotes. As the effector RNAs are small in size, RNAi provides a specific response distinct from that of the non-specific interferon-mediated destruction of RNAs. Using standard transfection methods, synthetic siRNAs can now be delivered into a broad spectrum of cell types including mammalian cells to achieve targeted gene silencing.

A number of recent reports have demonstrated the utility of siRNA in inhibiting HIV-1 replication.[5-9] Using direct transfection of siRNAs and/or siRNAs generated by expression plasmids in transfected cells, marked viral inhibition was achieved. Most of these constructs were designed to interfere with viral replication in infected cells by targeting HIV regulatory or accessory protein coding mRNAs. Infection of a susceptible cell by HIV-1 requires viral envelope interactions with the primary cell surface receptor, CD4, and a co-receptor, either CCR5, CXCR4, or other minor receptors.[10] Therefore, an even more attractive strategy is to prevent viral entry at the cell surface. Towards this end, the recent work of Novina *et al.*, 2002, demonstrated the utility of CD4 disruption by

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siRNA in preventing viral entry. Although important in providing the proof of concept in preventing viral entry, disruption of CD4 surface molecules on helper T cells will have serious physiological consequences since CD4 is indispensable for normal immunological functions. To circumvent this drawback, an alternative strategy is to target the cellular co-receptors CCR5 and CXCR4, the disruption of which may not have physiological effects. Primary infection is established by the macrophage tropic HIV-1 that uses CCR5 co-receptor whereas X4-tropic viruses that predominate late in infection use CXCR4 as co-receptor.[10] In the case of HIV-2, CXCR4 alone was reported to be adequate for infection in the absence of CD4.[11] This has been affirmed by the recent work utilizing a siRNA directed against the co-receptor CCR5.[9] However, disruption of CCR5 alone will not be adequate for complete protection against HIV-1 as all X4 tropic and/or X4/R5 tropic viral strains circulating *in vivo* use CXCR4 as the co-receptor for entry into cells.

Based on this premise, we targeted CXCR4 for disruption by siRNA to inhibit HIV-1 entry as a first step with the long-range goal of translating this into stem cell based gene therapy. Using a stem-loop structured construct, we show here that siRNA transfected cells exhibit a marked reduction of cell surface CXCR4 with a consequent potent suppression of viral entry, thus affording anti-viral protection.

MATERIALS AND METHODS

Design and synthesis of anti-CXCR4 siRNA:

To design the siRNAs, we chose an accessible sequence in the 5' region of the CXCR4 mRNA based on our previous experience with ribozymes to disrupt co-receptor

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molecules. The first base, guanosine, of CXCR4wt siRNA corresponds to the guanosine at the initiation codon of CXCR4 mRNA. A stem-loop hairpin structured design was utilized. Recent observations described by various groups were taken into account in constructing the siRNAs.[12-13] A long oligonucleotide consisting of sense and anti-sense sequences that constitute the siRNA with an intervening stretch of 9 nucleotides to create a loop structure was synthesized. The loop structure sequence used, UUCAAGAGA, was reported to have an enhanced activity compared to other loop structures.[12] The oligo also incorporated a T7 promoter sequence to permit *in vitro* transcription of the siRNA by the T7 polymerase. The schematics depicting the template oligo incorporating these sequences, panel A, and the expected products of *in vitro* synthesis, panels B and C, are presented in Fig. 1. The sequences of the different siRNA templates are as follows.

1) CXCR4wt-siRNA (wild-type): 5'AAGGAGGGGATCAGTATATAC/TCTCTTGAA/GTATATACTGATCCCCTC/ctatagtgagtcgtatta-3'. 2) CXCR4mt-siRNA (mutant) containing a nucleotide substitution at position 9 (bold lowercase): 5'-AAGGAGGGGA**g**CAGTATATAC/ TCTCTTGAA/ GTATATACTG**c**TCCCCTC/ ctatagtgagtcgtatta-3'. 3) CXCR4ur-siRNA (unrelated siRNA targeted against CCR5): 5'-AAGGATTATCAAGTGTCAAGT/ TCTCTTGAA/ ACTTGACACTTGATAATCC/ ctatagtgagtcgtatta-3'. Each of the synthetic templates contains a T7 promoter complement (lowercase) used in priming for T7 transcription (Fig. 1, panel A). The T7 promoter oligo used for priming *in vitro* transcription has the sequence: 5'-TAATACGACTCACTATAG-3'. PAGE purified oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA.

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The three siRNA transcripts, designated as CXCR4wt, CXCR4mt, and CXCR4ur were synthesized *in vitro* by T7 polymerase mediated transcription using a T7 primer from their respective templates as described.[14] The reaction mixture contained 0.1 μg of the template together with other components. The *in vitro* derived siRNA products (average yield of 40 μg per reaction) were analyzed by gel electrophoresis to confirm their sizes. The siRNAs were dissolved in nuclease-free water and stored at -70°C until further use.

Cell cultures and siRNA transfection:

U373-Magi-CXCR4 indicator cells[15-16] that constitutively express the HIV-1 co-receptor CXCR4 on their cell surface were obtained from the AIDS Reference and Reagent Program. They were maintained in DMEM medium containing 10% heat inactivated 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 hours before transfection. Lipid-RNA complexes were prepared by incubating 1 μg of appropriate siRNA with 1.5 μl lipofectamine (Gibco-Invitrogen, Rockville, MD) at room temperature for 25 minutes. Complexes were then added to the cells in a final volume of 350 μl. Cells were incubated with transfection mixture at 37°C overnight followed by the addition of 1 ml DMEM containing 10% heat inactivated FBS. Transfections were repeated on days 2 and 3. After allowing the siRNAs to induce their effect, the transfected cells were then used for flow cytometric and western blotting analysis to determine the down regulation of CXCR4. Peripheral blood mononuclear cells (PBMC) were stimulated in the presence of

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3 ug/ml PHA for three days to generate blasts and transfected with different siRNAs like above.

Flow cytometry:

To determine the effect of siRNA on the cell surface expression of the CXCR4 co-receptor, FACS analysis was performed. Forty-eight hours post-transfection, cells were washed twice with 0.5% BSA/PBS. Phycoerythrin (PE)-conjugated mouse anti-human CXCR4 and matching isotype (Pharmingen, San Jose, CA) were used for staining. Cell surface expression of an unrelated surface molecule, CD4, was also assayed to confirm the specificity of CXCR4 siRNA. Cells were resuspended in 45 µl 0.5% BSA/PBS and stained with 5 µl of appropriate antibody at 4°C for 2 hours. Cells were then washed 3 times with 0.5% BSA/PBS, resuspended in the same buffer and subjected to FACS analyses. Data was obtained and analyzed on Coulter EPICS®XL-MCL with EXPO32 ADC software (Coulter Corporation, Miami, FL).

Western blotting analysis:

To demonstrate the down regulation of the CXCR4 protein levels in siRNA transfected cells, western blotting analysis was performed using antibody specific for CXCR4. Cell lysates obtained at 48 hours post-transfection were resolved on 10% SDS-PAGE gels and blotted on to Immobilon™-P membranes (Millipore, Bedford, MA). After the blocking step, the transferred proteins were probed with rabbit anti-human N-terminal Fusin (BioChain Institute Inc., Hayward, CA) at 4°C overnight and later reacted with an alkaline phosphatase conjugated anti-rabbit secondary antibody (Santa Cruz

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Biotechnology, Santa Cruz, CA). Western Blue® substrate (Promega, Madison, WI) was added to visualize proteins as previously described. Human actin protein was used as an indicator for internal cell protein control and rabbit anti-actin (Sigma-Aldrich, St. Louis, MO) was used for its detection. Intensity of protein bands was determined by densitometry.

HIV-1 challenge of siRNA transfected cells:

U373-Magi-CXCR4 cells are susceptible to infection with X4-tropic HIV-1 NL4-3, as they express the respective co-receptor. To determine the resistance conferred by the down regulation of the cell surface co-receptor, the siRNA transfected cells were subjected to viral challenge *in vitro*. At 48 hours post-transfection, culture media was removed and 150 μl (10 ng p24 gag antigen) of virus was added to each well in the presence of polybrene (8 μg/ml). Virus was allowed to adsorb for 2 hours at 37°C. Cells were then washed twice with PBS and 1 ml of complete DMEM was added. Cells were incubated for 48 hours at 37°C for viral infection to progress. Since Magi cells contain an inducible β-galactosidase gene under the control of HIV-1 LTR, they serve as good indicator cells for HIV-1 infection by producing a blue color after appropriate staining. Accordingly, to detect virus infected cell foci, cultures were then fixed with 1% formaldehyde and 0.2% glutaraldehyde followed by staining for the detection of β-galactosidase as described.[15-16] Culture supernatants collected at different days post-challenge were assayed for p24 antigen by ELISA (Beckman-Coulter). SiRNA transfected primary lymphocytes were also challenged with HIV-1 NL4-3. Viral supernatants collected on different days, post-infection, were assayed for p24 antigen.

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RESULTS

Down regulation of CXCR4 by siRNA:

Various siRNAs (shown in Fig. 1) were synthesized *in vitro* by T7 polymerase-mediated transcription as described in the Materials and Methods section, and analyzed by gel electrophoresis. The siRNA products were found to correspond to their expected sizes (data not shown). Following siRNA transfection of Magi cells, two different methods were used to determine the down regulation of the CXCR4 co-receptor. To demonstrate specificity, two control siRNAs, one containing a single mutation in the 9th position (CXCR4mt) (Fig. 1, panel B) and another containing an unrelated sequence (CXCR4ur) were also used in these
experiments.

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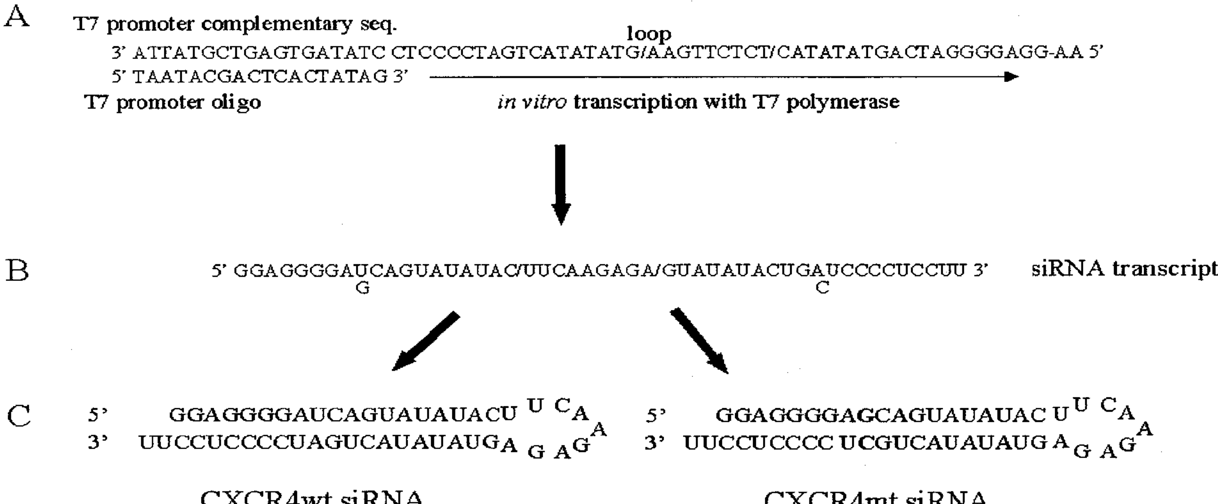

Figure 1: Design and synthesis of anti-CXCR4 siRNA: Sequence of the template oligo showing the T7 promoter, siRNA sense and antisense sequences with an intervening loop region. Complimentary T7 promoter oligo for priming and the direction of synthesis are also shown (A). Transcribed sequence of the predicted anti-CXCR4 siRNA. Mutated positions are shown below the transcript (mutations from A to C and U to G) (B). Predicted double stranded structures of the wild type and mutant siRNAs following transcription and self-annealing (C).

Flow cytometry analysis of siRNA transfected cells at 48 hours post-transfection revealed substantial reduction in CXCR4 staining amounting to 74% with the wild type construct compared with no reduction with the unrelated siRNA or mock transfected cells (Fig. 2). The 74% inhibition by CXCR4wt siRNA is also significantly higher than a low level inhibition (10%) shown by the CXCR4mt siRNA. The low but notable level of inhibition shown by the mutant siRNA with a single nucleotide mismatch is consistent

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with recent observations that such siRNAs are still functional to a certain degree.[18] CXCR4 siRNA had no inhibitory effect on the cell surface expression of an unrelated molecule CD4 (Fig. 2D). Thus, the flow cytometry data indicated that down regulation of CXCR4 by homologous siRNA was specific and highly significant.

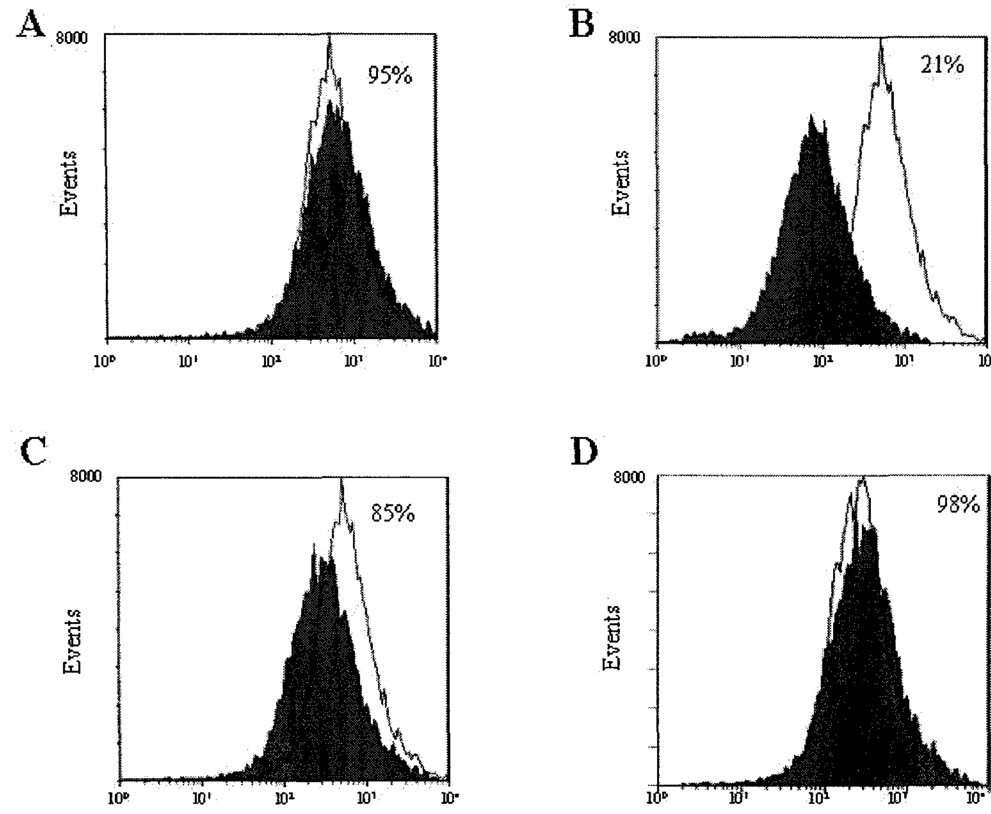

Figure 2: Cell surface down regulation of CXCR4 by siRNA: Magi-CXCR4 cells that constitutively express the HIV co-receptor CXCR4 and the primary receptor CD4 were transfected with synthetic siRNAs as described in methods. At 48 hours post-transfection, cells were stained with PE-conjugated antibodies to CXCR4 or CD4 and analyzed by FACS. Levels of CXCR4 in mock transfected cells (unshaded areas) are superimposed in each panel. Percent CXCR4 positive cells in each siRNA treatment in the shaded areas are indicated. A. CXCR4ur siRNA, B. CXCR4 wt siRNA, C. CXCR4mt siRNA. D. CD4 staining of mock and CXCR4wt siRNA transfected cells.

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To confirm these results, total levels of CXCR4 co-receptor protein in siRNA transfected cells were evaluated by immunoblotting analysis. The western blot data also showed a significant decrease (42%) in the levels of CXCR4 in wild type siRNA transfected cells consistent with FACS data (Fig. 3, lane 3). Both mock (lane 1) and unrelated siRNA (lane 4) transfected cells showed comparable levels of CXCR4 protein. Mutant siRNA transfected cells (lane 2) showed a 22% decrease in the amount of CXCR4 compared with the mock and and unrelated siRNA controls. Levels of cellular actin protein, used as an internal control, remained constant in all samples.

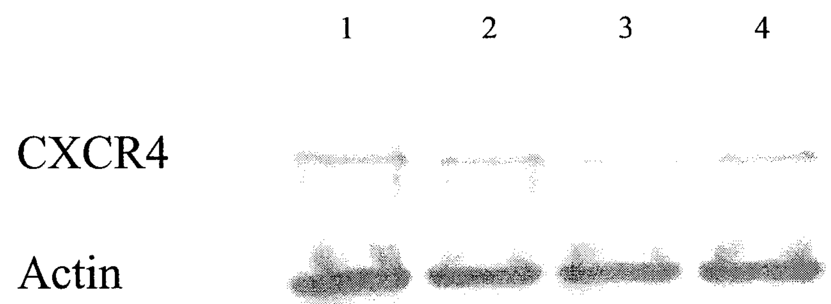

Figure 3: Western blot analysis of CXCR4 levels in siRNA transfected cells: Magi-CXCR4 cells transfected with respective synthetic siRNAs were collected at 48 hours post-transfection and subjected to immunoblotting detection as described in methods. Mock transfected cell lysates (1), CXCR4mt siRNA (2), CXCR4wt siRNA (3), CXCR4ur siRNA (4). Cellular actin was used as an internal control for quantitation. Intensity of bands was determined by densitometry.

Resistance of siRNA transfected cells to HIV-1 challenge:

The above results established the effectiveness of the siRNA in down regulating the co-receptor CXCR4 to a significant level. To determine if the co-receptor down regulation resulted in inhibition of HIV-1 entry, siRNA transfected Magi cells were

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challenged with an X4 tropic virus, HIV-1 NL4-3, 48hrs post-transfection. Magi cells are HeLa cell derivatives with an integrated silent LTR-β-galactosidase cassette, which is induced when Tat is produced upon HIV infection.[15-16] Control cells transfected with either mutant siRNA or unrelated siRNA construct were also similarly challenged. To detect cells that are productively infected during primary infection, cells were stained for *β*-galactosidase at 48hrs post-infection and blue cells were counted to quantify infection. CXCR4wt siRNA transfected cells were found to be markedly resistant to viral challenge as demonstrated by a twenty fold reduction in the number of infected cells (n=150) compared to that of mock transfected (n=3000) or CXCR4ur siRNA transfected cells (n=3120) (Fig. 4). Mutant siRNA transfected cultures displayed partial resistance (n=1600) consistent with FACS and western blot data in which a small reduction in CXCR4 expression was observed.

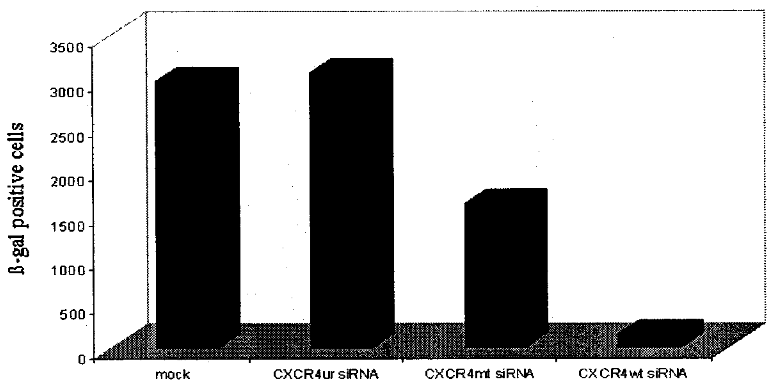

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Figure 4: Decreased frequency of infected cells in siRNA transfected cultures: Magi-CXCR4 cells transfected with respective siRNAs were challenged with HIV-1 NL4-3 at 48hrs. Two days after challenge, cultures were stained for ß-galactosidase production to detect infected cells. Individual infected cells were counted two days post-infection after staining (B). These results are representative of 3 independent experiments.

Results of the p24 assay of viral supernatants collected at different days post-infection of Magi-CXCR4 cells also showed marked suppression of viral infection by CXCR4wt siRNA. Moderate inhibition was shown by CXCR4mt siRNA (Fig.5).

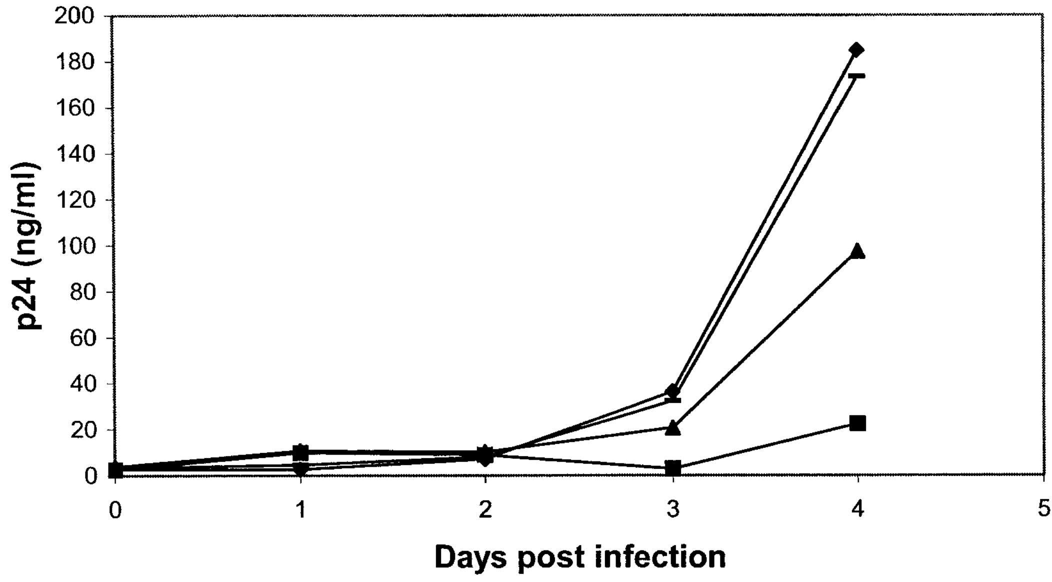

Figure 5: CXCR4 siRNA conferred resistance to HIV-1 challenge: Magi-CXCR4 cells transfected with respective siRNAs were infected with HIV-1 NL4-3. On days 1-4 post-infection, viral supernatants were collected and assayed for viral p24 antigen by ELISA. Mock (♦), CXCR4ur (-), CXCR4mt (▲), CXCR4wt (■).

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To confirm these results in a physiologically relevant setting, siRNA transfected primary lymphocytes were subjected to viral challenge. A three-fold reduction in viral production was seen on day three with viral levels showing an upward trend on day five most likely due to the transient effect of the transfected siRNAs. Similar to the data above, the mutant siRNA also exhibited partial protection (Fig. 6).

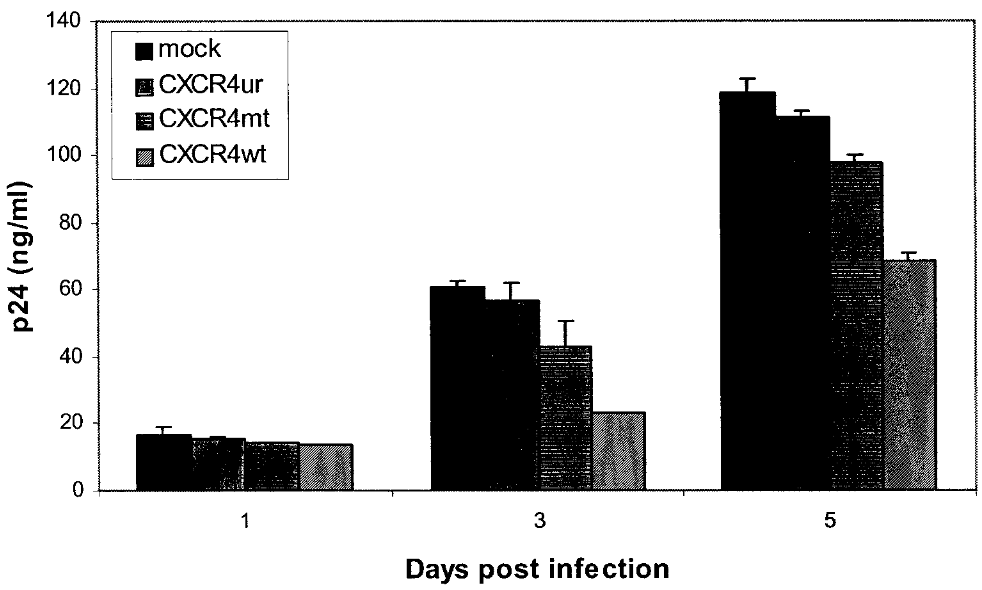

Figure 6: HIV-1 challenge of CXCR4 siRNA transfected PBMCs: PHA stimulated PBMCs were transfected with respective siRNAs as described in methods and challenged with HIV-1 NL4-3. Culture supernatants were assayed for p24 antigen by ELISA on different days post-infection.

DISCUSSION

The recent phenomenon of RNAi has reinvigorated research in targeted gene silencing and has paved the way for novel applications in gene therapy. The relatively simple methodology lends itself with ease for practical applications. As noted above, a number of recent reports documented the effectiveness of siRNAs in inhibiting HIV-1

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specific RNAs involved in viral replication at multiple levels including destruction of the incoming viral genome before reverse transcription. Approaches targeting essential cellular molecules necessary for viral entry also established the utility of down-regulating the cell surface primary receptor CD4 and co-receptor CCR5 in preventing HIV-1 infection. A notable advantage with targeting the cellular receptors is that it will be effective against a broad range of HIV strains, and is likely to minimize problems associated with viral mutation and consequent resistance.

In the present proof-of-concept studies we demonstrated the efficacy of a siRNA targeted to an essential cellular co-receptor CXCR4 in protecting against HIV-1 infection. All the approaches we used attest to the efficacy of siRNA in down regulating CXCR4 expression. FACS analyses indicated a decrease of 74% on cell surface expression which is also confirmed by marked decreased levels as seen in the western blot analysis when compared to that of levels seen in mock or unrelated siRNA tranfected cells. With regard to the mutant siRNA harboring a single nucleotide change, a 10% decrease was seen in cell surface expression together with a corresponding noticeable decrease in the protein level detected in the western blot. A possible reason for this could be an antisense effect since the construct is targeted to the 5' end, and may therefore interfere with the assembly of translation apparatus to some degree. Alternatively, it could be due to persistence of the RNAi effect even in the absence of a completely matched effector sequence in the siRNA. The specific inhibition by the wild type siRNA is clearly significant as demonstrated by much higher levels of down regulation (74%). In addition, the data also showed that siRNA transfected cells are markedly resistant to HIV-1 challenge. Inhibition of CXCR4 expression by wt siRNA resulted in a 20-fold reduction in viral

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infectivity. Interestingly, cells transfected with the mutant siRNA also showed significant viral inhibition (53%) in challenge assays despite displaying only a moderate 10% down regulation of CXCR4 on the cell surface. This suggests that even a small decrease in the CXCR4 surface expression can pose a dramatic hindrance to viral infection. The results of viral challenge experiments in primary lymphocytes also reflected the efficacy of CXCR4 siRNA in inhibiting viral entry, thus suggesting that the antiviral effect is applicable to a physiologically relevant setting. However, the levels of inhibition were substantially lower amounting to a three-fold reduction compared to a much higher level of inhibition seen in the Magi cell line. This could be due to sub-optimal levels of transfection of siRNAs into the primary lymphocytes. Nevertheless, further improvements in siRNA delivery and simultaneous targeting of multiple regions of the CXCR4 transcript are likely to increase efficacy.

Using non-lymphoid cell lines, a recent report has also shown the efficacy of a chemically synthesized CXCR4 siRNA in inhibiting HIV-1 entry.[17] Our present results confirm and extend these findings using a different construct and in primary lymphocytes. Furthermore, a stem-loop structured construct incorporating an improved design with inherent advantages was evaluated. Based on the remarkable efficacy shown *in vitro*, the anti-CXCR4 siRNA construct described here can be potentially harnessed for *in vivo* use. Although ideal for quick evaluation of different constructs *in vitro*, exogenous transfection of cells with pre-synthesized siRNAs is neither practical nor suitable for long-term gene therapy as the anti-viral effect is short lived. Endogenous expression is therefore necessary for sustained activity. Delivery of siRNAs via retroviral vectors will satisfy these criteria. Various configurations are possible to assemble siRNA

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motifs into retroviral vectors. Most of the recent studies using synthetic siRNAs employed preformed duplexes. In our current work, we transcribed a single RNA strand consisting of both sense and anti-sense sequences interrupted by a stretch of residues to yield a connecting loop. A recent study demonstrated the intracellular processing of stem loop structured transcripts and established their utility in facilitating the design and assembly of bi-specific or multi-specific constructs.[18] Therefore, a single promoter can be used in the vector construct to drive a transcript that has multiple siRNA motifs, which can be processed *in vivo*. Furthermore, it is likely that the stem loop structures will have an additional advantage of conferring a higher intracellular stability for siRNAs. Since both the sense and antisense strands are expressed as a single transcript with a connecting loop, an immediate annealing process is enforced spontaneously compared to transcripts expressed separately.[7] A number of unique features of HIV-1 that include high mutability pose considerable challenges in the design of completely efficacious gene therapeutic constructs. Therefore approaches that target different stages of the viral life cycle that include viral entry, pre- and post-integration steps are desirable. The stem loop structured transcript design will permit assembly of multiple siRNA motifs such as those incorporating anti-CXCR4, anti-CCR5, and anti-Rev siRNA constructs. Experiments utilizing such constructs are currently in progress.

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CHAPTER 3

POTENT SUPPRESSION OF CCR5 EXPRESSION AND HIV-1 INFECTION BY TRANSFECTED AND LENTIVIRAL VECTOR EXPRESSED shRNAs

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ABSTRACT

The recently discovered phenomenon of RNA interference has been shown to be highly potent and sequence specific in targeted gene silencing. Due to the mechanism of action of homologous mRNA degradation, RNAi has the potential for use as a therapuetic in combating HIV infections. By targeting the critical coreceptors CCR5, infections can be inhibited at the level of viral entry. CCR5 has been shown to be dispensable for normal physiology and is, therefore, an excellent target for gene therapy applications. In the present study, CCR5 expression was targeted by highly potent siRNAs that were capable of almost complete knockdown. These observations were confirmed by both FACS analysis for cell surface expression and quantitative real time PCR to detect intracellular transcript levels. When challenged with R5-tropic BaL-1 HIV-1, cells exhibited significant resistance to infection. These results demonstrate the effectiveness of these specific siRNAs targeting CCR5 for down regulation and their subsequent ability to protect cells from R5-tropic HIV-1 infections.

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INTRODUCTION

The use of intracellular immunization against HIV infections holds considerable promise as an antiviral therapeutic. With the recent discovery of RNAi and its highly potent and specific mechanism of post-transcriptional gene silencing, a novel tool can be added to the arsenal of anti-HIV genes. In the initial stage of HIV infection, virus particles have to attach and fuse with the target cell through the interaction with a major receptor, CD4, and a critical coreceptor, CCR5 or CXCR4.[1] Primary infection is established by R5 macrophage tropic HIV-1 that uses CCR5 as a coreceptor. These R5-tropic strains are mainly non-syncytia inducing and predominantly infect macrophages.[1] Targeting CCR5 for down regulation using siRNAs should theoretically inhibit HIV entry into host cells. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of this coreceptor thus conferring significant resistance to HIV infection.[2-4] Homozygous or heterozygous individuals for this mutation remain physiologically normal. These findings suggest that CCR5 is an excellent candidate for gene knockdown to inhibit HIV infections. Here I show the results of si/shRNAs that are highly effective in their ability to knockdown almost complete expression of CCR5 cell surface expression as well as intracellular transcript levels. Through standard transfection methods and lentiviral transduction of HIV susceptible cells, the subsequent down regulation of CCR5 surface expression conferred HIV resistance. Thus, these findings suggest that modulating CCR5 expression through the use of siRNAs holds promise for inhibiting HIV infections in a gene therapy setting.

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MATERIALS AND METHODS

***CCR5 siRNA design and transfection* :**

siRNAs targeting CCR5 were designed by Dharmacon (Lafayette, CO) using a proprietary algoritham for detecting highly effective siRNAs. The first set of siRNAs are 19mers consisting of a sense and antisense strand annealed together with two 3' uracil overhangs (#B, #4, #5, #6, #7). The target sequences are as follows: #B-5'-GUGUCAAGUCCAAUCUAUG-3', #4-5'-GAAGGUGUCAGAAUAAUAA-3', #5-5'-GUUCAGAAACUACCUCUUA-3', #6-5'-GAGCAUGACUGACAUCUAC-3', #7-5'-UCUUUGGCCUGAAUAAUUG-3'. The second set of siRNAs are 28mers with the sense and antisense sequences connected by the loop sequence AUAUGUG (#BB, #4B, #5B, #6B, #7B). These siRNAs target the same regions as stated above.

Cell cultures and siRNA transfection:

Ghost R5/X4/R3 cells that constitutively express the HIV-1 co-receptor CCR5 on their cell surface were obtained from the AIDS Reference and Reagent Program. They were maintained in DMEM medium containing 10% heat inactivated 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 hours before transfection. Lipid-RNA complexes were prepared by incubating various amounts of the appropriate siRNA with 4 μl lipofectamine (Gibco-Invitrogen, Rockville, MD) at room temperature for 25 minutes. 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 hours followed by the addition of 1 ml DMEM containing 10% heat inactivated FBS. Transfections were repeated on days 2 and 3.

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After allowing the siRNAs to induce their effect, the transfected cells were then used for flow cytometric and quantitative PCR analyses to determine the down regulation of CCR5 surface expression and transcript levels, respectively.

Flow cytometry:

To determine the effect of transfected siRNAs on the cell surface expression of CCR5, FACS analysis was performed. Seventy-two hours post-transfection, cells were washed twice with 0.5% BSA/PBS. Phycoerythrin (PE)-conjugated mouse anti-human CCR5 and matching isotype (Pharmingen, San Jose, CA) were used for staining. Data was obtained and analyzed on Coulter EPICS®XL-MCL with EXPO32 ADC software (Coulter Corporation, Miami, FL).

Quantitative PCR:

To confirm the results obtained by FACS analysis, quantitative PCR was performed on siRNA transfected cells to determine the levels of CCR5 transcripts present inside the cells. Total RNA was isolated from siRNA transfected cells using RNAzol as described by the manufacturer. Using a Superscript-III First Strand Synthesis kit (Invitrogen, Carlsbad, CA), 5ug cDNA was generated from cellular RNA. Quantitative real time PCR was then performed on 10ng cDNA using the SYBR Green kit (Invitrogen, Carlsbad, CA). QT-PCR was run and analyzed on the BioRad iCycler. Glyceraldehyde-6-phosphate was used as an internal control.

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HIV-1 challenge of siRNA transfected cells:

Ghost R5/X4/R3 cells are susceptible to infection with R5-tropic HIV-1 BaL-1, as they express the respective coreceptor, CCR5. To determine viral resistance conferred by the down regulation of CCR5, siRNA transfected cells were subjected to viral challenge *in vitro*. At 72 hours post-transfection, culture media was removed and virus was added to the cells (m.o.i. 0.01) in the presence of polybrene (4 μg/ml). Virus was allowed to adsorb for 2 hours at 37°C. Cells were then washed twice with PBS and 2 ml of complete DMEM was added. Culture supernatants collected at different days post-challenge were assayed for p24 antigen by ELISA (Beckman-Coulter, Fullerton, CA).

Plasmid and lentiviral vector construction:

A third generation lentiviral vector backbone was employed to derive the CCR5 constructs. An siRNA expression cassette targeting CCR5 under the control of the Pol-III H1 promoter was PCR amplified from the plasmid pSUPER (Oligoengine, Seattle, WA) as described by Castanotto *et al.*[5] This cassette was cloned into the pHIV-7-GFP transfer vector in a *BamHI* site immediately upstream of the CMV-EGFP reporter gene. Sequencing and confirmation of candidate clones was performed by Laragen Inc. (Los Angeles, CA).

293T cells, used for vector production were maintained in DMEM supplemented with 10% FBS. To generate lentiviral vectors, fifteen micrograms of transfer vector either GFP-alone or one of the CCR5 constructs were transfected along with 15ug pCHGP-2, 5ug pCMV-Rev, and 5ug pCMV-VSVG into 293T cells at 60% confluency in 100mm culture dishes using a calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO). Six hours after transfection, fresh medium was exchanged. Cell culture

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supernatants containing the vector were collected at 24, 36, 48, and 60 hours post tranfection and pooled. Vector supernatants were concentrated by ultracentrifugation and later titrated on 293T cells using FACS analysis for GFP expression.

Lentiviral vector transduction and FACS analysis:

Ghost R5/X4/R3 cells were seeded in 6-well plates 24 hours prior to transduction, $5x10^5$ cells per well. Cells were transduced with lentiviral vectors at an m.o.i. of 20 in the presence of 4ug/ml polybrene for 2 hours. Seventy-two hours post transduction, cells were sorted for EGFP expression. FACS analysis was then performed to determine the levels of CCR5 cell surface expression. Non-transduced and transduced cells were stained with an anti-CCR5 antibody conjugated with PE (Pharmingen, San Diego, CA). FACS analysis was performed on the Beckman Coulter Epics XL using ADC software for analysis.

HIV-1 Challenge:

To determine if CCR5 down regulation conferred HIV-1 inhibition, non-transduced and transduced cells were challenged with BaL-1 (R5-tropic) HIV-1, at an m.o.i of 0.01, as described above. Viral supernatants were collected at various days post-infection and analyzed for p24 by ELISA employing a Coulter-p24 kit (Beckman Coulter, Fullerton, CA).

RESULTS

Down regulation of CCR5 in siRNA transfected cells:

Following transfection of Ghost R5/X4/R3 cells with 640nM of the respective siRNA constructs, cells were analyzed by flow cytometry to determine the down

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regulation of CCR5 surface expression. As shown in Figure 1, siRNAs #B, #5, #6, #BB, #5B, and #6B demonstrated significant down regulation of CCR5 surface expression. Only 7, 10, 9, 6, 2, and 3% of cells were positive for CCR5 expression, respectively. These values were determined in comparison to non-transfected and unrelated siRNA transfected control cells. Four of the siRNAs, #4, #7, #4B, and #7B did not work as effectively at down regulating expression with 71, 46, 73, and 33% of cells still expressing CCR5, respectively. These four siRNAs were disregarded for further analysis due to their inefficient activity.

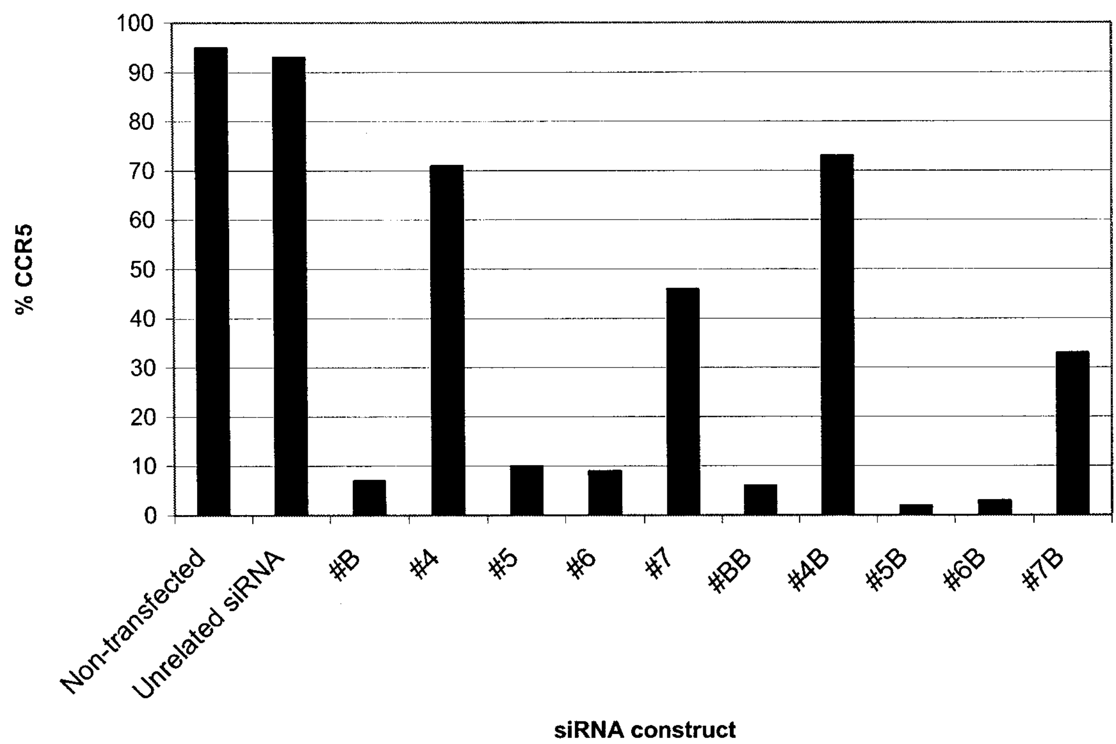

Fig 1. Down regulation of CCR5 in transfected cells at 640nM siRNA concentration: At 72 hours post-transfection, cells were stained with PE-conjugated antibodies to CCR5 and analyzed by FACS.

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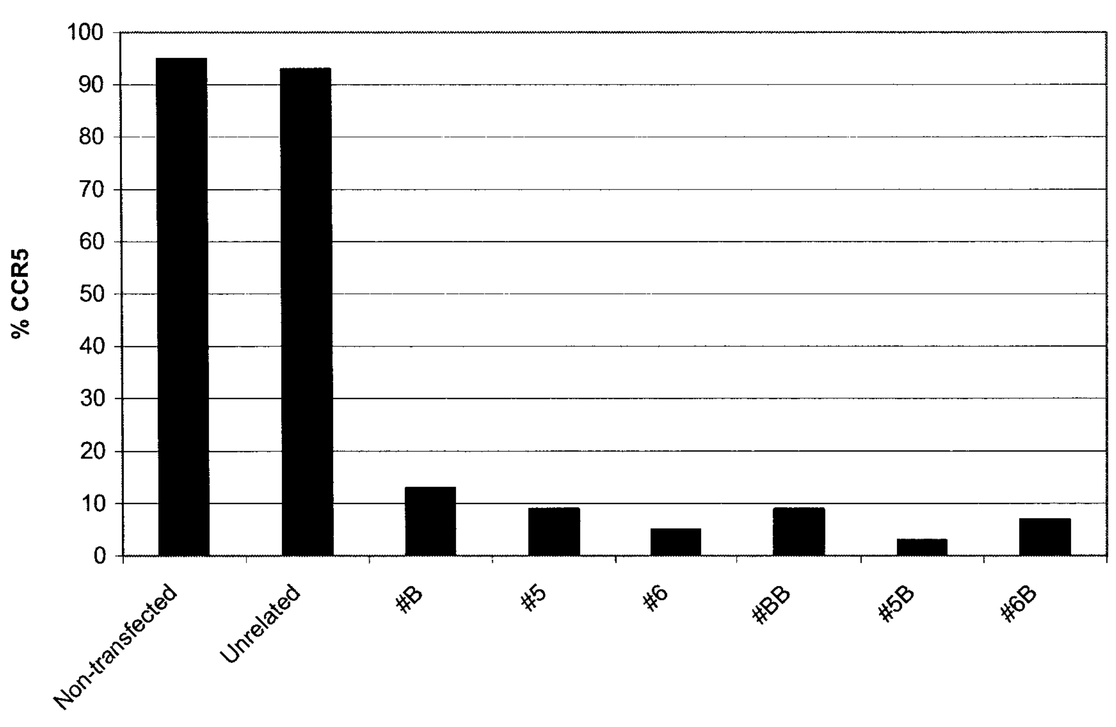
As these results demonstrated significant down regulation of CCR5 expression at a relatively high concentration of siRNAs (640nM), lower amounts were used to determine if knockdown ability was retained. Due to toxic effects that can be generated by large amounts of siRNAs as well as off-targeting effects, highly efficient siRNAs need to be discovered that work at minute concentrations. Decreasing concentrations, 320nM, 64nM, and 6.4nM, were then used via transfection to determine siRNA activity. FACS analysis of CCR5 surface expression demonstrated that, even at 100-fold lower concentrations, these siRNAs still significantly down regulated CCR5 surface expression (Fig 2).

A



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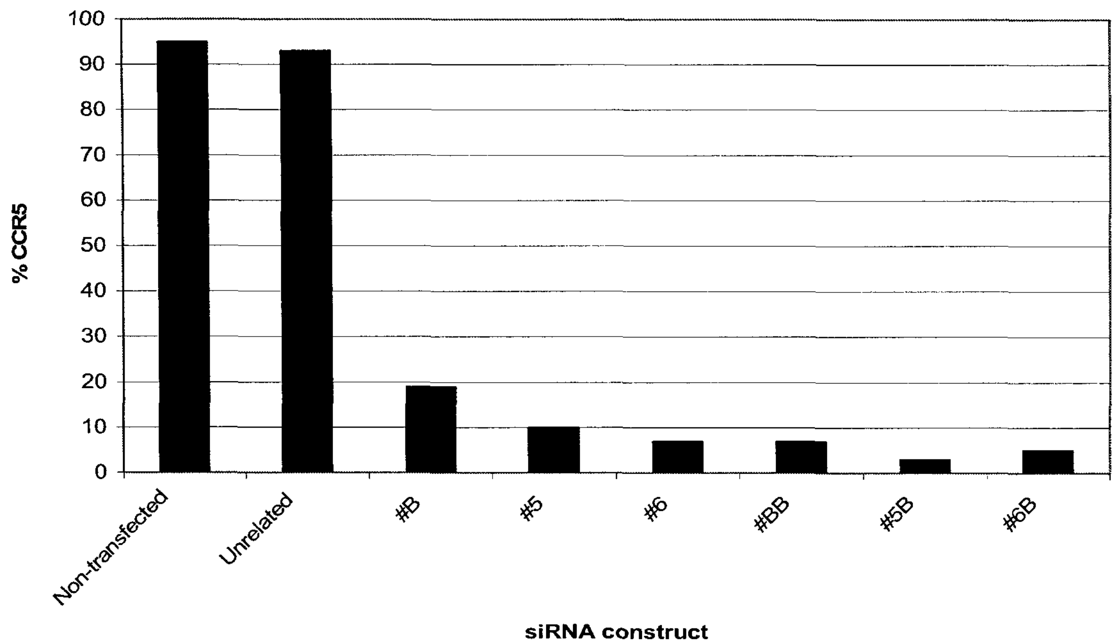
B



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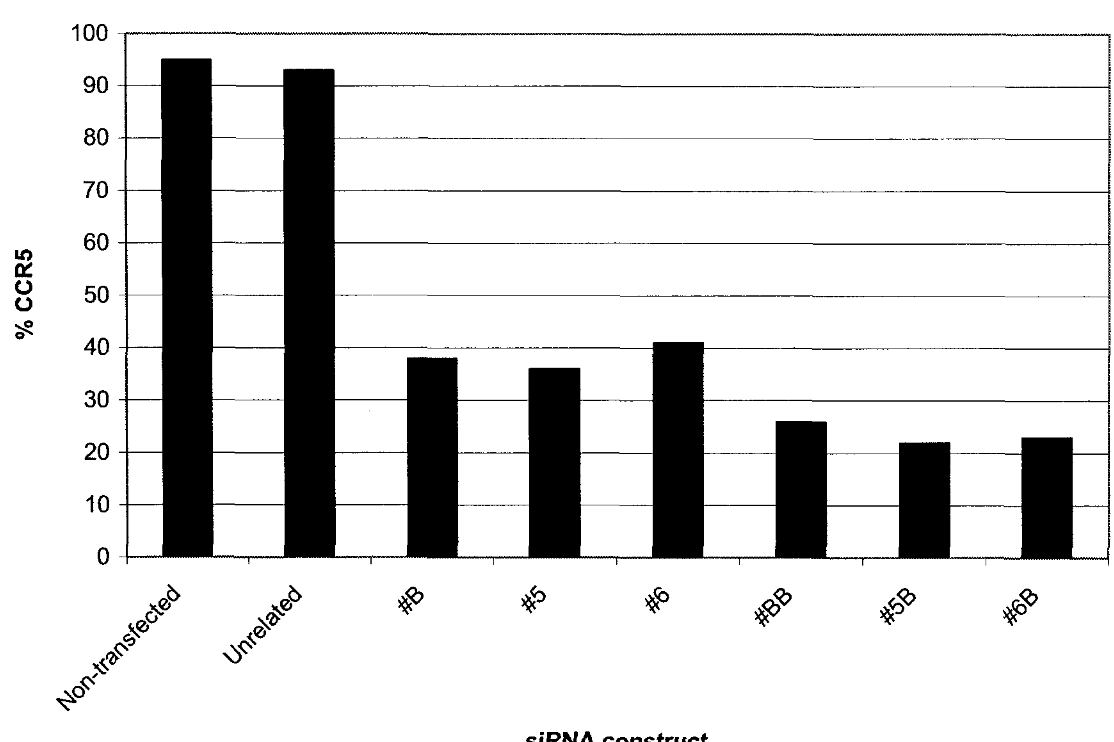

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Fig 2. Down regulation of CCR5 in transfected cells at decreased siRNA concentrations: At 72 hours post-transfection, cells were stained with PE-conjugated antibodies to CCR5 and analyzed by FACS. A) 320nM siRNA. B) 64nM siRNA. C) 6.4nM siRNA.

In order to investigate the down regulation of CCR5 expression with increased sensitivity, quantitative real time PCR was performed to quantitate transcript RNA levels. As shown in Figure 3, complete knockdown of CCR5 transcript expression was achieved using these shRNA constructs at a concentration of 64nM. Control, glyceraldehyde-6-phosphate transcript levels were similar for all samples tested.

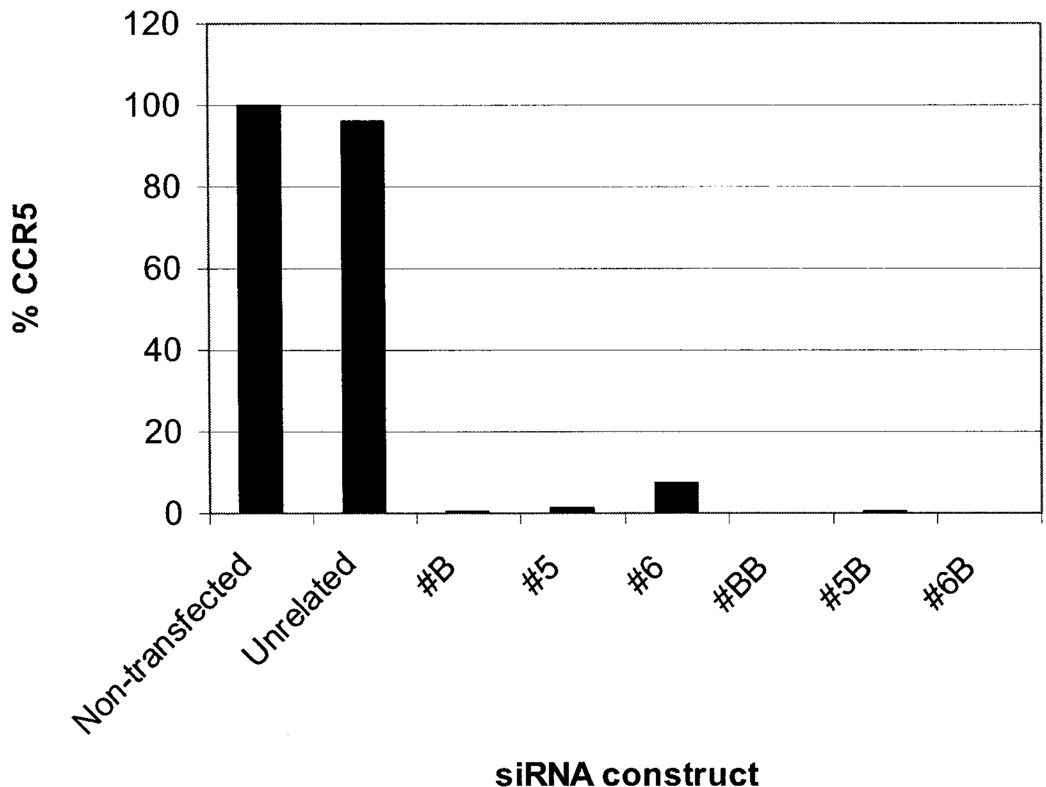

Fig 3. Quantification of CCR5 transcript down regulation. Total RNA was extracted from siRNA transfected cells and analyzed by quantitative real time PCR. Bar graphs represent percent CCR5 transcript levels.

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To determine if down regulation of the essential coreceptor CCR5 conferred viral resistance, transfected Ghost R5/X4/R3 cells were challenged with R5-tropic HIV-1 BaL-1. Viral p24 antigen levels at different days post-challenge were determined by ELISA to quantify levels of HIV-1 resistance. Over a 9-fold reduction in viral antigen levels was seen with #B, #5, #6, #BB, #5B, and #6B transfected ghost cells as compared to non-transfected, unrelated, #4, and #7 transfected cells (Fig 4).

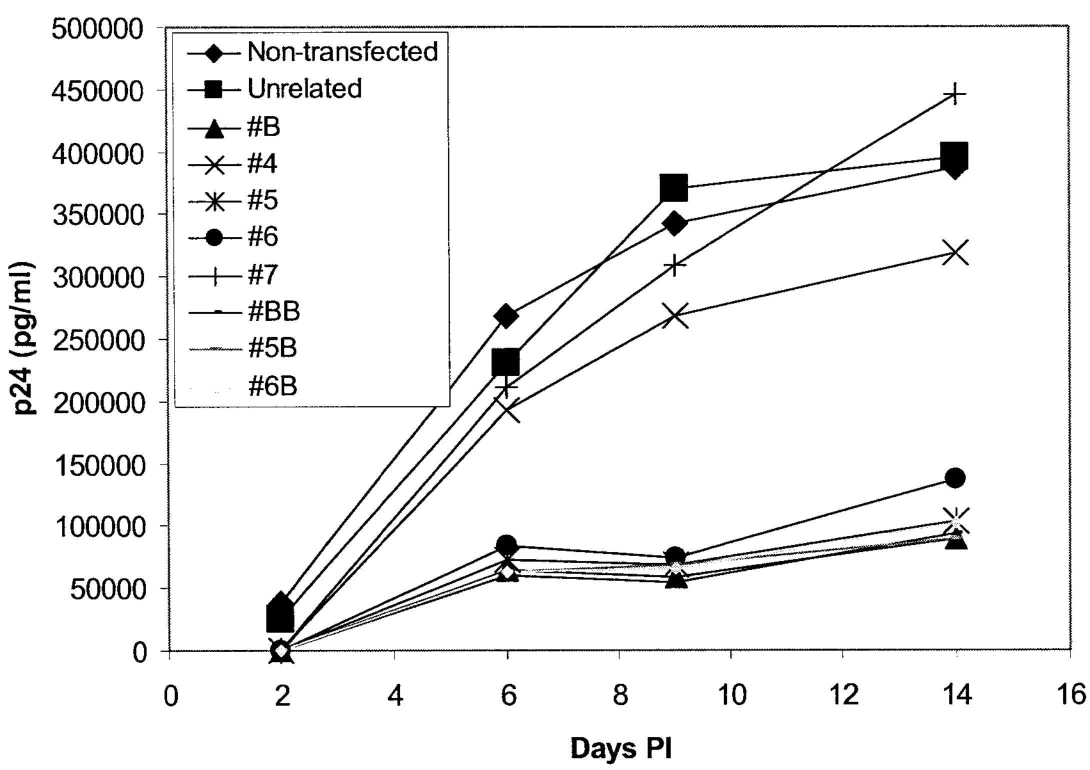

Fig 4. HIV-1 challenge of CCR5 siRNA transfected Ghost R5/X4/R3 cells. siRNA transfected cells were challenged with R5 tropic BaL-1 at an m.o.i of 0.01. Culture supernatants were collected at different days post challenge and p24 antigen was assayed by ELISA.

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As the effect of transfected siRNAs is only transient due to eventual degradation and dilution of siRNAs during cell division, constitutive expression via lentiviral transduction holds greater promise for siRNA therapy. Thus the major goal in these studies is to introduce highly effective CCR5 siRNAs into a single lentiviral construct to achieve stable expression in transduced cells. Lentiviral vectors offer advantages over conventional retroviral vectors as they can transduce dividing as well as nondividing cells and are less prone to transgene silencing.[6-9] Highly efficient siRNA constructs, #B, #5, #6, #BB, #5B, and #6B were cloned separately into a third generation lentiviral vector in which expression of the shRNA was driven by an H1 promoter. Downstream, the reporter gene, EGFP is driven by a CMV promoter (Fig 5.). The control GFP-alone vector, HIV-7-GFP, contains only the reporter gene and is used as a control for transduced cells.

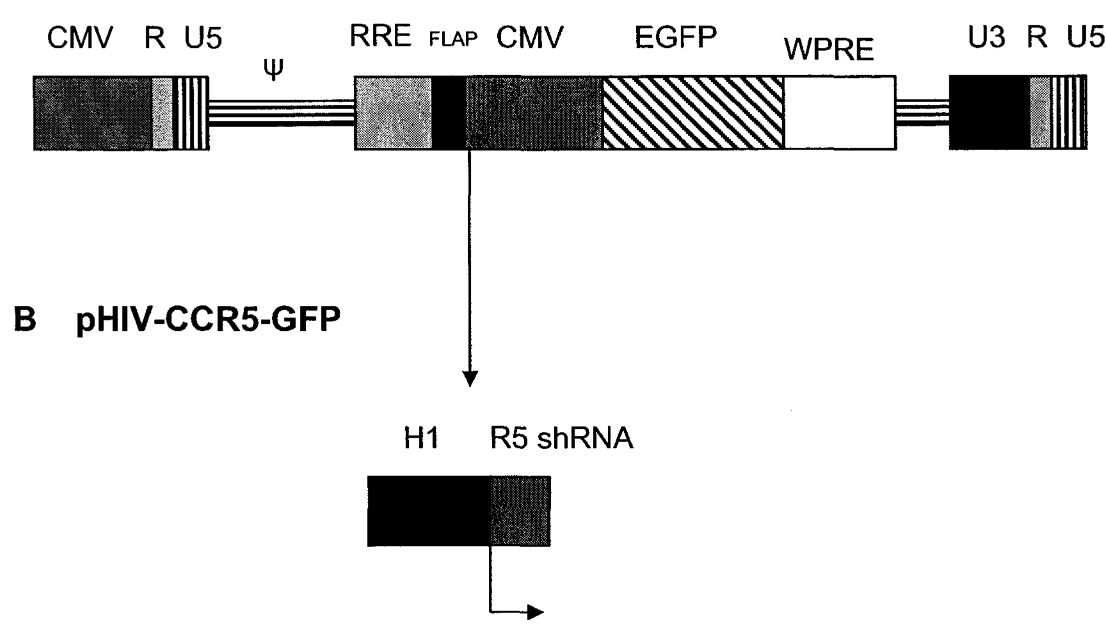

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Fig 5. Lentiviral vector encoding anti-CCR5 siRNAs. A) Control transfer vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. B) To derive the vectors containing anti-CCR5 siRNAs, highly effective siRNA constructs were cloned under the control of the H1 promoter upstream to the CMV-EGFP cassette.

Ghost R5/X4/R3 cells constitutively expressing CCR5 on the cell surface were transduced with the various lentiviral vectors constructed, both control GFP-alone and the six CCR5 vectors. Transduced cells were sorted by FACS for EGFP expression and analyzed for CCR5 surface expression by flow cytometry. Substantial down regulation was seen for all CCR5 vector transduced cell populations as compared to non-transduced and GFP-alone transduced cells (Fig 6.).

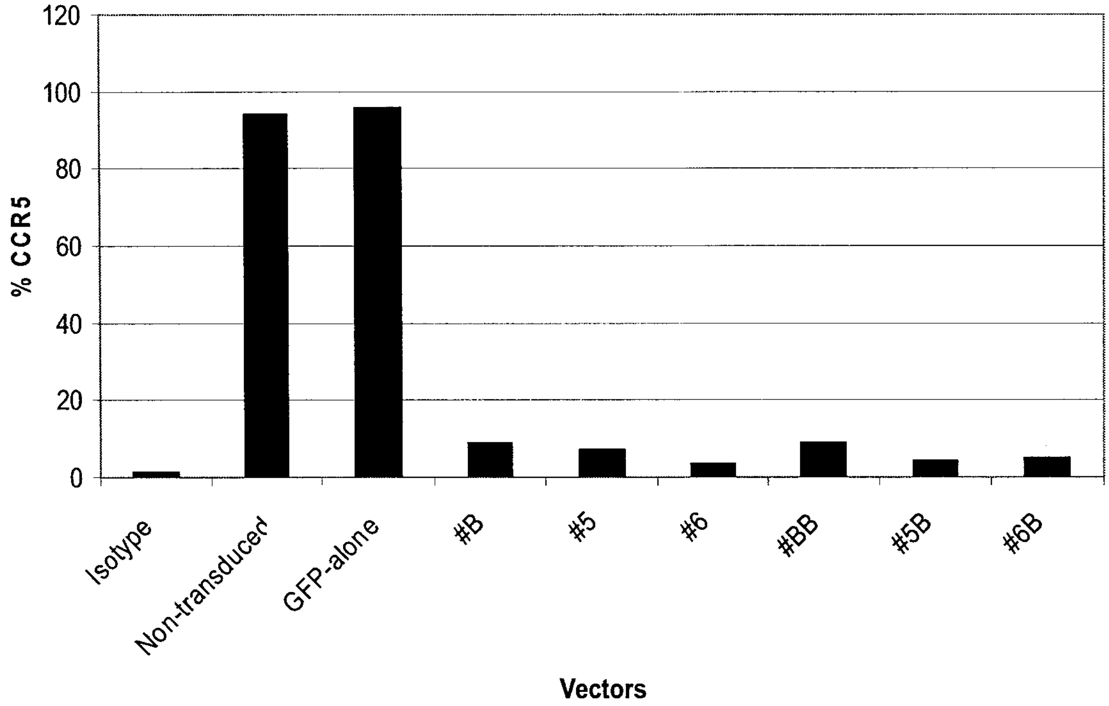

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Fig 6. Down regulation of CCR5 surface expression from lentiviral vector transduced cells. Ghost R5/X4/R3 cells were transduced with various vectors containing anti-CCR5 siRNA expression cassettes. Cells were analyzed for CCR5 surface expression by FACS.

To determine whether the down regulation of CCR5 surface expression conferred viral resistance, transduced cells were challenged with R5-tropic BaL-1 HIV. Viral p24 antigen levels at different days post-challenge were determined by ELISA to quantify levels of HIV-1 resistance. Over a 10-fold inhibition was seen with CCR5 vector transduced cells compared to non-transduced and GFP-alone transduced cells (Fig 7.)

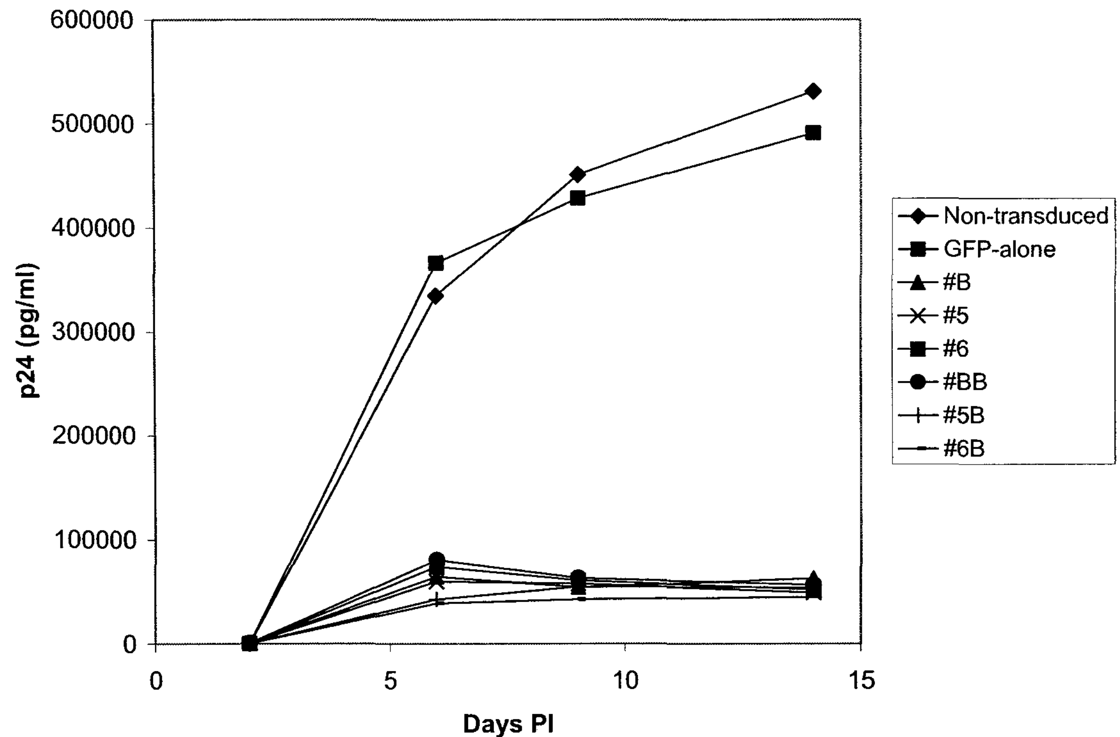

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Fig 7. HIV-1 challenge of CCR5 shRNA transduced cells. Ghost R5/X4/R3 cells were transduced with various CCR5 vectors and challenged with R5-tropic BaL-1 HIV-1 at an m.o.i. of 0.01. Cell culture supernatants were collected on various days post-infection and assayed for viral p24 via ELISA.

These results established that all of the CCR5 vectors analyzed were effective in inhibiting HIV-1.

DISCUSSION

In summary, these studies have shown that a lentiviral vector could be used to stably deliver highly effective siRNAs targeted to CCR5 and achieve protection against R5 tropic HIV-1. The short hairpin design allows for the use of a single promoter to transcribe both the sense and anti-sense strands of each of the siRNAs in the same transcript.

A major advantage in using a CCR5 siRNA construct to prevent HIV-1 infections is that it inhibits the entry of the virus. This provides protection from viral integration, latency, and the generation of resistant mutants. Another advantage of targeting CCR5 for down regulation comes from the fact that CCR5 is dispensable for normal physiology. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of coreceptor function thus conferring significant resistance to HIV infection.[2-4] Homozygous or heterozygous individuals with this mutation remain physiologically normal. These findings offer hope for the use of CCR5 knockdown in HIV gene therapy.

I have shown here that protection from HIV-1 infection can occur by targeting the cellular gene, CCR5, by siRNA mediated down regulation. By targeting CCR5, the virus

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is blocked at the cell surface and can not enter the cell. A number of CCR5 siRNAs were initially tested to determine their efficacy in down regulating CCR5 expression. Of these siRNAs, #B, #5, #6, #BB, #5B, and #6B showed significant reductions in CCR5 transcript levels and cell surface expression. The addition of a loop sequence connecting the sense and antisense strands allowed for their incorporation into a lentiviral vector expression cassette. As transfected siRNAs only create a transient effect due to dilution in cell division and eventual degradation, using lentiviral vectors for constitutive expression of siRNAs is more optimal for long term gene therapy.

In both transfected and transduced cells, greater than 97% of cellular CCR5 transcripts and 90% of CCR5 cell surface expression was abolished by the RNAi machinery. The low level expression of CCR5 seen by FACS analysis may be due to the half-life of the existing cell surface CCR5 or nonspecific binding of the FACS antibody. The substantial knockdown of CCR5 expression translated into significant viral inhibition. Over a 1-log inhibition was seen in viral infection for CCR5 lentiviral vector transduced cells compared to control cells. As seen in Fig. 7, viral replication was completely inhibited in CCR5 shRNA transduced cells.

Numerous studies have shown that both transfected and transduced CCR5 siRNAs can down regulate expression and inhibit HIV-1 infection.[10-13] However, the siRNAs used were not effective at down regulating CCR5 expression completely. Here we describe the use of three siRNAs that work at almost complete knockdown of both transcript and cell surface expression of CCR5. Finding the most efficacious siRNA for CCR5 down regulation is important as to not leave any surface expression possible which

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may lead to a low level of infection. Achieving complete knockdown is essential if CCR5 siRNAs are going to be used for future gene therapy applications.

As HIV can be both R5 and X4-tropic in nature, when designing anti-HIV constructs, care should be taken as to not select for a particular strain. Therefore, targeting both CCR5 and CXCR4 by siRNA knockdown will offer protection from both X4 and R5-tropic strains of HIV-1. Adding additional transgenes that target HIV transcripts will offer additional protection and help avoid selection of a particular strain.

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CHAPTER 4

BISPECIFIC SHORT HAIRPIN siRNA CONSTRUCTS TARGETED TO CD4, CXCR4, AND CCR5 CONFER HIV-1 RESISTANCE

Anderson J, Banerjea A, Akkina R: Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. Oligonucleotides 2003, 13:303-312.

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ABSTRACT

Exploiting the phenomenon of RNA interference, recent studies established the utility of mono-specific siRNAs in suppressing HIV-1 infection. However, due to the high mutation rate of the HIV genome, considerable challenges exist in the design of fully efficacious gene therapeutic constructs. Therefore, approaches that simultaneously target different stages of the viral life cycle are desirable. In our current studies, we designed bi-specific combinatorial siRNA constructs against HIV-1 cell surface receptors to inhibit viral entry. Dual specific short hairpin siRNA constructs, containing an 8-nucleotide intervening spacer, targeted against either CXCR4 and CD4 or CCR5 and CXCR4 were synthesized by *in vitro* transcription. Cleavage of the combinatorial constructs yielding mono-specific siRNAs was shown to occur in cell extracts. Magi-CXCR4 and CCR5 cells transfected with the transcribed siRNAs showed significant down regulation of their respective co-receptors as determined by FACS analysis. This suggested that combinatorial constructs comprising of multiple effector motifs were processed in transfected cells into their respective functional siRNAs. Transfected cells were challenged with either X4 (NL4-3) or R5-tropic (BaL-1) strains of HIV-1. Down regulation of the cell surface receptors coincided with resistance to *in vitro* viral challenge in both Magi cell lines and in PBMCs. These results demonstrated the practical utility of short hairpin siRNA combinatorial constructs synthesized as a single transcript. Since the short hairpin design will permit tandem assembly of multiple effector motifs, it is now possible to introduce promising multi-specific siRNA constructs into retro- and lentiviral vectors for *in vivo* gene therapeutic applications.

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INTRODUCTION

HIV continues to be a global health threat as an estimated 42 million people are currently living with the virus. In view of the lack of effective vaccines and the development of resistant strains to current drug-based therapies, alternative treatment strategies need to be developed. Intracellular immunization against HIV through gene therapy strategies offers a promising approach. A number of approaches have previously been tried in experimental settings with varying efficacies. These consisted of RNA decoys[1-2], ribozymes[3-7], and transdominant proteins[8-10]. Although these strategies offered an initial promise, the levels of viral inhibition were not enough to confer optimal protection. Therefore, further refinements in these approaches are needed. The recently described phenomenon of RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) holds new promise in further expanding gene therapeutic strategies.

RNAi is a mechanism of post-transcriptional gene silencing mediated by double stranded siRNAs, ranging in size from 19-24 nt in length, which can be targeted to any gene of interest[11-14]. Silencing is carried out by an endogenous RNase III-like endonuclease, dicer, that uses specific siRNAs as triggers for cleaving the target homologous mRNA. As the effector RNAs are small in size, RNAi provides a specific response distinct from that of the nonspecific interferon-mediated response. Several recent reports showed its efficacy in inhibiting HIV replication at different stages of the viral life cycle in infected cells[15-22]. The levels of viral suppression mediated by siRNAs appear to be much higher than achieved with previous approaches.

Most of the anti-HIV siRNA constructs introduced into the target cells were either directly transfected or generated endogenously by expression plasmids[15-18]. They

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were designed to interfere with viral replication by targeting HIV regulatory or accessory protein coding mRNAs. Other studies have targeted host cell surface proteins such as the primary receptor, CD4[18], and the co-receptors CCR5[19-21] and CXCR4[20,22], which are required for HIV entry[23-24]. The propensity of HIV to mutate and become resistant to mono-specific constructs highlights the necessity for a combinatorial approach that incorporates constructs effective against multiple targets.

Primary infections are established by macrophage tropic HIV-1 that uses CCR5 as the co-receptor, whereas X4-tropic viruses that predominate late in infections utilize CXCR4 as the major co-receptor[23-24]. Strategies that are designed to inhibit viral entry by focusing on a single receptor will not be completely effective due to the existence of dual tropic viruses and evolution of viral tropism during disease progression. To overcome this limitation, in the current studies we targeted both co-receptors, CCR5 and CXCR4, with the aid of a combinatorial construct. Using yet another combinatorial construct, both the primary receptor CD4 and a co-receptor, CXCR4, were targeted. To generate these constructs, we employed a short hairpin design that allowed linking of two mono-specific siRNA constructs. Using this strategy we show that a marked down regulation of the respective cell surface receptors could be achieved with consequent inhibition of HIV entry, thus affording antiviral protection.

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MATERIALS AND METHODS

Design and synthesis of combinatorial siRNA constructs:

In designing the combinatorial constructs, we employed the strategy of using a T7 polymerase driven transcription system to yield the siRNA products[25]. The synthetic oligonucleotide templates contained a T7 promoter sequence allowing *in vitro* transcription of the siRNA (Fig. 1) using Riboprobe® System-T7 (Promega, Madison, WI). The sequences contained both sense and antisense siRNA motifs with an intervening loop sequence representing a short hairpin siRNA structure[26-27]. Mutant constructs containing four point mutations were also designed to test the specificity of wild type constructs. In the combinatorial constructs containing two siRNAs, the siRNA sequences were arranged in tandem separated by an 8-nucleotide spacer sequence (Fig. 2). The spacer sequence used by Leirdal and Sioud, in a bi-specific short hairpin construct targeted to Protein Kinase C and GFP consisted of a string of eight uridines[28]. As such a spacer sequence is not suitable for use in expression vectors, we experimented with three different spacer sequences, namely 1) UUCAAGAGA, 2) UUCAAGAGAUU, and 3) UUCAACUU of which the third sequence was found to be optimal. The siRNA constructs were targeted against sequences in the HIV co-receptors CXCR4 and CCR5 as well as the primary receptor CD4. The combinatorial constructs consisted of combinations CXCR4/CD4 and CCR5/CXCR4.

The T7 promoter complimentary sequence (lowercase) is positioned at the 3' end of the oligo. Nucleotide changes in mutant siRNAs are also shown in lowercase in the sense and antisense sequences, and the spacer sequence in the combinatorial construct is in lowercase and italicized: The schematics depicting the template oligo incorporating

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these sequences, panel A, and the expected products of *in vitro* synthesis, panels B and C, are presented in Fig. 2. The sequences of the different siRNA templates are as follows: 1) CXCR4wt (wild-type): 5'- AAGGAGGGGATCAGTATATACAC/TCTCTTGAA/ GTGTATATACTGATCCCC TC/ctatagtgagtcgtatta- 3'. 2) CXCR4mt (consisting of 4 point mutations at positions 5, 7, 12, and 15): 5'- AAGGAGtGaATCAcTAgATACAC/ TCTCTTGAA/ GTGTATcTAgTGATtCaCTC/ctatagtgagtcgtatta- 3'. 3) CCR5wt: 5'- AAGGGCTCTATTTTATAGGCTTC/TCTCTTGAA/GAAGCCTATAAAATAGAGCC /ctatagtgagtcgtatta- 3'. 4) CCR5mt (consisting of 4 point mutations at positions 3, 6, 9, and 12): 5'- AAGGcCTaTAgTTgATAGGCTTC/TCTCTTGAA/GAAGCCTATcAA cTAtAGgC/ ctatagtgagtcgtatta- 3'. 5) R5/X4 (CCR5/CXCR4 combinatorial construct): 5'- AAGGAGGGGATCAGTATATACAC/TCTCTTGAA/GTGTATATACTGATCCC CTCC/*aagttgaa*/GGGCTCTATTTTATAGGCTTC/TCTCTTGAA/GAAGCCTATAAA ATAGAGCC/ctatagtgagtcgtatta- 3'. 6) X4/CD4 (CXCR4/CD4 combinatorial construct): 5'- AAGATCAAGAGACTCCTCAGTGA/TCTCTTGAA/TCACTGAGGAGTCTCT TGATC/*aagttgaa*/GGAGGGGATCAGTATATACAC/TCTCTTGAA/GTGTATATACT GATCCCCTC/ctatagtgagtcgtatta- 3'. The T7 promoter oligo used for priming *in vitro* transcription has the sequence: 5'-TAATACGACTCACTATAG-3'. The siRNA construct targeted against CD4 was shown previously to be effective in down regulating expressionof CD4[18]. PAGE purified synthetic oligos were obtained from Integrated DNA Technologies, Coralville, IA.

The respective siRNA transcripts were synthesized *in vitro* by T7 polymerase mediated transcription using a T7 primer from their respective templates as previously described.[25] The *in vitro* derived siRNA products (yielding ~20 μg/reaction) were

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analyzed by gel electrophoresis to confirm their sizes. The siRNAs were dissolved in nuclease-free water and stored at -70°C until further use.

Cell culture, siRNA transfection and intracellular cleavage:

U373-Magi-$CXCR4_{CEM}$[29] and CCR5[30] indicator cells that constitutively express the respective HIV-1 co-receptors were obtained from the AIDS Reference and Reagent Program. Magi-CXCR4 cells were maintained in DMEM containing 10% heat inactivated FBS, 0.2 mg/ml G418, 0.1 mg/ml hygromycin B, and 1.0 μg/ml puromycin. Magi-CCR5 cells were maintained in DMEM containing 10% heat inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml glutamine, 0.2 mg/ml G418, 0.1 mg/ml hygromycin B, and 1.0 μg/ml puromycin. Cells were plated 24 hours prior to transfection in 3.5 cm wells at 1.0×10^6 cells/well. Lipid-RNA complexes were prepared by mixing 3.0 μl Lipofectamine (Gibco-Invitrogen, Rockville, MD) with 2 μg of appropriate siRNA at room temperature for 25 minutes. These complexes were added to the cells in a final volume of 700 μl and incubated overnight at 37°C. Cells were then washed and 2 ml of complete medium was added. Transfections were repeated on days 2 and 3. Transfected cells were tested by flow cytometry to determine cell surface down regulation of the respective receptors. For both FACS analysis and HIV-1 challenge experiments, the X4/CD4 construct was tested in Magi-CXCR4 cells and the R5/X4 construct was tested in both Magi-CCR5 and Magi-CXCR4 cells. Peripheral blood mononuclear cells (PBMCs) were also used to determine the efficacy of these constructs in human lymphocytes. PBMCs were stimulated in the presence of 3.0 μg/ml PHA and

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1.0 μg/ml IL-2 for 3 days and transfected with siRNAs as described above. These cells were then used for HIV-1 challenge experiments.

To determine if cleavage of combinatorial constructs into monomeric siRNAs had occured, the full length transcripts were incubated in Magi cell extracts as described previously[28]. The cleaved products were then visualized by gel electrophoresis on a 15% TBE Urea gel (BioRad, Hercules, CA)(Fig. 3).

Flow cytometry:

To determine the effect of siRNA on the cell surface expression of CXCR4, CD4 or CCR5, transfected cells were analyzed by flow cytometry. Forty-eight hours post-transfection, cells were washed twice with 0.5% BSA/PBS and stained with the appropriate conjugated antibody. Phycoerythrin (PE)-conjugated mouse anti-human CCR5, CXCR4 (Pharmingen, San Jose, CA), and CD4 (Caltag, Burlingame, CA) were used for staining. Matching isotypes (Caltag, Burlingame, CA) were used as negative controls. CXCR4 and CD4 siRNAs were used as cross-specificity controls to confirm that they had no effect on the expression on CD4 and CXCR4, respectively. CCR5 and CXCR4 siRNAs were also used as cross-specificity controls to show they had no effect on the expression of CXCR4 and CCR5, respectively. Cells were resuspended in 45 μl 0.5% BSA/PBS and stained with 5 μl of appropriate antibody at 4°C for 2 hours. Cells were then washed 3 times with 0.5% BSA/PBS and analyzed by flow cytometry on Coulter EPICS®XL-MCL with EXPO32 ADC software (Coulter Corporation, Miami, FL).

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HIV-1 challenge of siRNA transfected cells:

Magi-CXCR4 and CCR5 cells are susceptible to infection with HIV-1 X4-tropic NL4-3 or R5-tropic BaL-1 viruses respectively, as they express the appropriate co-receptors. To determine the resistance conferred by the down regulation of cell surface co-receptors, the siRNA transfected cells were subjected to viral challenge *in vitro*. At 48 hours post-transfection, culture media was removed and either 150 μl (10 ng p24 antigen) of NL4-3 or 150 μl (40 ng p24 antigen) of BaL-1 was added to each well in the presence of polybrene (8 μg/ml). Virus was allowed to adsorb for 2 hours at 37°C. Cells were then washed twice with PBS and 1 ml of complete DMEM was added. Cells were incubated for 48 hours at 37°C for viral infection to progress. Since Magi cells contain an inducible *β*-galactosidase gene under the control of HIV-1 LTR, they serve as good indicator cells for HIV-1 infection by producing a blue color after appropriate staining[31]. Accordingly, to detect virus infected cells, cultures were fixed with 1% formaldehyde and 0.2% glutaraldehyde followed by staining for the detection of *β*-galactosidase as described.[29] Culture supernatants collected at different days post-challenge were assayed for p24 antigen by ELISA (Beckman-Coulter). SiRNA transfected PBMCs were also challenged like above with HIV-1 NL4-3 and BaL-1. Viral supernatants collected at different days post-infection were assayed for p24 antigen.

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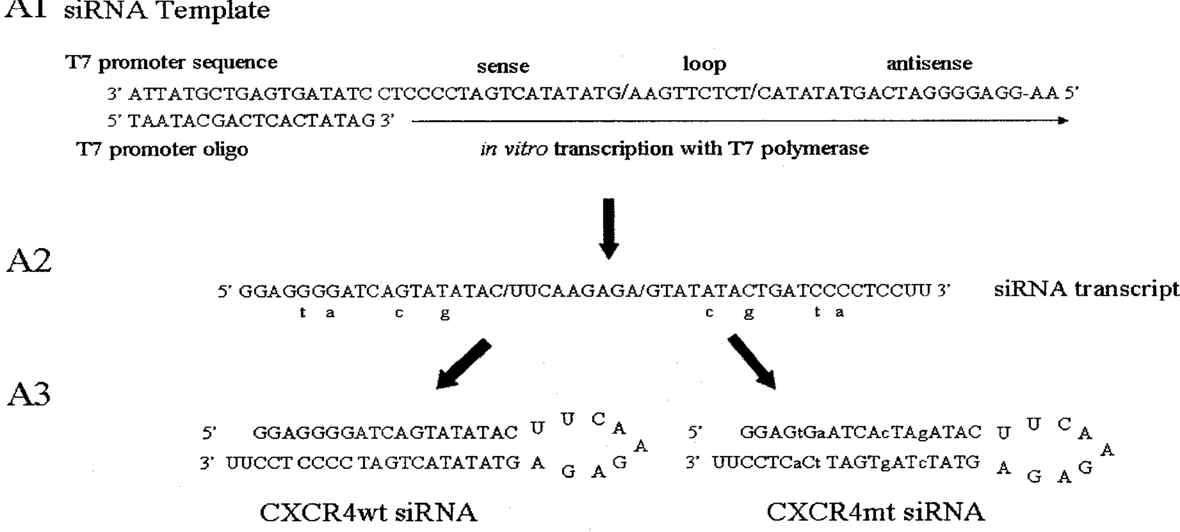
RESULTS

Synthesis of monospecific and combinatorial siRNA constructs and processing into monomeric siRNAs:

The design and *in vitro* strategies of generating mono-specific and combinatorial siRNA constructs are shown in Figures 1A and 1B. The T7 polymerase-mediated transcription products were analyzed by gel electrophoresis, and were found to correspond to their expected sizes (data not shown).



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B1 Bispecific siRNA Template

3' T7 promoter sequence — siRNA #1 — spacer — siRNA #2 5'

in vitro transcription

B2 CXCR4/CD4 siRNA (X4/CD4)

UCACUGAGGAGUCUCUUGAUC UU 3' 5' GGAGGGGAUCAGUAUAUACAC
AGUGACUCCUCAGAGAACUAG UUCAACUUCCUCCCCUAG UCAUAUAUGUG

Loops: GAAGA / AACUU; UCAAG / AGA
anti-CD4 anti-CXCR4

B3 CCR5/CXCR4 siRNA (R5/X4)

GUGUAUAUACUGAUCCCCUCC UU 3' 5' GGGCUCUAUUUUAUAGGCUUC
CACAUAUAUGACUAGGGGAGGUUCAACUUCCCGAGAUAAAAUAUCCGAAG

Loops: GAAGA / AACUU; UCAAG / AGA
anti-CXCR4 anti-CCR5

Figure 1: A: General design and synthesis of mono-specific siRNAs: Schematic for deriving anti-CXCR4 siRNA and its mutant, presented as a representative example (A1). Transcribed sequence of the predicted anti-CXCR4 siRNA. Mutated positions (lowercase) are shown below the transcript (positions 5,7,12, and 15) (A2). Predicted double stranded structures of the wild type (wt) and mutant (mt) siRNAs following transcription and self-annealing (A3). B: Bispecific siRNA constructs: Two mono-specific siRNA sequences are arranged in tandem separated by an intervening spacer sequence (B1). Predicted structure of the bispecific siRNA construct targeted to CXCR4 and CD4 (B2). Bispecific construct targeted to CCR5 and CXCR4 (B3).

For the combinatorial siRNA constructs that consist of two siRNAs, it is essential for cleavage to occur in the transfected cell for each mono-specific siRNA to be functional. When the combinatorial siRNA transcripts of X4/CD4 and R5/X4 were incubated with Magi cell extracts, mono-specific products of expected size were detected

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in the digestion products as analyzed by gel electrophoresis (Fig. 2). After one hour incubation, the ~75bp combinatorial transcripts yielded products ~20bp, the expected size of monomeric siRNAs (Fig. 2b and d). This data suggested that the target cells have the capacity to completely process the combinatorial siRNA constructs. Small amounts of lower size transcripts were also detected in the untreated control lanes (Fig. 2b and d). This could be due to high sensitivity of the long transcript for cleavage or, alternatively, the smaller products could be due to premature transcription termination.

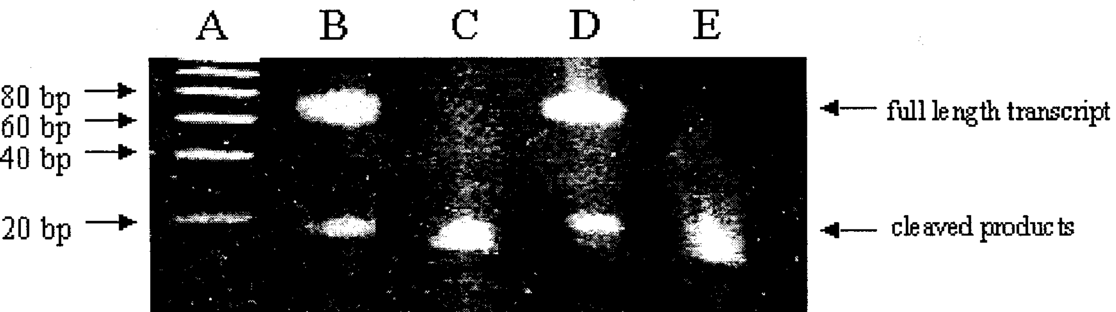

Figure 2: Cellular processing of bispecific siRNAs into monomers: To demonstrate the intracellular cleavage of the bispecific construct into the respective monomers, the T7 *in vitro* transcription product was incubated with cell extracts as described in methods. One hour later the mixture was run on a 15% TBE Urea gel. (A) size marker 20bp ladder. (B and C) CXCR4/CD4 siRNA transcript before and after digestion. (D and E) CCR5/CXCR4 siRNA transcript before and after digestion.

Of the three different spacer sequences tested, as described in materials and methods, one was found to be more effective for *in vitro* cleavage of the long transcript. This also coincided with a more effective down regulation of the targeted receptors (data not shown). Therefore, this spacer sequence, UUCAACUU, was used in the design of combinatorial constructs for this study.

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Down regulation of CXCR4, CCR5 and CD4 by combinatorial siRNA constructs:

Following transfection of Magi-CXCR4 and CCR5 cells with the respective siRNA constructs, cells were analyzed by flow cytometry to determine the down regulation of the respective HIV-1 cell surface receptors. Two control siRNAs, one with four point mutations and another with an unrelated sequence, were also included in these experiments to confirm the specificity and cross-specificity of respective constructs. FACS data for X4/CD4 combinatorial siRNA is presented in Figure 3. There was a 59% reduction in CXCR4 expression and a 42% reduction in CD4 expression as compared to mock transfected cells (Fig. 3A and B). The CXCR4 four-point mutant had little effect when compared to mock transfected cells (Fig. 3C). Other controls utilizing mono-specific CD4 siRNA on CXCR4 expression and mono-specific CXCR4 siRNA on CD4 expression confirmed no cross-specific effects with these siRNAs (Fig. 3D and E).

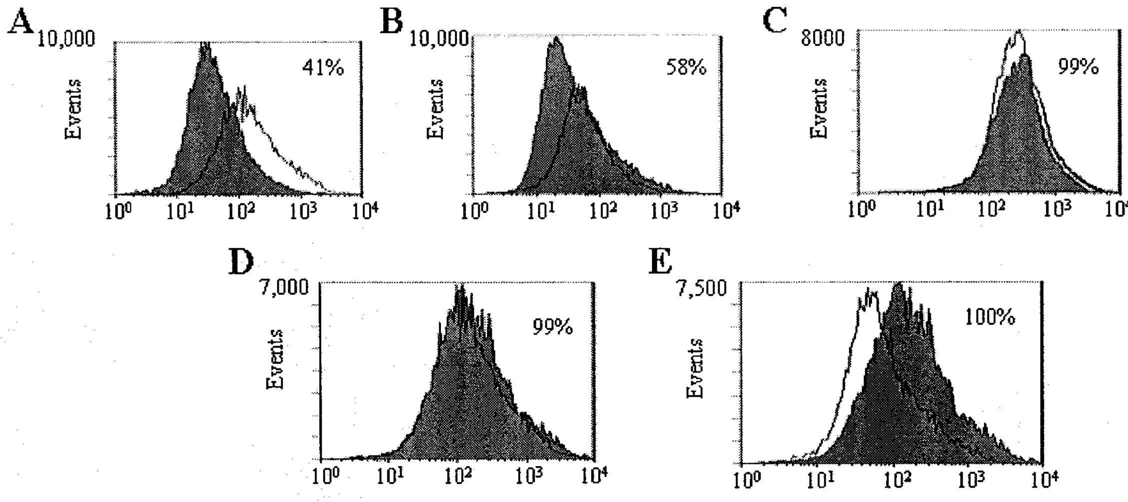

Figure 3: Cell surface down regulation of CXCR4 and CD4 by bispecific X4/CD4 siRNA: Magi-CXCR4 cells that constitutively express the HIV co-receptor CXCR4 and the primary receptor CD4 were transfected with synthetic siRNAs as described in methods. At 48 hours post-transfection, cells were stained with PE-conjugated antibodies to CXCR4 or CD4 and analyzed by FACS. Levels of CXCR4 in

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mock transfected cells (unshaded areas) are superimposed in each panel. Percent CXCR4 positive cells in each siRNA treatment in the shaded areas are indicated. (A) X4/CD4 siRNA with CXCR4 staining (B) X4/CD4 siRNA with CD4 staining (C) CXCR4 mt (D) CXCR4 siRNA with CD4 staining (E) CD4 siRNA with CXCR4 staining.

Down regulation of cell surface receptors by the second combinatorial construct, R5/X4 siRNA, are presented in Figure 4. The R5/X4 siRNA transfected cells showed a 45% reduction in the expression of CCR5 and a 53% reduction in CXCR4 compared to mock transfected cells (Fig. 4A and B). The CCR5 four-point mutant had no effect on CCR5 expression (Fig. 4C). Cross-specificity controls showed no effect of CXCR4 siRNA on CCR5 expression and CCR5 siRNA on CXCR4 expression (Fig. 4D and E).

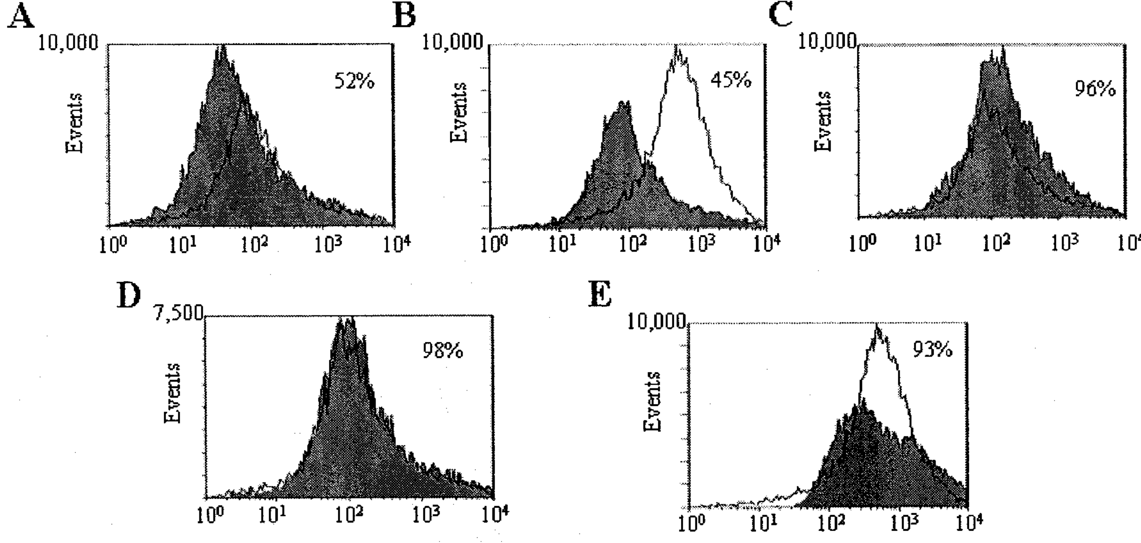

Figure 4: Cell surface down regulation of CCR5 and CXCR4 by bispecific R5/XR4 siRNA: Magi-CCR5 and CXCR4 cells that constitutively express the HIV co-receptors CCR5 and CXCR4, respectively, and the primary receptor CD4 were transfected with synthetic siRNAs as described in methods. At 48 hours post-transfection, cells were stained with PE-conjugated antibodies to CCR5 or CXCR4 and analyzed by FACS. Levels of CCR5 and CXCR4 in mock transfected cells (unshaded areas) are superimposed in each panel. Percent CCR5 and CXCR4 positive cells in each siRNA treatment in the shaded areas are

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indicated. (A) R5/X4 siRNA transfected Magi-CCR5 cells stained for CCR5 (B) R5/X4 siRNA transfected Magi-CXCR4 cells stained for CXCR4 (C) CCR5 mt siRNA (D) CXCR4 siRNA with CCR5 staining (E) CCR5 siRNA with CXCR4 staining.

This data, taken together, demonstrated a marked effect on the down regulation of CXCR4 and CD4 by the X4/CD4 combinatorial siRNA. Similarly, the R5/X4 construct also demonstrated its efficacy in co-suppression of both CCR5 and CXCR4 in transfected cells. Thus, these results established the ability of the combinatorial constructs to effectively down regulate two cell surface receptors simultaneously.

Resistance of siRNA transfected cells to HIV-1 challenge:

FACS data demonstrated the effectiveness of the combinatorial constructs to down regulate the cell surface receptors to a significant level. To determine if cell surface down regulation of HIV-1 receptors leads to protection from viral infection, challenge experiments were performed. Forty-eight hours post-transfection, Magi-CXCR4 and CCR5 cells were infected with HIV-1 strains NL4-3 and BaL-1, respectively. Magi cells are HeLa cell derivatives with an integrated silent LTR-β-galactosidase cassette, which is induced when Tat is produced upon infection with HIV-1[29-30]. Productively infected cells were detected by staining for β-galactosidase 48 hours post-infection. Both X4/CD4 and R5/X4 siRNA transfected Magi-CXCR4 cells were found to be markedly resistant to NL4-3 infection as determined by the significantly reduced number of virus positive blue cells (Fig. 5C and D) compared with mock and mutant siRNA transfected cells (Fig. 5A and B). Similarly, R5/X4 siRNA transfected

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Magi-CCR5 cells also showed resistance to R5 tropic BaL-1 viral challenge (Fig. 5G) compared to mock and mutant siRNA transfected cells (Fig. 5E and F).

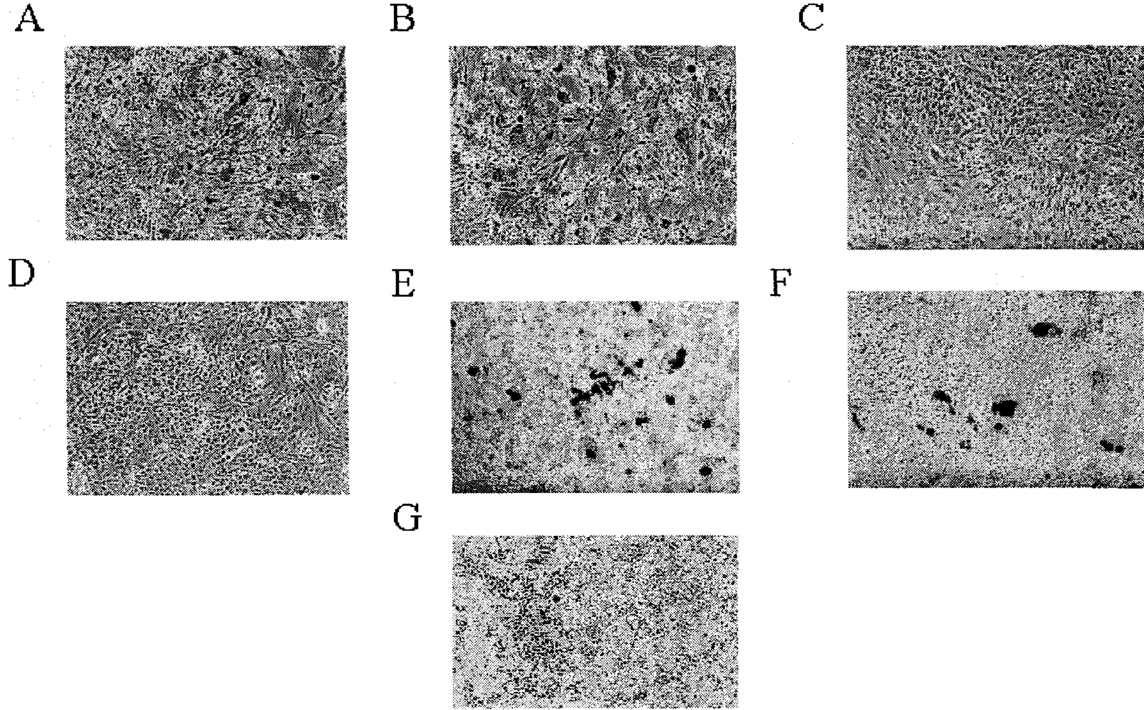

Figure 5: Decreased frequency of infected cells in siRNA transfected cultures: Magi-CXCR4 and CCR5 cells transfected with respective siRNAs were challenged with X4 tropic NL4-3 and R5 tropic BaL-1, respectively, at 48 hours. Two days after challenge, cultures were stained for ß-galactosidase production to detect infected cells. The relative frequency of infected cells is shown in each designated panel. (A) Mock transfected Magi-CXCR4 cells (B) Magi-CXCR4 cells with CXCR4 mt siRNA (C) Magi-CXCR4 cells with X4/CD4 siRNA (D) Magi-CXCR4 cells with R5/X4 siRNA (E) Mock transfected Magi-CCR5 cells (F) Magi-CCR5 cells with CCR5 mt siRNA (G) Magi-CCR5 cells with R5/X4 siRNA. These results are representative of 3 independent experiments.

Virus challenged Magi-CXCR4 and CCR5 cells were also monitored at different days post-infection by assaying for viral p24 production to determine the levels of virus in culture supernatants. Magi-CXCR4 cells transfected with combinatorial siRNA constructs X4/CD4 and R5/X4 and challenged with X4 tropic NL4-3 exhibited marked

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protection compared with mock and CXCR4 mutant siRNA transfected cells (Fig. 6A). Magi-CCR5 cells transfected with R5/X4 siRNA and challenged with R5 tropic BaL-1 also exhibited resistance to HIV-1 infection compared to mock and mutant siRNA transfected cells (Fig. 6B). These results demonstrated the efficacy of the dual siRNA constructs in HIV-1 susceptible cell lines to afford resistance to infection.

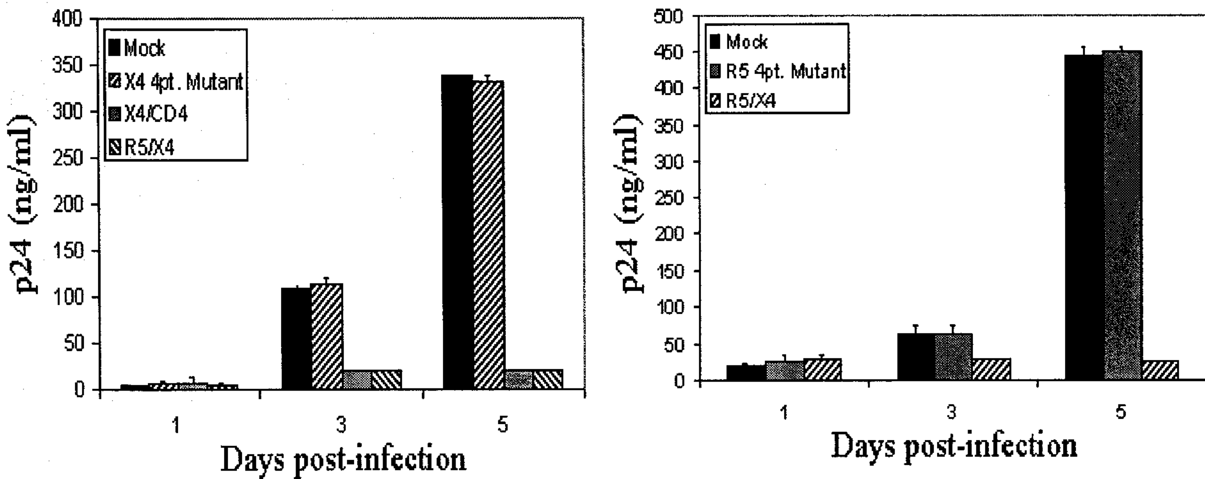

Figure 6: Bispecific siRNA constructs confer resistance to X4 AND R5 tropic HIV-1 challenge in Magi-CXCR4 and CCR5 cells. (A) Magi-CXCR4 cells transfected with respective siRNAs were infected with HIV-1 NL4-3. (B) Magi-CCR5 cells transfected with respective siRNAs were infected with HIV-1 BaL. On days 1-5 post-infection, viral supernatants were collected and assayed for viral p24 antigen by ELISA.

To further confirm the above results in a physiologically relevant setting, siRNA transfected PBMCs were also subjected to viral challenge and p24 production was assayed at different days post-infection. In PBMCs challenged with R5-tropic BaL-1, no protection was seen in either mock or CCR5 mutant siRNA transfected cells, whereas, the combinatorial R5/X4 siRNA tranfected cells showed significant protection (Fig. 7B). A three-fold reduction in virus antigen was seen on day three with viral levels showing an upward trend on day 5 most likely due to the transient effect of the transfected siRNAs.

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Similarly, PBMCs challenged with X4-tropic NL4-3 also showed significant protection when transfected with either X4/CD4 or R5/X4 combinatorial siRNA constructs compared to either mock or CXCR4 mutant siRNA transfected cells (Fig. 7A).

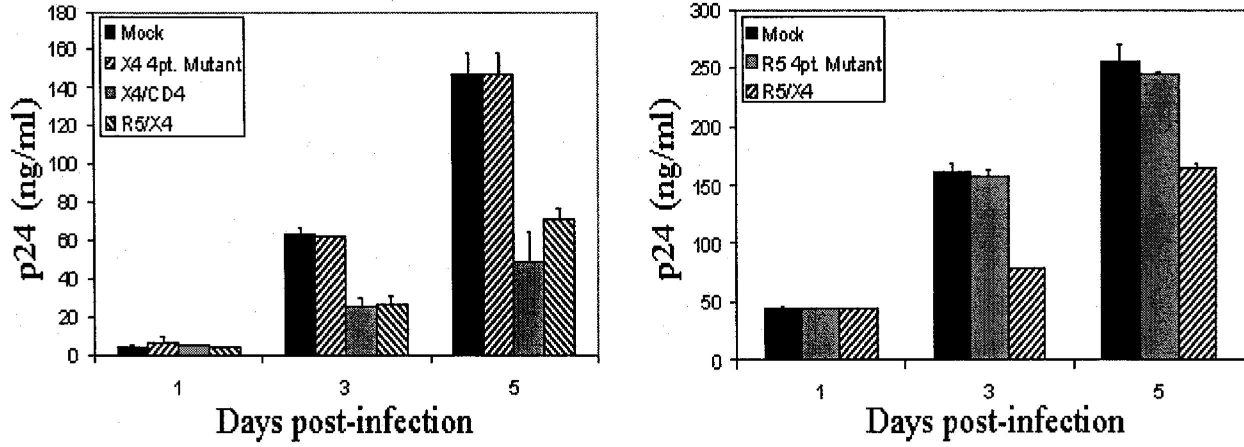

Figure 7: Bispecific siRNA constructs confer resistance to X4 and R5 tropic HIV-1 challenge in PBMCs. (A) PBMCs transfected with respective siRNAs were infected with HIV-1 NL4-3 (B) PBMCs transfected with respective siRNAs were infected with HIV-1 BaL-1. Culture supernatants were assayed for p24 antigen by ELISA on days 1-5 post-infection.

By comparision, X4/CD4 siRNA exhibited a higher level of protection than the R5/X4 construct. This is consistent with the ability of X4/CD4 siRNA to down regulate both viral receptors, CXCR4 and CD4, which are relevant to NL4-3 infection, whereas R5/X4 down regulates only a single receptor, CXCR4. This data showed the efficacy of the combinatorial constructs to confer resistance in PBMCs, which are the primary targets for HIV-1 infection *in vivo*.

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DISCUSSION

Targeting of essential cellular molecules involved in viral entry by siRNAs demonstrated their efficacy in down regulating the cell surface primary receptor CD4[18] and the co-receptors CCR5[19-21] and CXCR4[20,22], and in protecting against HIV-1 infection. A clear advantage in targeting cellular receptors is the broad range of protection that can be achieved against multiple HIV-1 strains. All previous studies, including ours targeting CXCR4[22], have utilized individual mono-specific siRNA constructs to establish their efficacy. However, as the HIV-1 genome is highly mutatable, it is likely to acquire resistance to individual siRNAs during prolonged therapy, as seen recently with poliovirus[32-33]. With regard to siRNAs targeted to individual co-receptors, sustained use of a single mono-specific siRNA against either CXCR4 or CCR5 during therapy is also likely to promote the selection and emergence of viral populations that can infect via the non-targeted co-receptor. Therefore, dual specific siRNA constructs that can down regulate both co-receptors simultaneously are highly desirable.

In the present proof-of-concept studies we demonstrated the feasibility of assembling bi-specific siRNA constructs directed against different targets, and have shown their efficacy in down regulating two cell surface molecules simultaneously. The short hairpin design employed here allowed assembly of two siRNAs in tandem in a single transcript. Incubation of the long primary transcript with cell extracts yielded smaller RNA products corresponding to the size of the individual siRNAs. These results suggested that the down regulation seen for each of the cell surface receptor in cells

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transfected with the combinatorial constructs is due to the action of the intracellularly processed mono-specific siRNAs.

In Magi-CXCR4 cells transfected with the X4/CD4 combinatorial siRNA, there was a 59% reduction in CXCR4 and a 42% reduction in CD4 levels. The down regulaton of these targets is specific since there was no reduction in CXCR4 staining in cells treated with the CXCR4 four-point mutant. Additionally, in experiments designed to test cross-specificity, CD4 siRNA had no effect on CXCR4 expression and CXCR4 siRNA did not interfere with CD4 expression. These data established that both the primary receptor CD4 and a co-receptor CXCR4 can be down regulated simultaneously with the aid of a combinatorial construct. As sustained down regulation of CD4, an essential cell surface molecule important in immunological function, is likely to have physiological consequences, combinatorial constructs consisting of siRNAs against CCR5 and CXCR4 are likely to be more practical for long-range therapy. Accordingly, when such a construct was tested in Magi-CXCR4 and CCR5 cells and analyzed by FACS, levels of CCR5 were reduced by 45% whereas CXCR4 expression decreased by 53%. Specificity and mutant CCR5 siRNA control transfected cells showed no decrease in the respective cell surface receptors.

The above results showed the capacity of combinatorial siRNA constructs to significantly down regulate the expression of respective HIV-1 receptors. *In vitro* challenge of combinatorial siRNA transfected Magi-CXCR4 and CCR5 cells showed very few infected cells compared to mock transfected cells after staining for β-galactosidase production. This suggested that very few of the transfected cells were initially infected during viral challenge. In experiments designed to evaluate viral spread

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and amplification during the 5-day post-challenge period, p24 antigen assay was used. Consistent with the above data, both Magi-CXC4 and CCR5 cells exhibited marked resistance to challenge with X4 and R5-tropic viruses respectively, as p24 production remained at basal levels. The results of viral challenge experiments performed in primary PBMCs also corraborated the efficacy of combinatorial siRNA constructs in inhibiting viral entry, thus suggesting that the antiviral effect is applicable to a physiologically relevant setting. In X4/CD4 and R5/X4 siRNA transfected PBMCs challenged with NL4-3, the former of these two siRNAs showed a higher protective effect compared with lower protection afforded by the CXCR4 siRNA component of the R5/X4 construct. Thus, these data clearly showed the added protective effects of targeting two relevant receptors when compared to down regulation of a single receptor. However, the overall levels of viral inhibition were substantially lower in virus challenged PBMCs compared to a much higher level of inhibition seen in the Magi cell line. This is likely due to the sub-optimal levels of transfection of siRNAs into primary lymphocytes[18-19]. Regardless, further improvements in siRNA delivery and simultaneous targeting of multiple regions in each of the transcript are likely to increase efficacy.

Studies of Maritnez et al.[20] have shown the efficacy of chemically synthesized mono-specific CXCR4 and CCR5 siRNAs in inhibiting HIV-1 entry using non-lymphoid cell lines. Our recent work had improved upon these findings using a different mono-specific CXCR4 siRNA construct incorporating a short hairpin design and showing efficacy in primary lymphocytes[22.] As synthetic siRNAs delivered via transfection are likely to have only a transient effect, it will be necessary to use methodologies that mediate constitutive expression in target cells for a sustained effect. Towards this end,

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use of retro- or lentiviral vectors to deliver siRNAs into cells will be ideal. Such a goal has been accomplished in our most recent studies in which a lentiviral vector was used to transduce an anti-rev siRNA into hematopietic stem cells from which HIV-1 resistant T cells and macrophages were derived[34-35]. Similarly, a lentiviral vector was also used to transduce an anti-CCR5 siRNA into primary lymphocytes[19]. The vector delivered siRNAs in these two reports used two tandemly placed U6 promoters to drive the sense and anti-sense strands of the siRNA. In addition to the currently used siRNAs, other previous RNA based approaches have used ribozymes targeted to the CCR5 co-receptor[3-6]. In an innovative approach, Bai et al.[5] used combinatorial constructs incorporating three ribozymes targeted to three distinct regions of the CCR5 mRNA that showed additional protective effect. As can be expected, efficacy of the constructs can be greatly broadened by incorporating more than one target-specific effector siRNA as we have confirmed here with the combinatorial siRNA constructs. The stem loop structured transcript design allowed the assembly of dual specific siRNA constructs described here. Furthermore, this design is particularly suitable for incorporating multiple siRNAs into lentiviral vectors as a single promoter can be efficiently used to drive a single transcript harboring multiple siRNA motifs, which can be processed intracellularly.

Although it is apparent that multiple siRNAs directed against several targets can theoretically be assembled into a single construct based on the results presented here, caution should be exercised not to evoke the interferon pathway by the longer RNA transcripts. As we have demonstrated the efficient cleavage of the long combinatorial construct *in vitro* in cell extracts and the individual siRNAs are less than 30bp in size, it is unlikely that the interferon pathway was triggered in the above experiments. Another

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issue that should be considered is the upper limit to the number and levels of siRNAs constitutively produced in a cell such that the RNA-induced silencing complex (RISC) is not completely titrated out by such disproportionate siRNA expression. If it proves to be the case that the RISC complex is saturated, alternative combinatorial strategies may need to be employed in which a combination of different inhibitory RNA molecules with varied mechanisms of action might be of use to circumvent this limitation. Such constructs may employ an optimal combination of siRNAs, ribozymes and RNA aptamers.

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CHAPTER 5

HIV-1 RESISTANCE CONFERRED BY siRNA COSUPPRESSION OF CXCR4 AND CCR5 CORECEPTORS BY A BISPECIFIC LENTIVIRAL VECTOR

Anderson J, Akkina R: HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. AIDS Res Therapy 2005, 2:1-12.

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ABSTRACT

RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) has proved to be a highly effective gene silencing mechanism with great potential for HIV/AIDS gene therapy. Previous work with siRNAs against cellular coreceptors CXCR4 and CCR5 had shown that down regulation of these surface molecules could prevent HIV-1 entry and confer viral resistance. Since monospecific siRNAs targeting individual coreceptors are inadequate in protecting against both T cell tropic (X4) and monocyte tropic (R5) viral strains simultaneously, bispecific constructs with dual specificity are required. For effective long range therapy, the bispecific constructs need to be stably transduced into HIV-1 target cells via integrating viral vectors. To achieve this goal, lentiviral vectors incorporating both CXCR4 and CCR5 siRNAs of short hairpin design were constructed. The CXCR4 siRNA was driven by a U6 promoter whereas the CCR5 siRNA was driven by an H1 promoter. A CMV promoter driven EGFP reporter gene is also incorporated in the bispecific construct. High efficiency transduction into coreceptor expressing Magi and Ghost cell lines with a concomitant down regulation of respective coreceptors was achieved with lentiviral vectors. When the siRNA expressing transduced cells were challenged with X4 and R5 tropic HIV-1, they demonstrated marked viral resistance. HIV-1 resistance was also observed in bispecific lentiviral vector transduced primary PBMCs. Both CXCR4 and CCR5 coreceptors could be simultaneously targeted for down regulation by a single combinatorial lentiviral vector incorporating respective anti-coreceptor siRNAs. Stable down regulation of both the coreceptors protects cells against infection by both X4 and R5 tropic HIV-1. Stable

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down regulation of cellular molecules that aid in HIV-1 infection will be an effective strategy for long range HIV gene therapy.

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INTRODUCTION

HIV/AIDS continues to be a major public health problem worldwide with millions of people currently infected and new infections being on the rise. As no effective vaccines are currently available for prevention, new and innovative therapies need to be developed. Although combinatorial therapies such as HAART have proven to be effective in prolonging life, they do not afford a complete cure. Other constraints with HAART therapy are the development of drug resistant viral mutants and toxicity after prolonged therapy. Intracellular immunization by gene therapy strategies offers a promising alternative approach for controlling and managing HIV disease. A number of previous approaches that involved the use of transdominant proteins,[1-3] decoys,[3-7] and ribozymes[5,8-12] had shown initial promise but fell short of practical utility in providing adequate protection. With the discovery that the RNA interference phenomenon operates in mammalian cells and is highly effective in selective gene silencing, new potent small interfering RNA (siRNA) molecules have become available to add to the anti-HIV arsenal.[13]

RNAi is a highly potent and specific mechanism of post-transcriptional gene silencing. Mediated by sequence specific siRNAs, it can effectively down regulate expression of either viral or cellular RNA target molecules by selective degradation of mRNAs.[13-16] Mechanism of destruction involves an endonuclease present in the RISC complex which is guided by the antisense component of the siRNA for target recognition. A number of reports have shown that delivery of siRNAs by transfection of presynthesized or plasmids encoding siRNAs into cultured cells can effectively inhibit HIV-1 infections.[17-26] Antiviral effects of these delivery methods are only transient due to

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eventual degradation and dilution of siRNAs during cell division. For HIV gene therapy strategies to succeed in long range, it is necessary that siRNA coding transgenes be maintained and expressed long term in a virus susceptible target cell. In this regard, lentiviral vectors have proven to be highly effective in high efficiency gene transduction and sustained gene expression.

A number of previous approaches using either synthetic siRNAs or plasmid expressed constructs have successfully targeted viral transcripts and achieved effective viral inhibition. Of these, some anti-HIV-1 siRNAs, such as siRNAs against tat , tat-rev had been introduced into lentiviral vectors and their efficacy was demonstrated both in cell lines and primary T cells and macrophages.[27-28] Promising data was also obtained in experiments showing that anti-rev siRNAs against HIV-1 were functional in conferring viral resistance in differentiated T cells and macrophages derived from lentiviral transduced CD34+ hematopoietic progenitor cells.[29]

In addition to targeting viral transcripts, many studies including ours also investigated the efficacy of siRNAs in down regulating host cell molecules necessary for HIV-1 infection.[18,21,23,24,30,31] An advantage in targeting cellular molecules is that efficacy will be more broad spectrum against all the clades of the virus and the frequency of escape mutants will be lower. Down regulation of the primary cell surface receptor CD4 and consequent inhibition of HIV-1 infection was shown using synthetic siRNAs. However, since CD4 is an essential cell surface molecule for immunological function, it is not a practical target for HIV gene therapy. Chemokine receptors CCR5 or CXCR4 play an essential role as coreceptors for HIV-1 entry, thus are suitable targets for siRNA mediated down regulation. During the initial stages of HIV infection, macrophage tropic

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(R5) viral strains predominate followed by gradual evolution of T cell tropic (X4) viral strains towards the later stages of disease.[32-33] X4-tropic strains appear to be responsible for the major damage to the immune system by destroying T cells and thus enabling opportunistic infections. Therefore, blocking both coreceptors needs to be considered when developing effective therapeutics. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of this coreceptor thus conferring significant resistance to HIV infection.[34-36] Homozygous or heterozygous individuals for this mutation remain physiologically normal. With regard to the CXCR4 coreceptor, it was found to be dispensable for T cell development and maturation in murine studies.[37] These findings suggest that CCR5 and CXCR4 are promising targets for HIV therapies.

Based on this rationale, recent work with synthetic siRNAs demonstrated that down regulating either CXCR4 or CCR5 will protect cells from X4 or R5 HIV-1 strains respectively at the level of viral entry.[18,21,23,24] Although stable expression of an anti-CCR5 siRNA was achieved using a lentiviral vector in one study, down regulating CCR5 alone in the face of an HIV-1 infection is insufficient.[31] Therefore, we recently experimented with synthetic bispecific combinatorial constructs targeted to both CXCR4 and CCR5 and have shown their efficacy in cultured cells.[24] To make further progress, our present studies are directed towards constructing a single bispecific lentiviral vector expressing both CXCR4 and CCR5 siRNAs. Using this combinatorial construct, here we show high efficiency transduction, simultaneous down regulation of both coreceptors resulting in HIV-1 resistance.

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MATERIALS AND METHODS

Plasmid and lentiviral vector construction:

Previously characterized siRNAs against CXCR4 and CCR5 were used in generating the bispecific lentiviral vector.[23-24,30] A third generation lentiviral vector backbone was employed to derive the bispecific constructs. The two *cis*-acting elements, namely, the central DNA flap consisting of cPPT and CTS (to facilitate the the nuclear import of the viral preintegration complex) and the WPRE (to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts), are engineered to enhance the performance of the vector.[38-39] An siRNA expression cassette targeting CXCR4 under the control of the Pol-III U6 promoter was PCR amplified from the plasmid pTZ-U6+1 as described by Castanotto *et al.*[40] This cassette was cloned into pHIV-7-GFP transfer vector in the *BamH*I site immediately upstream of the CMV-EGFP gene. This cassette contained an *Mlu*I restriction site downstream from the CXCR4 siRNA sequence for subsequent cloning of the H1 promoter driven CCR5 siRNA cassette. The H1-CCR5 siRNA expression cassette was also generated as described above using the plasmid pSUPER (Oligoengine, Seattle, WA). Sequencing and confirmation of candidate clones was performed by Laragen Inc. (Los Angeles, CA). The transfer vector containing the inserts U6-X4 siRNA and H1-CCR5 siRNA is termed pHIV-XHR-GFP.

Cell culture and vector production:

293T cells and PBMCs were maintained in DMEM media supplemented with 10% FBS. Magi-CXCR4 cells obtained from the AIDS Reference and Reagent Program were maintained in media as previously described.[41-42] Ghost-R5 cells obtained from the

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AIDS Reference and Reagent Program were maintained in media as previously described.[43] To generate lentiviral vectors, fifteen micrograms of transfer vector with either GFP-alone or XHR were transfected along with 15ug pCHGP-2, 5ug pCMV-Rev, and 5ug pCMV-VSVG into 293T cells at 60% confluency in 100mm culture dishes using a calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO). Six hours after transfection, fresh medium was exchanged. Cell culture supernatants containing the vector were collected at 24, 36, 48, and 60 hours post tranfection and pooled. Vector supernatants were concentrated by ultracentrifugation and later titrated on 293T cells using FACS analysis for GFP expression.

Lentiviral vector transduction and FACS analysis:

Magi-CXCR4 and Ghost-CCR5 cells were seeded in 6-well plates 24 hours prior to transduction, $5x10^5$ cells per well. Cells were transduced with lentiviral vectors at an m.o.i. of 10 in the presence of 4ug/ml polybrene for 2 hours. For transduction of PBMCs, cells were first isolated from whole blood by Histopaque®-1077 (Sigma-Aldrich), and then cultured in CD3 and CD28 antibody coated plates. Three days after stimulation, PBMCs were transduced at an m.o.i of 20 in the presence of 4ug/ml polybrene. PBMC transduction was repeated the following day. Seventy-two hours post transduction with siRNA containing lentiviral vectors, FACS analysis was performed to determine the levels of cell surface expression of CXCR4 and CCR5. Non-transduced and transduced cells were stained with appropriate antibodies conjugated with PE-Cy 5 (Pharmingen, San Diego, CA) namely, anti-CXCR4 for Magi-CXCR4 cells and anti-CCR5 for Ghost-CCR5 cells. Transduction efficiency was determined by assaying for

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EGFP expression. FACS analysis was performed on the Beckman Coulter Epics XL using ADC software for analysis.

Northern analysis for shRNA expression:

Total RNA was extracted from non-transduced and transduced Magi-CXCR4 and Ghost-CCR5 cells using the RNA-STAT-60 reagent (Tel-Test, Friendswood, TX). Small RNAs, <200 nt, were separated and concentrated using the *mir*Vana™ miRNA Isolation Kit (Ambion, Austin, TX). Twenty micrograms of small RNAs were hybridized overnight at 37°C using the *mir*Vana™ miRNA Detection Kit (Ambion) with γ-^{32}P labeled probes made using the *mir*Vana™ Probe & Marker Kit (Ambion). Probes were complementary to the antisense strands of CXCR4 and CCR5 siRNAs. Hybridization reactions were processed according to the manufacturer's protocol and run on 15% polyacrylamide TBE-Urea gels. Gels were then exposed to X-ray film. A probe complementary to miRNA-16 supplied with the miRNA detection kit was used as an internal control.

Western Blot analysis of phosphorylated PKR:

Cell lysates of non-transduced and transduced cells were run on 10%-polyacrylamide-SDS TBE gels. Proteins were immunoblotted onto Immobilon™-P membranes (Millipore, Bedford, MA) and incubated with antibody specific for phosphorylated-PKR (Sigma-Aldrich), while anti-actin antibody (Sigma-Aldrich) was used to detect cellular actin as an internal control. A secondary antibody, goat anti-rabbit IgG conjugated with alkaline phophatase (Promega, Madison, WI), was then added. An

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alkaline phophatase substrate reagent, Western Blue (Promega), was used to visualize the bands.

RT-PCR:

Total RNA was extracted from non-transduced and transduced cells. Primers specific for CXCR4 (forward: 5'-ggaggggatcagtatatacacttc and reverse: 5'-cgccaacatagaccaccttttc) and CCR5 (forward: 5'-caaaaagaaggtcttcattacacc and reverse: 5'-cttgctcgctcgggagcctc) (IDT, Coralsville, IA) were used to determine transcript levels while GAPDH (forward: 5'-ctgagaacgggaagcttgtcatcaa and reverse: 5'-gcctgcttcaccaccttcttgatg) primers were used as an internal control. One-step RT-PCR reactions were performed using the Superscript™ III One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Reactions were run on 1% agarose gels and appropriate bands were visualized with UV light.

HIV-1 Challenge:

To determine if down-regulation of CXCR4 and CCR5 transcript levels and cell surface expression inhibited HIV-1 infection, non-transduced and transduced cells were challenged with NL4-3 (X4-tropic) and BaL-1 (R5-tropic) strains of HIV-1, at an m.o.i of 0.01, as previously described.[24] Viral supernatants were collected daily from infected Magi-CXCR4 and Ghost-CCR5 cells for p24 assay. ELISA was used to determine p24 values employing a Coulter-p24 kit (Beckman Coulter, Fullerton, CA). For PBMC challenge experiments, non-transduced and transduced cells were infected with NL4-3

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and Bal-1 strains and cell culture supernatants were collected on days 1,3,5, and 7 post-infection to measure p24 levels.

RESULTS

Coreceptor down regulation by a bispecific lentiviral vector:

Our major goal in these studies is to introduce both CXCR4 and CCR5 siRNAs into a single lentiviral construct to achieve their stable expression in transduced cells. Lentiviral vectors offer advantages over conventional retroviral vector systems since they can transduce dividing as well as nondividing cells and are less prone to transgene silencing.[44-47] The transfer vector HIV-7-GFP-XHR (referred to as XHR) contained a short hairpin type anti-CXCR4 siRNA driven by a Pol-III U6 promoter followed by a short hairpin anti-CCR5 siRNA driven by a different Pol-III promoter, H1. Downstream, the reporter gene, EGFP is driven by a CMV promoter. The control GFP-alone vector, HIV-7-GFP, contained only the reporter gene EGFP (Fig 1).

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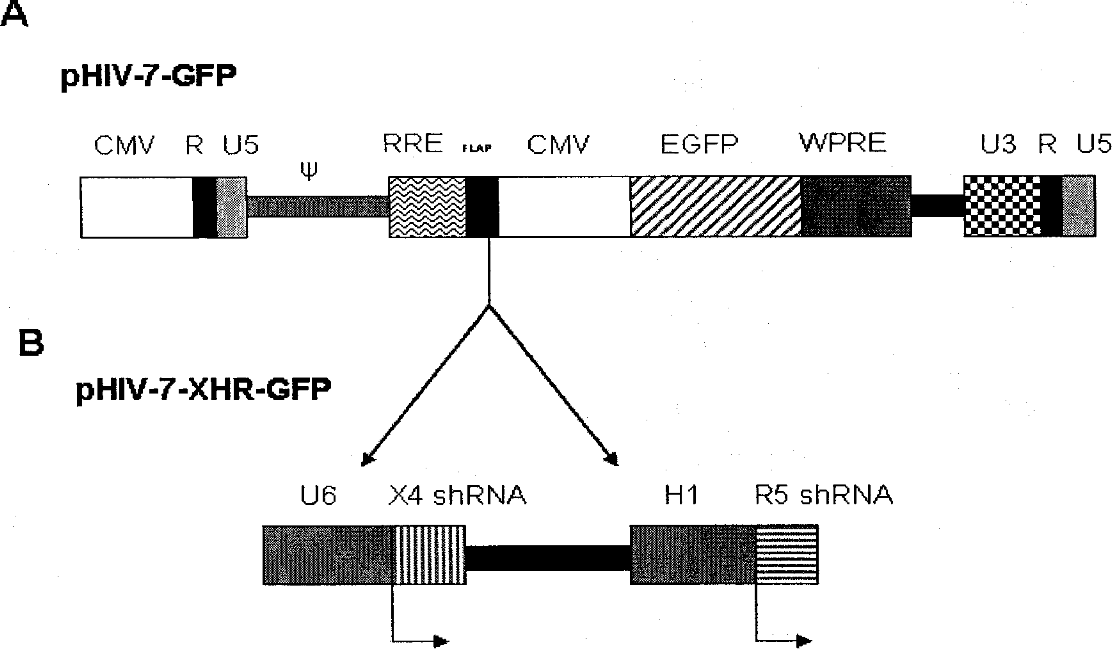

Fig 1. Bispecific lentiviral vector (XHR) encoding anti-CXCR4 and CCR5 siRNAs. A) Control transfer vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. B) To derive the bispecific vector pHIV-XHR-GFP, a U6 promoter driven short hairpin CXCR4 siRNA cassette was cloned into the *BamHI* site upstream to the CMV-EGFP cassette. The H1-CCR5 siRNA cassette was inserted into an *MluI* site downstream to the U6-CXCR4 siRNA cassette.

Magi-CXCR4 cells constitutively expressing CXCR4 on the cell surface when transduced with the control vector or XHR vector had shown 97% and 83% EGFP expression respectively as measured by FACS analysis indicating high efficiency of transduction (Fig 2, A and C). To determine if CXCR4 was down regulated by the respective siRNA in the XHR construct, the transduced cells were analyzed for CXCR4 surface expression. The surface levels of CXCR4 were reduced significantly in XHR transduced cells (73% lower) compared to the cells transduced with control vector (Fig 2, B and D) indicating the efficacy of the CXCR4 siRNA on its target.

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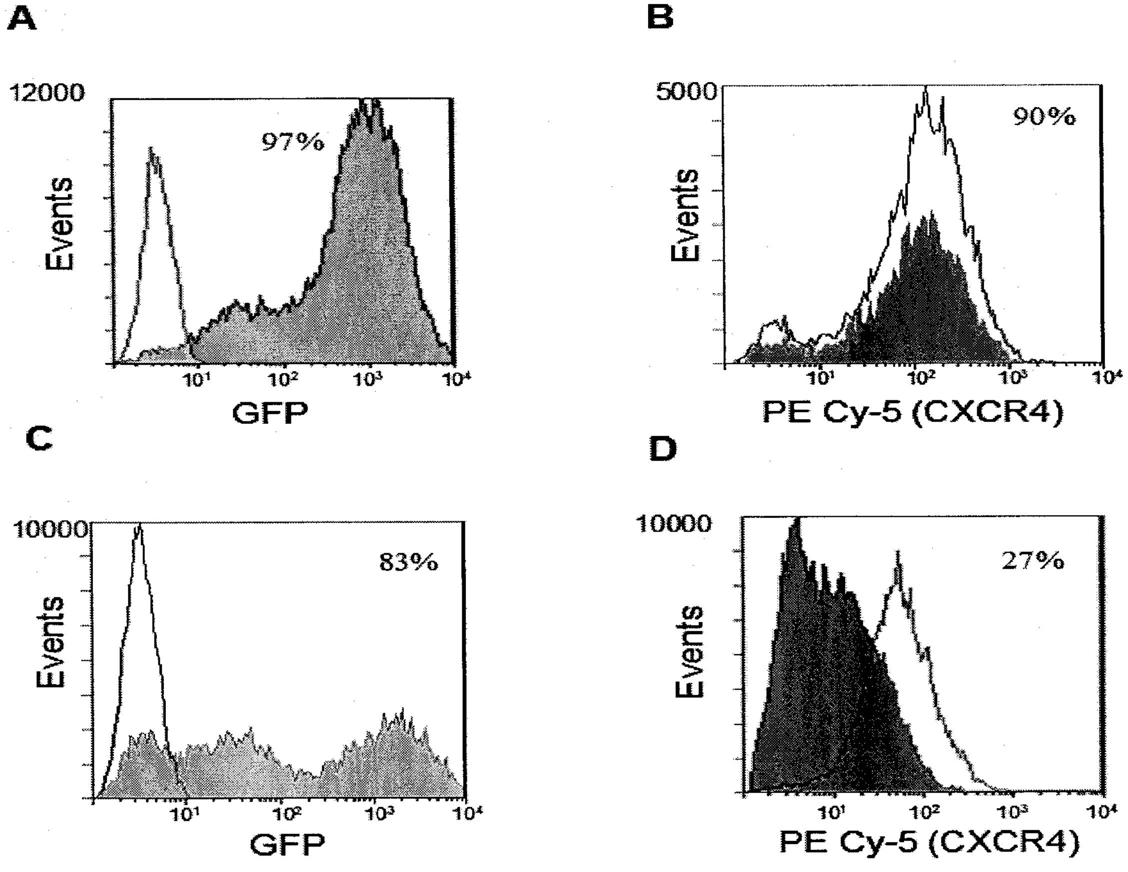

Fig 2. Cell surface down regulation of CXCR4 in XHR transduced Magi-CXCR4 cells. Magi-CXCR4 cells that constitutively express CXCR4 were transduced with control GFP or XHR vectors. Cells were stained with PECy5-conjugated antibodies to CXCR4 and analyzed by FACS 72 hours post-transduction. Levels of CXCR4 in non-transduced cells are superimposed (unshaded areas). Transduction efficiency was determined by FACS for EGFP expression. Levels of EGFP in control GFP-alone vector (A) and XHR vector (C) transduced cells. Levels of CXCR4 expression in GFP-alone (B) and XHR (D) vector transduced cells. Percent positive cells are indicated.

Similarly, to determine the activity of the anti-CCR5 siRNA in the XHR vector, transduced Ghost R5 cells that constitutively express CCR5 were evaluated. As seen in Fig 3 A and B, high levels of transduction (84% and 83%) were seen in Ghost-R5 cells

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with either the control vector or XHR vector, respectively. When the transduced cells were analyzed for CCR5 expression, a dramatic decrease in CCR5 expression was seen in XHR cells (72%) compared to control vector transduced cells (Fig 3, B and D).

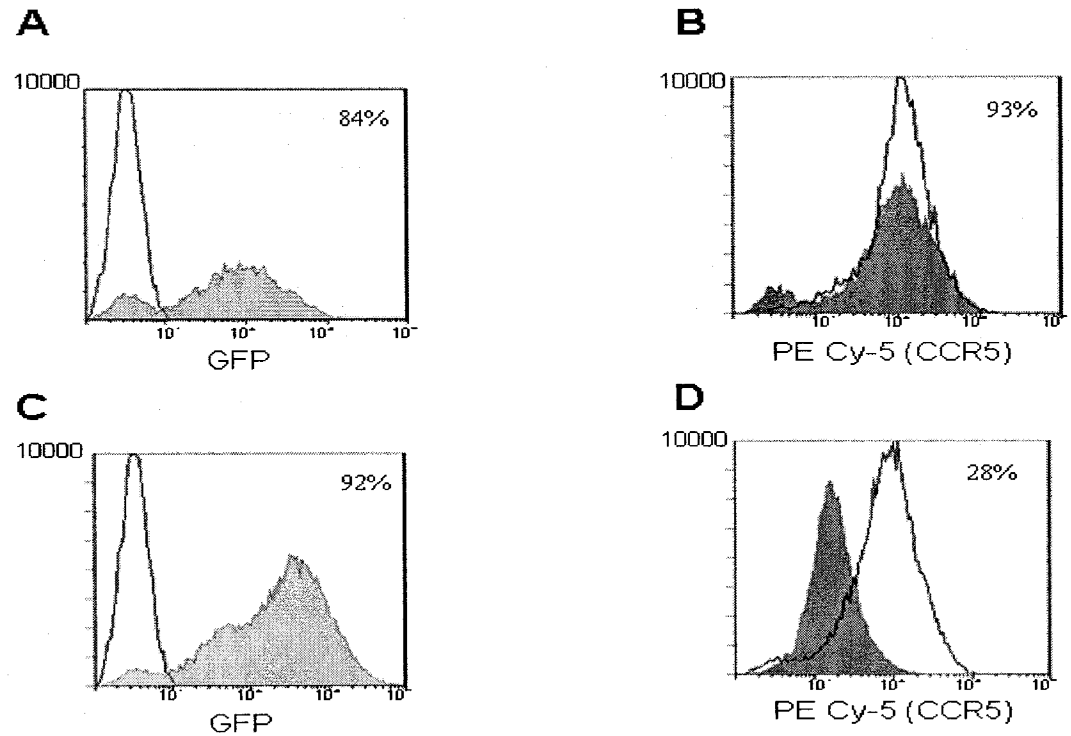

Fig 3. Cell surface down regulation of CCR5 in XHR transduced Ghost-R5 cells. Ghost-R5 cells that constitutively express CCR5 were transduced with GFP-alone or XHR vectors. Cells were stained with PECy5-conjugated antibodies to CCR5 and analyzed by FACS 72 hours post-transduction. Levels of CCR5 in non-transduced cells are superimposed (unshaded areas). Transduction efficiency was measured by FACS for EGFP expression. Levels of EGFP in control GFP-alone vector (A) and XHR vector (C) transduced cells. Levels of CCR5 expression in GFP-alone (B) and XHR (D) vector transduced cells. Percent positive cells are indicated.

These results had shown that the bispecific lentiviral vector XHR efficiently down regulates both CXCR4 and CCR5 targets in respective cells.

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Expression of siRNAs and down regulation of CXCR4 and CCR5 transcripts:

To confirm that the down regulation of both CXCR4 and CCR5 coreceptors as seen by FACS analysis is due to reduced levels of the corresponding mRNAs, vector transduced cells were analyzed by RT-PCR. As an internal control, GAPDH mRNA was also analyzed. XHR vector transduced cells showed considerable reduction in transcript levels for both CXCR4 and CCR5 as compared to non-transduced and control GFP vector transduced cells. The levels of GAPDH control mRNA remained unchanged in all samples (Fig 4).

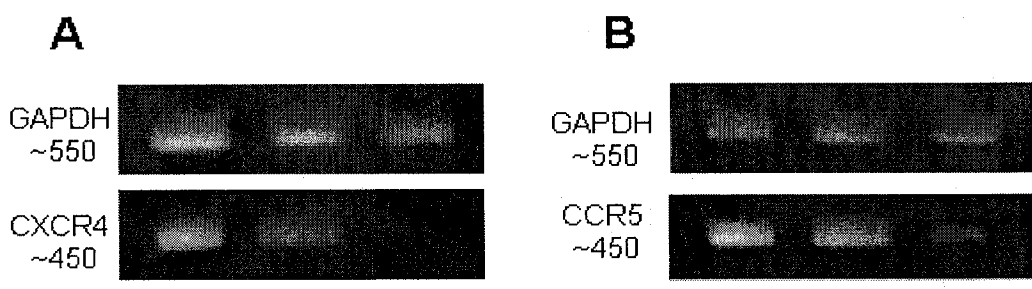

Fig 4. RT-PCR detection of CXCR4 and CCR5 mRNA down regulation. Total RNA was extracted from vector transduced cells and one-step RT-PCR was performed. PCR products of 450 bp were amplified to detect the coreceptor transcripts. A) Levels of CXCR4 mRNA in non-transduced (lane 1), GFP-alone (lane 2), and XHR (lane 3) vector transduced Magi-X4 cells. B) CCR5 transcript levels in non-transduced (lane 1), GFP-alone (lane 2), and XHR vector transduced Ghost-R5 cells. GAPDH transcript levels were used as internal controls (PCR product size ~550 bp).

To validate the expression of individual siRNAs in transduced Magi-CXCR4 and Ghost R5 cells, cellular RNA was analyzed by northern analysis for their presence. As internal controls, the presence of constitutively expressed miRNA-16 RNAs were also

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analyzed in parallel. As expected, comparable levels of miRNA-16 RNAs (22bp in length)were detected in GFP control vector transduced as well as in XHR vector transduced cells (Fig 5A). RNAs corresponding to CXCR4 and CCR5 shRNAs (representing the 21nt antisense strand of each shRNA) were seen in XHR transduced but not in GFP control vector transduced cells (Fig 5B).

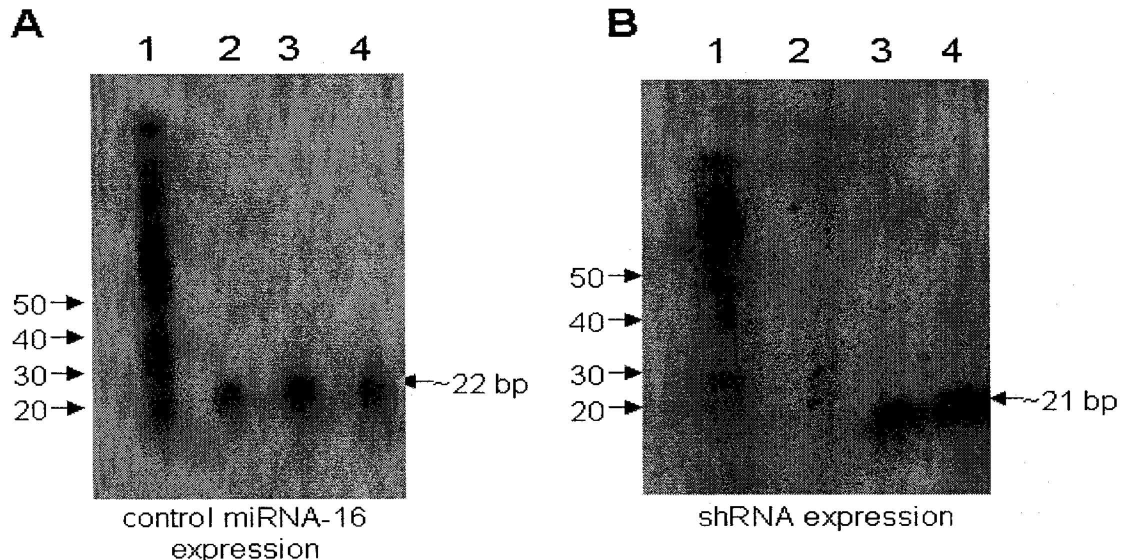

Fig 5. Northern analysis to detect siRNA expression in transduced cells. Small RNAs (<200 nt) were extracted from transduced cells and probed with specific primers to detect the expression of siRNAs as described in materials and methods. A) Northern blot to detect the presence of miRNA-16 (~22 bp) as an internal control in GFP-alone vector transduced (lane 2) and XHR transduced Magi-X4 (lane 3) and Ghost-R5 (lane 4) cells. B) siRNA (~21 bp) detection in GFP-alone vector transduced (lane 2) and XHR transduced Magi-X4 (lane 3) and Ghost-R5 (lane 4) cells. Decade markers (lanes A1 and B1).

Bispecific siRNA vector does not induce interferon:

Double stranded RNA molecules longer then ~30bp are known to induce the interferon pathway in response to viral infections. As siRNAs are generally comprised of 19-24bp in length, they are not expected to activate such a response that mediates a non-specific down regulation of cellular or viral mRNAs. However, recent data had shown that in some circumstances, certain siRNAs might induce variable levels of interferon activation.[48-50] To rule out such a possibility with the present siRNAs, we looked for

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upregulation of phosphorylated-PKR by western blot analysis. PKR is a protein kinase that becomes activated through phosphorylation in the presence of dsRNA and is involved during the interferon response. Our results have shown that the levels of phosphorylated PKR remain unchanged in XHR transduced cells similar to mock and GFP vector transduced cells. In contrast, elevated levels of phosphorylated PKR could be seen in poly I:C transfected cells used as positive controls (Fig 6). These data exclude the possibility of non-specific interferon activation by the combinatorial lentiviral construct.

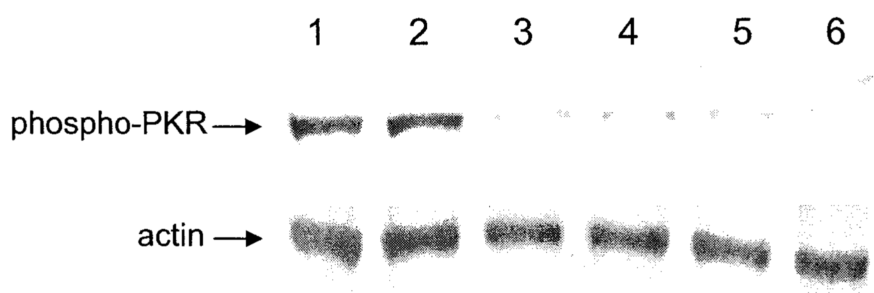

Fig 6. Lack of interferon induction in siRNA transduced cells. To detect interferon induction in siRNA vector transduced cells, western blot analysis was performed to detect elevated levels of phophorylated PKR. Poly I:C was used to induce interferon as a positive control. Transduced cell extracts were run on 10% SDS-PAGE gels, transferred, and probed with an anti-phospho-PKR antibody. Positive control poly I:C transfected (lanes 1 and 2), non-transduced (lane 3), GFP-alone vector (lane 4), and XHR transduced (lane 5) Magi-X4 cells and XHR transduced Ghost-R5 cells (lane 6). An anti-actin antibody was used as an internal control.

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Resistance of siRNA transduced cells to HIV-1 infection:

To determine if down regulation of the essential coreceptors, CXCR4 and CCR5, translated to virus resistance, transduced Magi-CXCR4 and Ghost R5 cells were challenged with X4 (NL4-3) and R5 (BaL1)-tropic strains of HIV-1, respectively. Viral p24 antigen levels at different days post-challenge were determined by ELISA to quantify levels of HIV-1 resistance. Over a 10-fold reduction in viral antigen levels was seen with both XHR transduced Magi-CXCR4 and Ghost-R5 cells as compared to non-transduced and GFP-alone vector transduced cells (Fig 7).

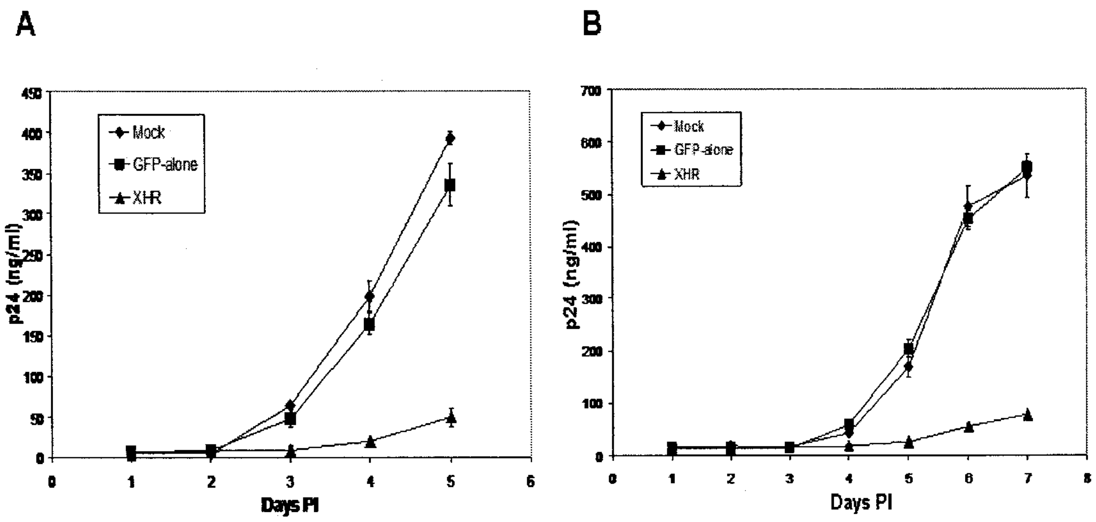

Fig 7. HIV-1 challenge of XHR transduced Magi-X4 and Ghost-R5 cells. Vector transduced cells were challenged with either X4 tropic or R5 tropic viruses at an m.o.i of 0.01. Culture supernatants were collected at different days post challenge and p24 antigen was assayed by ELISA. A) Transduced Magi-X4 cells challenged with X4 tropic HIV-1 NL4-3. B) Transduced Ghost-R5 cells challenged with R5 tropic HIV-1 BaL-1.

We next wanted to determine if the XHR vector expressing CXCR4 and CCR5 siRNAs is effective in physiologically relevant cells for gene therapy. Accordingly,

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PBMCs transduced with vectors were challenged in the same manner as above. A 3-fold level of inhibition was seen on days 3,5, and 7 (Fig 8).

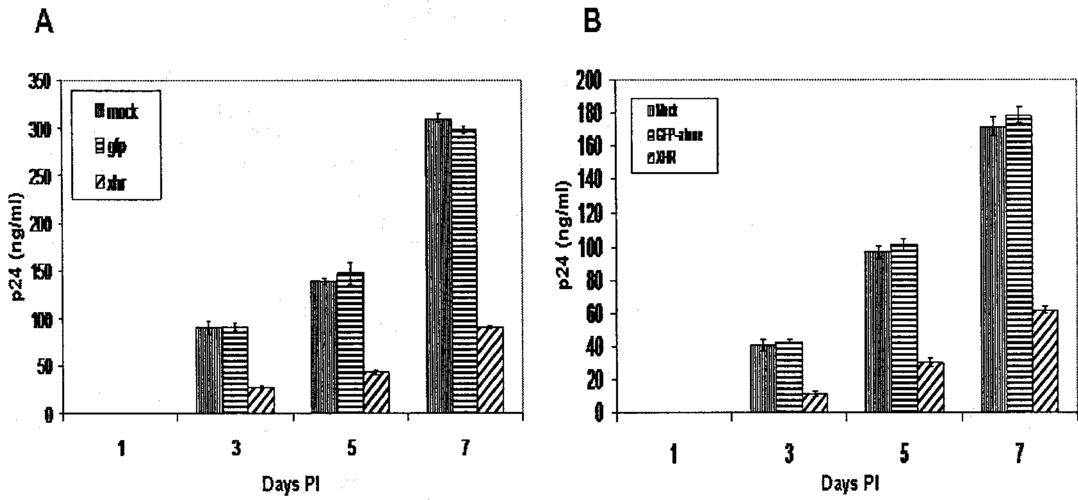

Fig 8. HIV-1 challenge of XHR transduced PBMCs. Vector transduced PBMCs were challenged with either X4 tropic or R5 tropic viruses. Culture supernatants were collected at different days post challenge and p24 antigen was assayed by ELISA. Transduced PBMCs challenged with either HIV-1 NL4-3 (A) or BaL-1 (B).

These results established that the XHR vector is also effective in primary cells in inhibiting HIV-1. Although clearly significant, the levels of virus inhibition were not as dramatic as seen with Magi and Ghost cell lines. The observed levels of viral inhibition in primary PBMC are similar to those observed in a recent report.[31] Lower levels of protection in PBMCs was likely due to the lower levels of transduction. Future studies that are aimed at increasing transduction efficiencies into primary lymphocytes and macrophages are likely to overcome this hurdle.

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DISCUSSION

In summary, our studies have shown for the first time that a single lentiviral vector could be used to stably deliver two different siRNAs targeted to two different cell surface co-receptor molecules and achieve protection against both X4 and R5 tropic HIV-1 viral strains. The short hairpin design permitted use of a single promoter to transcribe both the sense and anti-sense strands of each of the siRNAs. No promoter interference was observed between the U6 promoter driving the transcription of CXCR4 siRNA and the H1 promoter driving the CCR5 siRNA since comparable amounts of both the siRNAs could be seen in transduced cells. Furthermore, possible interferon induction by the combinatorial construct was also ruled out.

A major advantage in using a combinatorial lentiviral construct targeted to both the coreceptors is that infection with either of the viral strains could be prevented at the entry step thus eliminating the possibility of proviral integration and viral latency. Given the success with the current bispecific construct, other novel constructs could be designed and experimented with that incorporate siRNAs targeted to both the cellular as well as viral targets. Based on the design employed here, it is possible to introduce more than two siRNAs in a single construct in the future. However caution should be exercised while incorporating multiple siRNAs in a single construct because the possibility exists that overexpression of foreign siRNAs in a cell may have undesirable effects such as saturating the endogenous RISC complex and consequent toxicity. Such a possibility needs to be tested in long range experiments *in vivo*. We previously have introduced a monospecific siRNA targeted to HIV-1 *rev* into CD34 hematopoietic progenitor cells via lentiviral vectors and derived transgenic macrophages *in vitro* and T cells *in vivo*.[29] The

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transgenic cells were found to be apparently normal while markedly resistant to HIV-1 infection.

No deleterious effects are expected by the stable knock down of the CCR5 coreceptor *in vivo* since individuals harboring a 32bp deletion in the corresponding gene are physiologically normal.[34-35] Although CXCR4 down regulation in circulating mature T cells in the periphery may not have any unsurmoutable ill effects, this may have possible drawbacks in a stem cell setting due to its role in cell homing into bone marrow.[51-52] Therefore, the present combinatorial construct targeted to both CXCR4 and CCR5 coreceptor molecules need to be tested in an *in vivo* system such as the SCID-hu mouse model to evaluate its efficacy and possible toxicity in differentiated cells before it can be used for gene therapy in human subjects. Such experiments are currently underway.

For HIV/AIDS gene therapy strategies to succeed, novel molecules need to be harnessed. In this regard, siRNAs offer great potential. Exploitation of these promising candidates to down regulate essential cellular coreceptors via the use of lentiviral vectors facilitates long term derivation of resistant T cells and macrophages which are the main targets for the virus. Our results showed for the first time that expression of both CXCR4 and CCR5 siRNAs in combination is possible by the use of lentiviral vectors. Coreceptor specific siRNAs stably transduced with the bispecific lentiviral vector showed marked resistance against both T cell tropic and monocyte tropic HIV-1 infection in cell lines and primary PBMCs. The newly developed bispecific vector shows promise for potential *in vivo* application.

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CHAPTER 6

CXCR4 AND CCR5 shRNA TRANSGENIC CD34+ CELL DERIVED MACROPHAGES ARE FUNCTIONALLY NORMAL AND RESIST HIV-1 INFECTION

Anderson J. and Akkina R. CXCR4 and CCR5 shRNA transgenic CD34+ cell derived macrophages are functionally normal and resist HIV-1 infection. Retrovirology. 2005. 2:53

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// ABSTRACT

Stable simultaneous knock down of the HIV-1 coreceptors CCR5 and CXCR4 is a promising strategy to protect cells from both R5 macrophage tropic and X4 T cell tropic as well as dual tropic viral infections. The potency of shRNAs in targeted gene silencing qualifies them as powerful tools for long term HIV gene therapy. Our previous work with a bispecific lentiviral vector containing CXCR4 and CCR5 shRNAs showed efficacy in down regulating both coreceptors and conferring viral resistance to both X4 and R5-tropic strains of HIV-1in cultured cell lines. To extend these results to a stem cell gene therapy setting, here we show transduction of primary CD34+ hematopoietic progenitor cells to derive normal end stage cells that are resistant to HIV-1 infection. The bispecific XHR lentiviral vector harboring CXCR4 and CCR5 shRNA expression cassettes was efficient in transducing CD34+ cells. The transduced cells gave rise to morphologically normal transgenic macrophages when cultured in cytokine media. There was a marked down regulation of both coreceptors in the stably transduced macrophages which showed resistance to both R5 and X4 HIV-1 strains upon *in vitro* challenge. Since off target effects by some shRNAs may have adverse effects on transgenic cells, the stably transduced macrophages were further analyzed to determine if they are phenotypically and functionally normal. FACS evaluation showed normal levels of the characteristic surface markers CD14, CD4, MHC class II, and B7.1. Phagocytic functions were also normal. The transgenic macrophages demonstrated normal abilities in up-regulating the costimulatoy molecule B7.1 upon LPS stimulation. Furthermore, IL-1 and TNFα cytokine secretion in response to LPS stimulation was also normal. Thus, the transgenic macrophages appear to be phenotypically and functionally normal. These studies have demonstrated for the first time that a bispecific lentiviral vector could be used to stably deliver shRNAs targeted to both CCR5 and CXCR4 coreceptors into CD34+ hematopoietic progenitor cells and derive transgenic macrophages. Transgenic macrophages with down regulated coreceptors were resistant to both R5 and X4 tropic HIV-1 infections. The differentiated cells were also phenotypically and functionally normal indicating no adverse effects of shRNAs on lineage specific differentiation of stem cells. It is now possible to construct gene therapeutic lentiviral vectors incorporating

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multiple shRNAs targeted to cellular molecules that aid in HIV-1 infection. Use of these vectors in a stem cell setting shows great promise for sustained HIV/AIDS gene therapy.

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INTRODUCTION

Gene therapy approaches using the strategy of intracellular immunization hold considerable promise towards controlling HIV infection. Previous attempts with anti-HIV molecules that employed RNA decoys, transdominant proteins, and ribozymes were promising towards developing novel therapies.[1-12] With the recent discovery of RNA interference (RNAi), a new and more powerful tool has become available to add to the growing anti-HIV arsenal. The phenomenon of RNA interference has proven to be highly potent in post-transcriptional gene silencing.[13-15] Mediated by sequence specific small-interfering RNAs (siRNAs), RNAi can effectively down regulate the expression of either viral or cellular RNA targets by selective degradation of homologous mRNAs.[16] The mechanism of mRNA degradation involves an endonuclease present in the RNA-induced silencing complex (RISC) which is guided by the antisense component of the siRNA for target recognition.[13-14] A number of reports have shown that delivery of siRNAs by transfection of presynthesized siRNAs or plasmids encoding siRNAs into cultured cells can effectively inhibit HIV-1 infections.[17-26] However, due to the transient nature of transfected nucleic acid, the antiviral effects are only temporary. For HIV gene therapy strategies to succeed long range, it is necessary that siRNA coding transgenes be maintained and expressed long term in a virus susceptible target cell. In this regard, lentiviral vectors have proven to be highly effective in high efficiency gene transduction and sustained gene expression.[27-32]

A number of studies using siRNAs have targeted HIV genes as well as the cellular molecules critical for HIV entry, namely CD4, CXCR4 and CCR5.[18-19,21,23-24,33-37] SiRNAs targeting HIV genes alone will not be sufficient to ward off chronic infection due to the high possibility of generating escape mutants.[38-39] Therefore by targeting host cellular genes critical for viral entry and/or replication, a more sustained efficacy of antiviral effects may be obtained. As a critical player in immunological function, CD4 is physiologically indispensable. The chemokine receptors CXCR4 and CCR5 also play critical roles as coreceptors for viral entry during infection with T cell tropic X4 and macrophage tropic R5 HIV-1 viral strains respectively.[40-41] Their sustained knock down may prove to be more efficacious for long range siRNA therapy.

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Since both R5 and X4-tropic viral strains are involved in disease pathogenesis, it is important to consider both coreceptors when developing effective therapeutics. In a segment of the human population, a naturally occurring 32-bp deletion in the CCR5 gene results in the loss of coreceptor function thus conferring significant resistance to HIV infection.[42-44] Homozygous or heterozygous individuals with this mutation remain physiologically normal. With regard to the CXCR4 coreceptor, it was found to be dispensable for T cell development and maturation in murine studies.[45]

Based on this rationale, recent work with synthetic siRNAs demonstrated that down regulating either CXCR4 or CCR5 will protect cells from X4 or R5 HIV-1 strains, respectively, at the level of viral entry.[18-19,21,23-24,33-37] Stable expression of an anti-CCR5 siRNA was also achieved using a lentiviral vector. However, down regulating CCR5 alone in the face of an HIV-1 infection is insufficient.[34] Therefore, we recently demonstrated that synthetic bispecific combinatorial constructs as well as a bispecific lentiviral vector targeting both CXCR4 and CCR5 showed efficacy in inhibiting HIV-1 infections in cell culture lines.[24,37] In translating these findings into a stem cell gene therapy setting, this bispecific lentiviral vector was used in the present studies to generate shRNA expressing transgenic macrophages.

Macrophages, along with T cells, are major cell targets of HIV infections. Programming these cells to express shRNAs targeted to the essential coreceptors, CXCR4 and CCR5, could confer resistance to HIV infection. Macrophages also have a significant role in immune system functions as antigen presenting cells and as major effector cells in inflammation. Therefore, protecting macrophages from HIV infection is important in maintaining immune system homeostasis. Since shRNAs can have possible off target effects thus dysregulating cellular physiology, transgenic macrophages also need to be assessed for proper functionality.[46] Here we show that CD34+ hematopoietic progenitor cell derived macrophages expressing shRNAs targeting CXCR4 and CCR5 are functionally normal and resist infection to both X4 and R5-tropic strains of HIV-1.

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MATERIALS AND METHODS

Generation of CXCR4 and CCR5 bispecific siRNA lentiviral vector XHR:

A third-generation lentiviral vector system was used to produce the bispecific shRNA-expressing lentiviral vector.[47] The transfer vector pHIV-7-GFP was designed to contain an anti-CXCR4 shRNA cassette under the control of the Pol-III U6 promoter and an anti-CCR5 shRNA cassette under the control of the Pol-III H1 promoter, as previously described.[37] The anti-CXCR4 shRNA targets the CXCR4 transcript at nucleotides 3-23 and the anti-CCR5 shRNA targets the CCR5 transcript at nucleotides 13-31. A depiction of this bispecific lentiviral vector along with two important *cis*-acting elements is shown (Fig. 1). The two *cis*-acting elements, namely, the central DNA flap consisting of the cPPT and CTS (to facilitate the nuclear import of the viral preintegration complex) and the WPRE (to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts), are used to enhance the performance of the vector.[47-48] To generate lentiviral vectors, 293T cells, maintained in complete DMEM containing 10% FBS, were transfected with the plasmids pCHGP-2, pCMV-Rev, pCMV-VSVG, and the appropriate transfer vector, GFP-alone or XHR, using a calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO). Cell culture supernatants were collected at 24, 36, 48, and 60 hours post-transfection, pooled, and concentrated by ultracentrifugation. Vector titers were then analyzed on 293T cells by FACS for EGFP expression. Concentrated vector titers ranged from 8.0×10^7 to 1.5×10^8 for XHR and GFP-alone vectors, respectively.

Transduction of CD34+ hematopoietic stem cells and derivation of macrophages:

CD34+ hematopoietic progenitor cells were purified from human fetal liver by selection with monoclonal antibody-conjugated immunomagnetic beads (Miltenyi Biotech, Auburn, CA).[8] The purity of CD34+ cells was determined by FACS using a PE conjugated CD34+ antibody. The purity of cells was routinely >93% (data not shown). CD34+ cells were maintained in Iscove's modified Dulbecco's growth medium containing IL-3, IL-6, and stem cell factor (SCF) each at 10 ng/ml (R&D Systems,

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Minneapolis, MN) supplemented with 10% FBS. Lentiviral vector transductions were performed on 2 consecutive days at an m.o.i. of 30 in the presence of polybrene (4 ug/ml). Transduced cells were then sorted by FACS for EGFP expression and subsequently placed in semi-solid 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) for 4 days to derive mature macrophages.

Phenotypic and functional analysis of transgenic macrophages:

To determine if stem cell derived anti-coreceptor shRNA transgenic macrophages were otherwise phenotypically normal, analysis of macrophage cell surface markers was performed by FACS with respective conjugated antibodies, PE-CD14 (Caltag, Burlingame, CA), PE-HLA-DR, PE-CY5-CD4, PE-CY5-CXCR4, and PE-CY5-CCR5 (BD Biosciences, San Jose, CA).

Activated macrophages up-regulate the expression of B7.1 co-stimulatory molecules upon stimulation with various stimuli. Accordingly, control non-transduced, GFP-alone, and XHR vector transduced macrophages were stimulated with LPS (5 μg/ml) (Sigma-Aldrich, St. Louis, MO). Twenty-four hours post-stimulation, macrophages were stained with PE-CY5 conjugated anti-B7.1 antibody (BD Biosciences, San Jose, CA) and analyzed by FACS. FACS analyses were performed on the Beckman Coulter Epics XL using ADC software for analysis.

Macrophages play an important role in the immune system as phagocytes. To determine if XHR transgenic macrophages retained the ability to phagocytose foreign material, a phagocytosis assay utilizing tetramethylrhodamine fluorescently labeled *E. coli* Bioparticles® (Invitrogen, Carlsbad, CA) were used. To the cell culture media, 5 ug/ml of LPS and 5 ug/ml of *E. coli* particles were added. Twenty-four hours post-addition, cells were analyzed by FACS. Transduced Magi-CXCR4, maintained as previously described [49-50], were used as a non-phagocytic cell control. Bioparticles® were detected in the PE (FL2) channel for FACS analysis.

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Transgenic macrophages were also analyzed for the secretion of two major cytokines, IL-1 and TNF-α. Macrophages were stimulated with 5 ug/ml of LPS. On days 1, 2, and 3 post-stimulation, cell culture supernatant samples were collected and analyzed by a Quantikine® ELISA kit (R&D Systems, Minneapolis, MN). Non-stimulated supernatants were also analyzed for basal levels of cytokine secretion.

HIV-1 Challenge of CXCR4 and CCR5 siRNA Transgenic Macrophages:

To determine if the stable down regulation of CXCR4 and CCR5 conferred resistance to HIV-1 infection in CD34+ derived macrophages, cells were challenged with X4 (NL4-3) or R5 (BaL-1) tropic strains of HIV-1. Both NL4-3 and BaL-1 challenge experiments were carried out at an m.o.i. of 0.01 for 2 hours in the presence of polybrene (4 ug/ml). Viral supernatants were collected on various days post-infection for p24 antigen ELISAs. To quantify viral p24 levels, a Coulter-p24 kit (Beckman Coulter, Fullerton, CA) was used.

RESULTS

Lentiviral vector transduction of CD34+ cells with CXCR4 and CCR5 shRNAs and derivation of mature macrophages:

A bispecific lentiviral vector XHR, coding for an shRNA targeting CXCR4 driven by a U6 promoter and a CCR5 shRNA under the control of an H1 promoter was designed as previously described (Fig. 1).[37] This vector also contains an EGFP reporter gene downstream from the shRNA cassettes.

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Figure 1. Bispecific lentiviral vector (XHR) encoding anti-CXCR4 and CCR5 shRNAs: A) Control transfer vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. B) To derive the bispecific vector pHIV-XHR-GFP, a U6 promoter driven short hairpin CXCR4 shRNA cassette was cloned into the *BamHI* site upstream of the CMV-EGFP cassette. The H1-CCR5 shRNA cassette was inserted into an *MluI* site downstream to the U6-CXCR4 shRNA cassette.

CD34+ hematopoietic progenitor cells were transduced with either control GFP or XHR vectors. Cells were then sorted for EGFP and driven towards a myeloid lineage in semi-solid methyl cellulose cytokine media to generate transgenic macrophages. No significant differences were found in the levels of macrophages obtained when compared between the control GFP vector and XHR vector transduced cells or control non-transduced CD34+ cells. The morphology of the transgenic macrophages also appeared normal (data not shown).

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Down regulation of HIV-1 coreceptors CXCR4 and CCR5 in transgenic macrophages:

CD34+ derived macrophages normally express both major HIV-1 coreceptors, CXCR4 and CCR5, albeit a lower level of CXCR4. In XHR transduced cells FACS analysis showed an 82% decrease in CXCR4 expression. GFP-alone control vector transduced cells and non-transduced cells displayed normal levels of CXCR4 expression (94%) (Fig. 2A). Similar analysis for CCR5 expression showed a 73% decrease in XHR transduced macrophages with normal levels seen in GFP-alone vector transduced cells similar to non-transduced cells (98%) (Fig. 2B). Thus, stably transduced macrophages exhibited significant down regulation of both the coreceptors CXCR4 and CCR5 due to shRNA targeting.

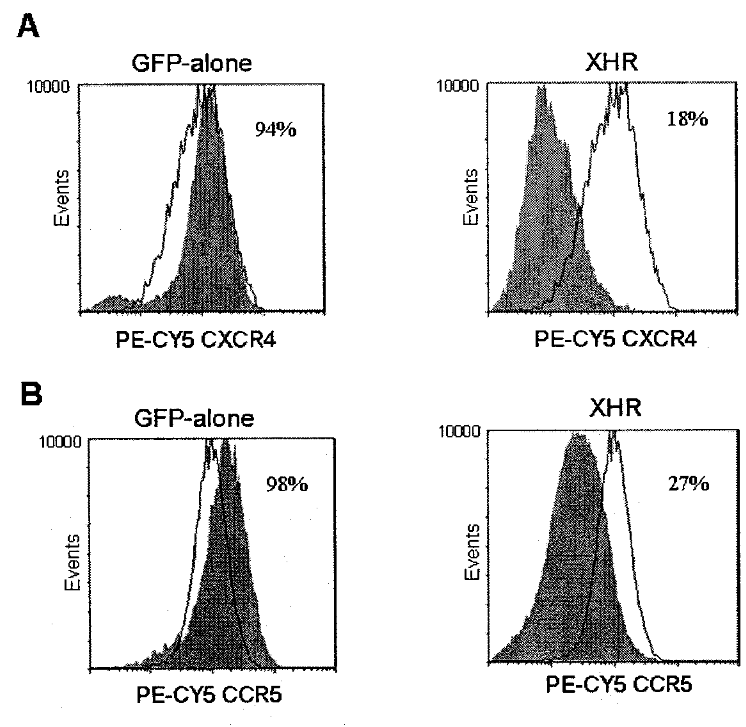

Figure 2. Down regulation of the coreceptors CXCR4 and CCR5 in XHR transgenic macrophages: GFP-alone and XHR transduced CD34+ derived macrophages were labeled with PE-CY5 conjugated antibodies specific for CXCR4 (A) and CCR5 (B) and analyzed by FACS. Control, nontransduced macrophages are shown superimposed as unshaded areas.

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XHR transgenic macrophages resist HIV-1 challenge:

To determine if down regulation of CXCR4 and CCR5 coreceptors conferred viral resistance, transduced macrophages were challenged with X4-tropic (NL4-3) and R5-tropic (BaL-1) strains of HIV-1. Antigen ELISAs to detect viral p24 in culture supernatants were performed on various days post-infection. Over a 2-log reduction in viral yield was seen in XHR transduced macrophages challenged with X4-tropic HIV-1 as compared to control cells (Fig. 3A). In BaL-1 challenge experiments, there was over a 1-log reduction in viral antigen in XHR transduced macrophages compared to control cells (Fig. 3B). Thus stable coreceptor down regulation by siRNAs resulted in marked protection of transgenic macrophages against viral challenge.

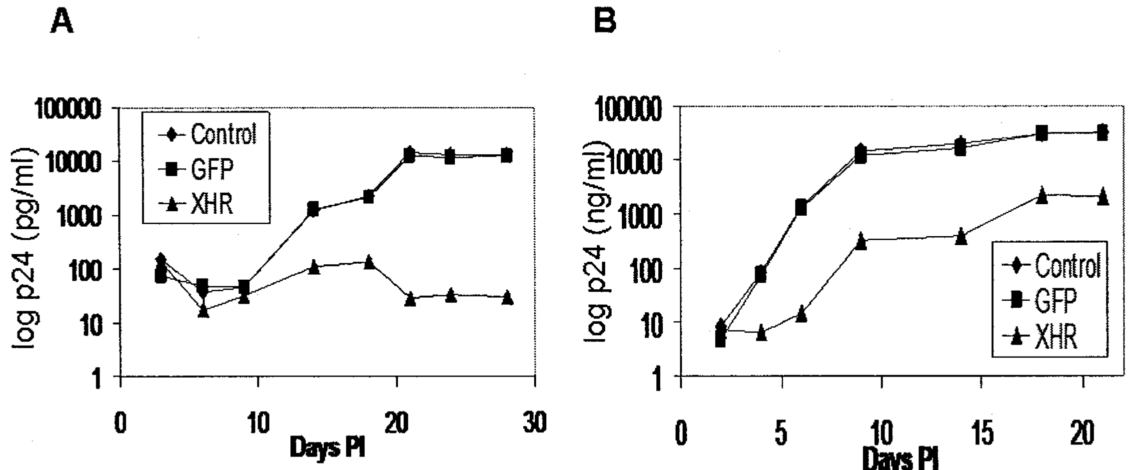

Figure 3. HIV-1 resistance of XHR transgenic macrophages: Control nontransduced (♦), GFP-alone (■), and XHR (▲) transduced CD34+ derived macrophages were challenged with (A) X4-tropic NL4-3 and (B) R5-tropic BaL-1 strains of HIV-1. p24 ELISAs were performed on culture supernatants taken at various time points post-infection. Experiments were performed in triplicate.

Transgenic macrophages display characteristic phenotypic cell surface markers:

Macrophages are critical players in the immune system and also participate in the inflammatory response. Recent work demonstrated possible off target effects of some siRNAs.[46] Such effects may disrupt the phenotypic properties of macrophages or alternatively, may interfere with their normal function. Therefore, transgenic macrophages were subjected to phenotypic analyses to assess their characteristic cell

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surface markers by FACS. Levels of the monocyte/macrophage marker CD14 in XHR macrophages were found to be similar to GFP-alone transduced or nontransduced cells (98% and 97% respectively) (Fig. 4A). Similarly the levels of CD4, a primary HIV-1 receptor, were found at comparable levels for XHR and GFP-alone transduced macrophages at 95% and 93% respectively, coinciding with levels in nontransduced cells (Fig. 4B). The antigen presenting cell surface specific marker, HLA-DR (MHC II) present on macrophages is critical for presenting antigen to CD4+ T cells. A second co-stimulatory molecule B7.1 needed to activate T cells is present at low levels on normal macrophages. Its expression is elevated upon activation with certain stimuli such as LPS. Our evaluation showed that XHR transgenic macrophages displayed similar levels of HLA-DR (92%) when compared to GFP-alone (89%) or with non transduced macrophages (Fig. 4C). The levels of the costimulatory molecule B7.1 were found to be normal at ~15% without stimulation. The transgenic macrophages also displayed capacity to upregulate B7.1 (65%) after LPS stimulation similar to that seen with vector alone and non-transduced control cells (Fig. 4D).

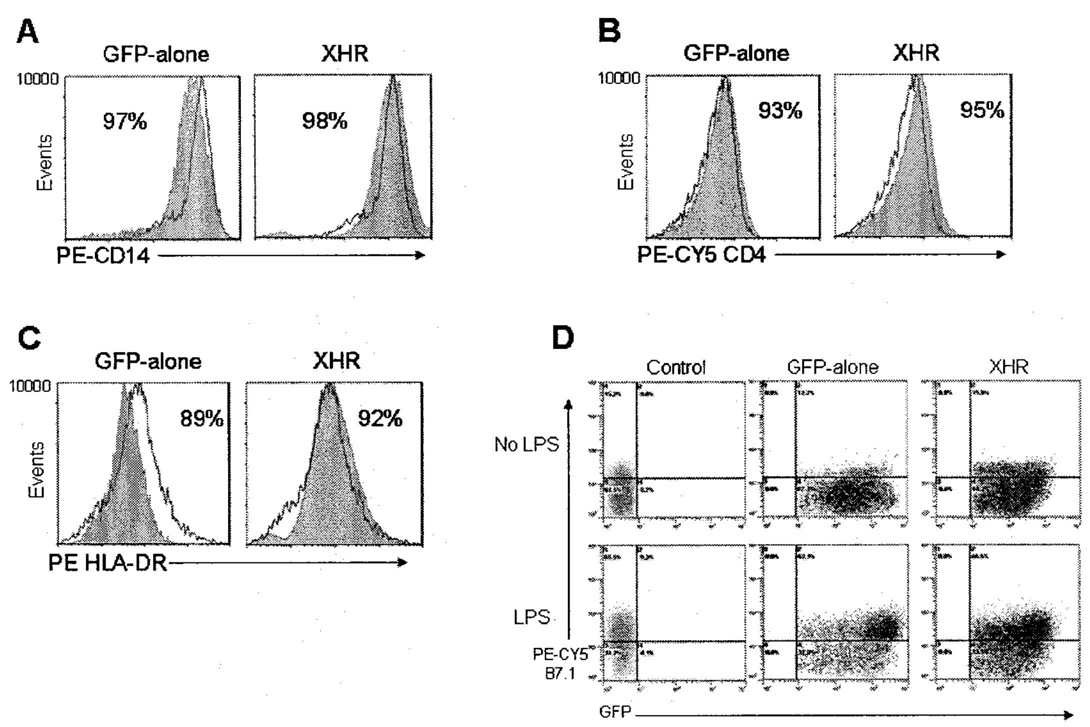

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Figure 4. Transgenic macrophages display normal cell surface markers: GFP-alone and XHR transduced CD34+ derived macrophages were labeled with antibodies specific for (A) CD14, (B) CD4, and (C) HLA-DR and analyzed by FACS. Control, nontransduced macrophages are shown superimposed as unshaded areas. (D) B7.1 upregulation of transgenic macrophages stimulated with LPS. Twenty-four hours post-stimulation, macrophages were labeled with a PE-CY5 conjugated anti-B7.1 antibody and analyzed by FACS. B7.1 upregulation data are representative of triplicate experiments.

Transgenic macrophages are functionally normal:

As stable expression of some shRNAs could have possible off-target global effects leading to disruption of normal cellular functions, we performed functional assays on transgenic macrophages to evaluate this possibilty. A typical function of macrophages is phagocytosis of foreign material and presentation of antigenic peptides. To determine if XHR transgenic macrophages retained the phagocytic function, they were presented with fluorescently labeled *E. coli* (Bioparticles®). Foreign cell uptake was measured by FACS. In comparing non-transduced, GFP-alone transduced, and XHR transduced macrophages, No significant differences in the phagocytic capacity were found between the transgenic macrophages and the vector alone transduced or non-transduced cells. Based on fluroecsecnce levels, XHR macrophage phagocytosis was quantified at 68.2% (Fig. 5E) compared to non transduced and GFP-alone cells at 63.5% and 61.5%, respectively (Fig. 5C and 5D). Transduced Magi-CXCR4 cells, serving as non-phagocytic cell controls did not display any phagocytic activity (Fig. 5B).

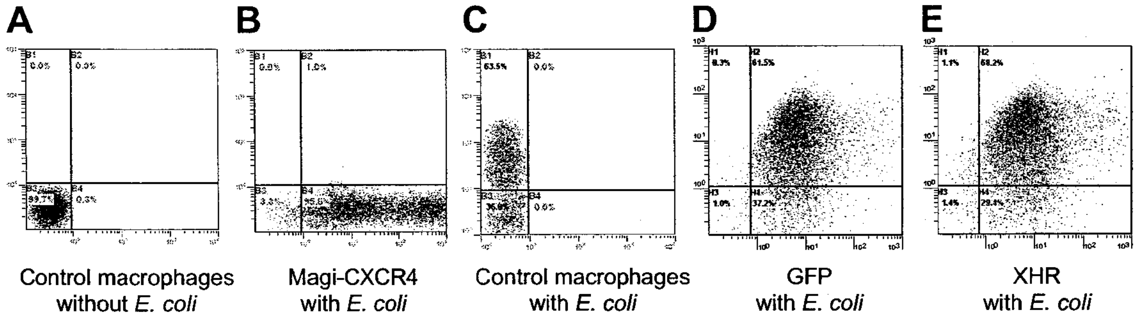

Figure 5. Phagocytosis of fluorescently labeled E.coli by CD34+ derived macrophages: E. coli Bioparticles® were added directly to the cultured macrophages along with 5 μg/ml LPS. Twenty four hours post-stimulation, cells were analyzed by FACS. (A) Control macrophages without Bioparticles®. Panels B-E show plots of cells incubated with Bioparticles® (B) Transduced Magi-CXCR4 (non-phagocytic cell culture), (C) nontransduced, (D) GFP-alone, and (E) XHR macrophages. These data are representative of triplicate experiments.

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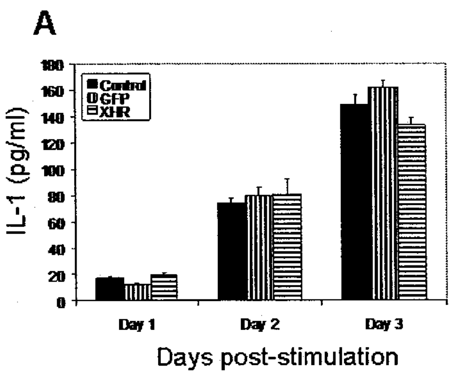
Due to their role in immunity and inflammatory response, macrophages secrete and respond to a number of important cytokines that include IL-1 and TNF-α. To determine if siRNA transgenic macrophages retained their functional capacity to secrete these cytokines at normal levels, they were stimulated with LPS. Levels of released cytokines were measured by ELISA. No significant differences were seen in levels of IL-1 and TNF-α cytokine secretion among the transgenic and control cell types (Fig. 6A and 6B). Basal levels of cytokine production were also detected without LPS stimulation with no differences seen between cell types (data not shown). Collectively the above data showed that coreceptor siRNA transgenic macrophages were phenotypically and functionally normal.



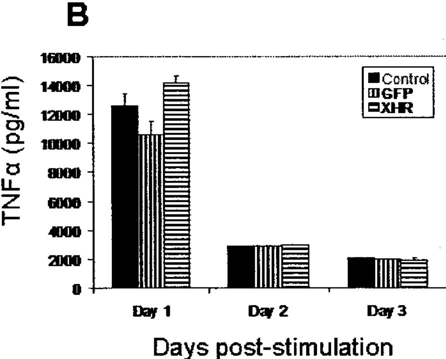


Figure 6. XHR transgenic macrophages secrete normal levels of the cytokines IL-1 and TNFα: Control nontransduced, GFP-alone, and XHR macrophages were stimulated with 5 μg/ml LPS. On days 1, 2, and 3 post-stimulation, supernatants were collected and assayed by ELISA for cytokine secretion of (A) IL-1 and (B) TNFα. Experiments were done in triplicate.

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DISCUSSION

Down regulation of the major HIV-1 coreceptors CXCR4 and CCR5 in virus susceptible cells is a promising approach to prevent viral entry and establishment of productive infection. As noted above, targeting both coreceptors simultaneously will have the added advantage of protecting cells from both X4 and R5 tropic viruses as well as dual tropic strains. In the present studies we have shown that a bispecific lentiviral vector was effective in transducing the respective siRNAs targeted to these coreceptors into primary CD34+ hematopoietic progenitor cells which can give rise to all the blood cell lineages including macrophages, T cells, and dendritic cells.

Since siRNAs are new tools being used for genetic manipulation, it is necessary that they be systematically evaluated in a stem cell setting for their long range utility in protecting end stage differentiated cells such as macrophages. Recent studies have demonstrated that some siRNA constructs may have off target effects.[46] This may adversely affect cell differentiation pathways. Our results have demonstrated that mature macrophages could be derived from lentivirally transduced shRNAs targeting both CXCR4 and CCR5. No significant differences were found in the yields of macrophages from control non-transduced, control GFP-alone vector, and the bispecific shRNA vector transduced CD34+ cells when cultured in cytokine media permitting cell differentiation. This suggests that the respective shRNAs did not interfere with the lineage specific differentiation of gene transduced CD34+ cells into macrophages.

The transgenic macrophages showed significant down regulation of the respective targeted coreceptors CXCR4 and CCR5. Thus, differentiated cells retained functional shRNAs that were effective against their respective target mRNAs. When challenged with HIV-1 *in vitro* they showed marked resistance to infection with both X4 and R5 tropic viral strains. Most primary infections with HIV-1 are believed to be caused by R5 tropic HIV-1 as it is transmitted with relative ease with macrophages as the initial *in vivo* target. During disease progression, X4 tropic viruses are believed to emerge. However recent studies showed that primary X4 HIV-1 isolates could also infect macrophages obtained from human tissue establishing that initial infection of these cells *in vivo* is not confined to R5 strains.[51] Therefore, protecting macrophages against both R5 and X4 tropic viruses is essential to prevent initial viral infection. Thus, the bispecific lentiviral

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vector harboring both CXCR4 and CCR5 shRNAs, described here, would be ideal in preventing HIV-1 infection at the cell entry stage.

A requirement for successful HIV-1 gene therapy is for transgenic virus resistant cells to be phenotypically and functionally normal to maintain and restore the body's immunological function. Accordingly, transgenic macrophages were evaluated to determine if they met these criteria. Although the levels of coreceptor expression diminished substantially as a result of shRNA targeting, phenotypic analyses of shRNA transgenic macrophages showed that they were otherwise phenotypically normal. This was shown by the comparable levels of CD14 and CD4 cell surface markers for both control cells and shRNA transgenic macrophages. Levels of the MHC class II molecule HLA-DR were also found to be normal. Upregulation of the costimulatory molecule B7.1 in response to LPS stimulation was comparable between shRNA transgenic and control vector containing cells. Furthermore, phagocytic functions were also found to be normal. To analyze the critical function of macrophages in secreting cytokines during the inflammatory response, the levels of IL-1 and TNF-α secretion were analyzed. Our results demonstrated that the expression of CXCR4 and CCR5 shRNAs and the subsequent downregulation of these chemokine receptors had no apparent effect on IL-1 or TNF-α secretion. These data collectively suggest that phenotypically and functionally normal macrophages could be obtained from CD34+ cells lentivirally transduced with CXCR4 and CCR5 shRNA constructs. These results establish for the first time that simultaneous knock down of both the chemokine receptors CXCR4 and CCR5 have no apparent adverse effects on macrophage differentiation, phenotype or function.

The above data showed the efficacy of this bispecific shRNA construct in deriving HIV-1 resistant macrophages *in vitro* in a stem cell setting. Further preclinical testing of this construct is needed *in vivo* to determine its suitability for use in the human. The SCID-hu mouse model that harbors a functional human thymus permits evaluation of vector transduced CD34+ cells to determine their capacity to give rise to mature T cells. The transgenic T lymphocytes so derived could be assessed for their functionality and viral resistance as we have shown previously.[29] Adverse effects are not expected by the stable knock down of CCR5 *in vivo* as it was previously documented in many studies that individuals harboring a 32bp deletion in the CCR5 gene do not exhibit any

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immunological abnormalities.[42-44] However, stable CXCR4 knock down may have possible side effects in a stem cell setting due to its role in cell homing.[52] Therefore, a systematic evaluation of the CCR5 and CXCR4 bispecific construct *in vivo* in the SCID-hu mouse model is necessary to determine its efficacy and possible toxicity in differentiated T cells prior to its evaluation in human subjects. Such studies are currently underway.

Stable simultaneous knock down of both the coreceptors CCR5 and CXCR4 is necessary to prevent HIV-1 infection at the entry level by both R5 and X4, as well as dual tropic viral strains. Our present studies have demonstrated for the first time that a bispecific lentiviral vector could be used to stably deliver shRNAs targeted to both CCR5 and CXCR4 coreceptors into CD34+ hematopoietic progenitor cells and derive transgenic macrophages. Stable down regulation of both the coreceptors was achieved in transgenic macrophages which displayed marked resistance to HIV-1 challenge in vitro. The siRNA expressing macrophages were also found to be phenotypically and functionally normal. It is now possible to construct gene therapeutic lentiviral vectors incorporating multiple siRNAs targeted to cellular molecules that aid in HIV-1 infection. Use of these vectors in a stem cell setting shows great promise for sustained HIV/AIDS gene therapy.

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CHAPTER 7

TRIM5α_{rh} EXPRESSION RESTRICTS HIV-1 INFECTION IN LENTIVIRAL VECTOR TRANSDUCED CD34+ CELL DERIVED MACROPHAGES

Anderson J. and Akkina R. TRIM5alpharh expression restricts HIV-1 infection lentiviral vector-transduced CD34+ cell derived macrophages. Mol Therapy. 2005. 4:687-696.

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ABSTRACT

Species specific innate resistance against viral infections offers novel avenues for antiviral therapeutic and prophylactic approaches. The retroviral and lentiviral restriction factors Ref1 and Lv1 are variants of the tripartite motif protein, TRIM5α, a component of cytoplasmic bodies. TRIM5α severely restricts productive retroviral infections at the post-entry and pre-integration steps by destabilizing the incoming viral capsid via ubiquitination. Using this approach, resistance to HIV-1 infection could be conferred by $TRIM5\alpha_{rh}$ expression in otherwise susceptible cells. Here we show that stable expression of simian $TRIM5\alpha_{rh}$ via a lentiviral vector in a permissive cell culture line, Magi-CXCR4, conferred resistance to HIV-1. To translate these findings into a stem cell gene therapy setting, the $TRIM5\alpha_{rh}$ transgene was stably introduced into CD34+ hematopoietic progenitor cells to derive transgenic macrophages. Upon viral challenge, $TRIM5\alpha_{rh}$ expressing macrophages were highly resistant to HIV-1 infection compared to control cells. Human macrophages expressing $TRIM5\alpha_{rh}$ were also found to be phenotypically and functionally normal expressing the characteristic surface markers CD14, CD4, CCR5, CXCR4, MHCII, and B7.1. These results demonstrate that the species-specific restriction factor $TRIM5\alpha_{rh}$ is effective in conferring HIV-1 resistance in a stem cell setting thus paving the way for its application in AIDS gene therapy.

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INTRODUCTION

To control and treat HIV/AIDS, novel and innovative therapies need to be developed as current therapies are toxic and eventually give rise to resistant viral strains with prolonged use[1-6]. Intracellular immunization through gene therapy offers a promising alternative approach in protecting target cells, thus preserving and restoring immune function. A number of gene therapeutic strategies have been attempted through the years which showed considerable promise. These approaches used effector molecules such as transdominant proteins[7,8], decoys, ribozymes, and siRNAs[9]. Since HIV replication could be blocked at different stages, both cellular and viral specific molecules have been targeted. Some of these have reached clinical trials[10].

As the replication cycle of HIV is better understood with recent advances, it has become increasingly clear that host specified proteins play an important role in aiding and regulating viral expression. Of the many therapeutic approaches, restricting viral replication at the stage of viral entry is of particular advantage since the cell is protected from the toxicity of virally expressed proteins and prevents proviral formation and eventual latency in a sub-population of cells. Recent work from many laboratories, including ours, has successfully targeted cell surface molecules that aid in viral entry. For example, the HIV-1 coreceptor CCR5 was effectively down regulated by the use of ribozymes and siRNAs[9,11-16]. Similarly, the primary receptor CD4 and the coreceptor CXCR4 were also effectively inhibited by the use of siRNAs[12-14,17,18]. Such down regulation of the cell surface receptors have provided the proof of concept that HIV-1 Infection could be prevented at the level of viral entry. This concept was further extended for use in a stem cell gene therapy setting by utilizing lentiviral vectors to transduce hematopoietic stem cells with various constructs[8,11,19,20]. However, as HIV-1 is a highly mutation prone virus with propensity to generate escape variants, targeting a limited number of either viral or cellular molecules will not be sufficient for long term therapy. Therefore, novel targets need to be identified and harnessed.

It has long been known that species-specific restriction factors exist that confer resistance to retroviral and lentiviral infections[21]. Therefore the restriction factors that block viral infection could also be exploited for HIV-1 gene therapy. Certain nonhuman primates are naturally resistant to HIV-1 infection due to restricted species tropism. HIV-

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1 is unable to infect rhesus macaque cells because of a restriction at the post-entry step[21-24]. Retroviral restriction was shown to be due to the action of factors Ref1 and Lv1[21,25-35]. Through a genetic screen of rhesus monkey lung fibroblasts it was recently discovered that the MLV restriction factor Ref1 and the lentiviral restriction factor Lv1 are encoded by the same gene, TRIM5α[36]. The rhesus macaque isoform of TRIM5α ($TRIM5\alpha_{rh}$) was shown to confer strong resistance to HIV-1 when expressed in cells otherwise susceptible to infection[29,31-33,37-39]. It was also shown that $TRIM5\alpha_{rh}$ which restricts HIV-1 infection in monkey cells shares an 87% amino acid homology with that of the human TRIM5α isoform ($TRIM5\alpha_{hu}$) [36].

A component of cell cytoplasmic bodies, TRIM5α is a member of the tripartite motif (TRIM) family of proteins that contain RING domains, B boxes, and coiled coils[40-42]. TRIM5α also contains a carboxy terminal B30.2 SPRY domain that was shown, along with the RING domain, to be critical for HIV-1 restriction[29,36-39]. HIV-1 restriction mediated by $TRIM5\alpha_{rh}$ at the level of post-entry and pre-integration, is believed to be due to its destabilizing action on the viral capsid[27,43-45]. It is presumed to occur through ubiquitination as one TRIM isoform was shown to have ubiquitin ligase activity that is common to RING containing proteins[46]. The antiviral activity of $TRIM5\alpha_{rh}$ has recently been narrowed down to a 13 amino acid patch of positively selected amino acids in the SPRY domain[38]. Further analysis of this domain revealed a single amino acid change in the human isoform of $TRIM5\alpha_{hu}$ that conferred resistance to HIV-1[37,39].

Based on these observations, $TRIM5\alpha_{rh}$ could be exploited for conferring genetic resistance to HIV-1 in otherwise susceptible cells. The goal in our present study is to stably transduce $TRIM5\alpha_{rh}$ into human cells to confer HIV-1 resistance. For long range gene therapy, CD34+ hematopoietic progenitor stem cells (HPCs) are ideal targets for transducing anti-HIV genes as they give rise to both T cells and macrophages which are the main viral targets. For stable transduction of these cells, retroviral vectors are commonly used. Of the retroviral vectors, lentiviral vectors have proven to be superior for transduction of mostly quiescent cells such as hematopoietic cells[19,47]. In these studies we used a third generation lentiviral vector for stable gene transfer and sustained gene expression. We wanted to determine the utility of $TRIM5\alpha_{rh}$ to derive HIV-1 resistant differentiated target cells. Using an *in vitro* cell differentiation system, we show

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that expression of TRIM5α_{rh} has no adverse effect on lineage specific differentiation of CD34+ cells into HIV-1 resistant macrophages.

MATERIALS AND METHODS

Construction and Production of a Lentiviral Vector Expressing TRIM5α_{rh}:

A cDNA clone of the previously characterized restriction factor, TRIM5α_{rh}, isolated from rhesus monkey lung fibroblasts was obtained from the AIDS Reference and Reagent Program. The plasmid vector construct pLPCX has a TRIM5α-HA tag fusion protein for immunodetection. This construct was used to generate the gene transfer lentiviral vector. A third generation lentiviral transfer vector backbone, HIV-7-GFP, containing an EGFP reporter gene was employed to derive the construct. The vector backbone also contains two *cis*-acting elements, namely, the central DNA flap consisting of cPPT and CTS (to facilitate the nuclear import of the viral preintegration complex) and the WPRE (to promote nuclear export of transcripts and/or increase the efficiency of polyadenylation of transcripts), to enhance the vector performance[50]. The TRIM5α_{rh}-HA cassette was PCR amplified from pLPCX-TRIM5α_{rh}-HA and inserted into the lentiviral vector upstream of the EGFP reporter gene (Fig. 1). The expression of TRIM5α_{rh}-HA is driven by a CMV promoter. Confirmation and sequencing of candidate clones was performed by Laragen (Los Angeles, CA).

To generate lentiviral vectors, 15 ug of transfer vector plasmid with either GFP-alone or TRIM5α_{rh}-HA were transfected along with 15 ug pCHGP-2, 5 ug pCMV-Rev, and 5 ug pCMV-VSVG into 293T cells at 60% confluency in 100 mm culture dishes using a calcium phosphate transfection kit (Sigma-Aldrich, St. Louis, MO). Six hours after transfection, fresh medium was exchanged. Cell culture supernatants containing the vector were collected at 24, 36, 48, and 60 hours post transfection and pooled. Vector supernatants were concentrated by ultracentrifugation and later titrated on 293T cells using FACS analysis for GFP expression as described previously. The titers of the T5A vector were generally lower than that of the control GFP-alone vector being $5.0*10^6$ and $1.0*10^8$ respectively. This could very likely be due to interference with vector packaging

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through interactions between TRIM5α_{rh} and the HIV *gag* capsid encoded in the packaging plasmid, pCHGP-2.

Lentiviral Vector Transduction and Derivation of Macrophages:

Initial experiments with the lentiviral vector were performed on HIV-1 susceptible cell lines followed by studies in hematopoietic stem cells. Magi-CXCR4 cells obtained from the AIDS Reference and Reagent Program were maintained in media as previously described[52-53]. Cells were transduced with either GFP-alone or TRIM5α_{rh}-HA vectors for two hours in the presence of 4 ug/ml polybrene. To enrich for transduced cells, forty-eight hours post-transduction, cells were sorted based on EGFP expression. The sorted cells were later cultured to obtain a pure population of transgenic cells for subsequent experiments.

CD34+ hematopoietic progenitor cells were purified from human fetal liver using monoclonal antibody-conjugated immunomagnetic beads (Miltenyi Biotech, Auburn, CA). The purity of CD34+ cells obtained was routinely >95%. Cells were cultured in Iscove's media containing 10% FBS and 10 ng/ml of each of the cytokines IL-3, IL-6, and SCF. For vector transduction, CD34+ cells were incubated with respective vectors for two hours in the presence of 4 ug/ml polybrene. Two rounds of transductions were performed on two consecutive days. Transduced cells were sorted by FACS for EGFP expression forty-eight hours post-transduction. To drive transduced CD34+ cells towards a myeloid lineage, sorted cells were differentiated in a semi-solid Methocult medium (Stem Cell Technologies) for 12 days. To derive monocytes/macrophages, the myeloid colonies were then pooled and cultured in cytokine media containing 10% FBS and 10 ng/ml each of the cytokines GM-CSF and M-CSF for five days. Subsequent experiments were performed on these *in vitro* derived pure populations of mature macrophages.

Detection of TRIM5α_{rh}-HA expression in transgenic cells by immunoblotting:

To determine the expression of TRIM5α_{rh}-HA in transduced cells, western blotting was performed using an antibody specific for the HA tag. Transduced Magi-

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CXCR4 cell lysates were resolved on a 10% polyacrylamide gel and blotted on to Immobilon™-P membranes (Millipore, Bedford, MA). After the blocking step, the transferred proteins were probed with rabbit anti-HA at 4°C overnight and later reacted with an alkaline phosphatase conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Western Blue® substrate (Promega, Madison, WI) was added to visualize proteins as previously described. Human actin protein was used as an internal cell protein control and rabbit anti-actin (Sigma-Aldrich, St. Louis, MO) was used for its detection.

HIV-1 Challenge of Transduced Magi-CXCR4 Cells:

To determine if the expression of TRIM5α_{rh}-HA conferred resistance to HIV-1 infection, transduced Magi-CXCR4 cells were challenged with X4-tropic (NL4-3) HIV-1 at an m.o.i. of 0.01. Infections were carried out in the presence of 4 ug/ml polybrene for two hours. Cell culture supernatants were collected on various days post-infection for p24 analysis. ELISA was performed to determine viral antigen levels using the Coulter-p24 kit (Beckman Coulter, Fullerton, CA). Transduced Magi-CXCR4 cells were also challenged with an X4-tropic, NL4-3 strain of HIV-1 that expresses the murine CD24 heat stable antigen (HSA). Infections were carried out in the presence of 4 ug/ml polybrene for two hours at various m.o.i of 0.01, 0.05, and 0.1. Cells were analyzed by FACS on days 2, 4, and 7 post-infection to detect expression of murine CD24 using a PE conjugated anti-mCD24 antibody (Biolegend, San Diego, CA).

HIV-1 Challenge of TRIM5α_{rh} Transgenic Macrophages Derived from CD34+ cells:

To determine if the expression of TRIM5α_{rh}-HA conferred resistance to HIV-1 infection in a stem cell setting, transduced CD34+ derived macrophages were challenged with X4 (NL4-3) or R5 (BaL-1) tropic strains of HIV-1. Infections were carried out like above at an m.o.i. of 0.01. Culture supernatants were collected on various days post-infection for p24 analysis. ELISA was performed to determine viral antigen levels using the Coulter-p24 kit (Beckman Coulter, Fullerton, CA).

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Phenotypic Analysis of TRIM5α_{rh} Transgenic Macrophages:

To determine if transgenic macrophages were phenotypically normal, analysis of macrophage cell surface markers was performed by FACS with respective conjugated antibodies, PE-CD14 (Caltag, Burlingame, CA), PE-HLA-DR, PE-CY5-CD4, PE-CY5-CXCR4, and PE-CY5-CCR5 (BD Biosciences, San Jose, CA).

Activated macrophages up-regulate the expression of B7.1 co-stimulatory molecules upon stimulation with various stimuli. Accordingly, control non-transduced, GFP-alone, and T5A vector transduced macrophages were stimulated with LPS (10 μg/ml) (Sigma-aldrich, St. Louis, MO). Twenty-four hours post-stimulation, macrophages were stained with PE-CY5 conjugated anti-B7.1 antibody (BD Biosciences, San Jose, CA) and analyzed by FACS. FACS analyses were performed on the Beckman Coulter Epics XL using ADC software for analysis.

RESULTS

Transduction and expression of TRIM5α_{rh} in human cells with a lentiviral vector:

To obtain stable transduction and expression of the TRIM5α_{rh} transgene in human cells, a third generation lentiviral vector was constructed as described in the methods (Fig. 1).

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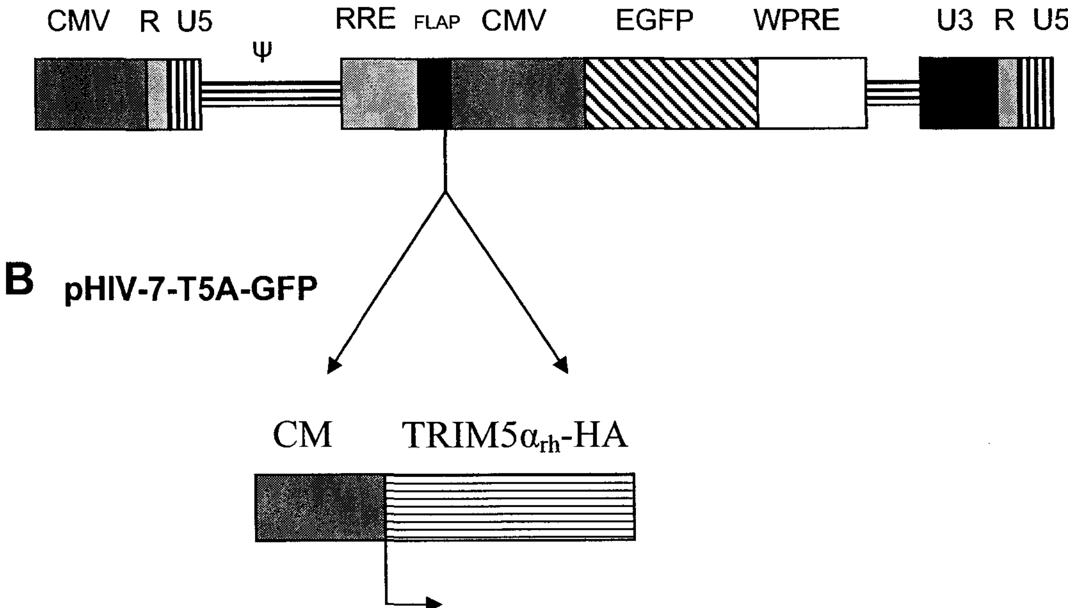

Figure 1. Lentiviral vector encoding the TRIM5αrh-HA transgene: A) Control transfer vector pHIV-7-GFP encoding a CMV promoter driven EGFP reporter gene. B) To derive the vector pHIV-T5A-GFP, a CMV driven TRIM5α_{rh}-HA cassette was cloned upstream of the CMV-EGFP cassette.

Vector titers obtained for T5A and GFP-alone were $5.0*10^6$ and $1.0*10^8$ respectively. The decreased levels of T5A may possibly be due to the interaction of TRIM5α_{rh} and the *gag* protein which is expressed from the packaging plasmid, pCHGP-2, during vector production. To obtain higher titers for T5A, the amount of pCHGP-2 was doubled during transfection of 293T cells. By adding additional packaging plasmid, the levels of T5A vector production increased 2-fold (data not shown). Human HeLa cell derived Magi-CXCR4 cells that are permissive to HIV-1 NL4-3 infections were transduced with respective vectors containing EGFP-alone (control vector) and a TRIM5α_{rh} expressing vector (T5A). To obtain a pure population of stably transduced cells, the cells were sorted based on EGFP expression and subsequently cultured. Representative FACS profiles showing percent EGFP positive cells in these cultures are shown in Fig. 2A and 2B. Control vector and T5A transduced cells were 94% and 85% positive for EGFP expression respectively. To detect the expression of TRIM5α_{rh}-HA in stably transduced cells, western blot analysis was performed. As seen in Fig. 2C, lane 3,

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T5A vector transduced cells were positive for TRIM5α_{rh}-HA expression as shown by antibody detection of the the HA epitope tag. Control non-transduced cells and control vector transduced cells, Fig. 2 lanes 1 and 2, respectively, were negative for TRIM5α_{rh}-HA expression. Cellular actin was used as an internal control and its levels were consistent for all samples.

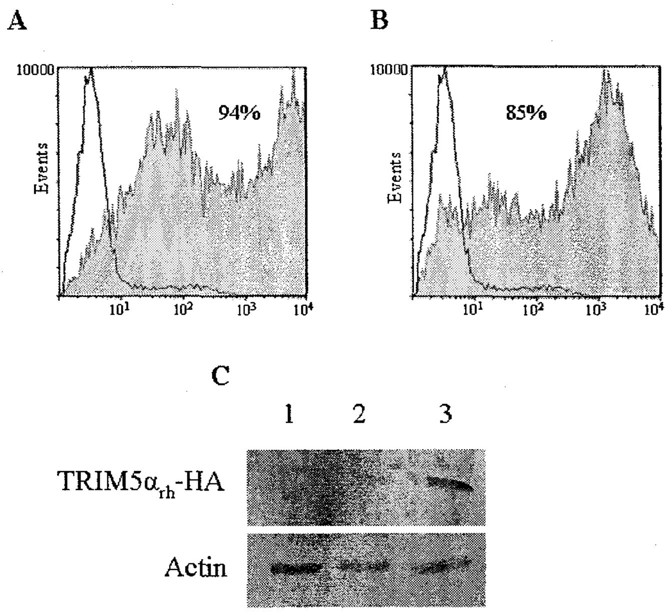

Figure 2. Vector transduction levels in Magi-CXCR4 cells and detection of TRIM5α_{rh}-HA expression by western blot: Magi-CXCR4 cells were transduced with a A) GFP-alone control vector and the B) T5A vector. Transduced cells were then sorted based on EGFP expression and cultured. Levels of EGFP from non-transduced cells are superimposed. C) Vector transduced Magi-CXCR4 cell lysates were subjected to immunoblotting using an anti-HA antibody as described in methods. Lane 1) Non-transduced cells, Lane 2) GFP-alone vector transduced cells, Lane 3) T5A vector transduced cells. Cellular actin was used as an internal control.

TRIM5α_{rh} restriction of HIV-1 in transduced Magi-CXCR4 cells:

Magi-CXCR4 cells, a HeLa derivative human cell line that constitutively expresses CXCR4 can be productively infected with X4-tropic strains of HIV-1[48-49]. To

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evaluate the restriction of HIV-1 by TRIM5α_{rh} in stably transduced cells, they were challenged with X4-tropic HIV-1 NL4-3. Viral p24 antigen levels were measured at various days post-infection. As seen in Fig. 3, viral replication was severely inhibited in T5A transduced cells compared to control non-transduced and control vector transduced cells. The level of inhibition was around 1.5 log.

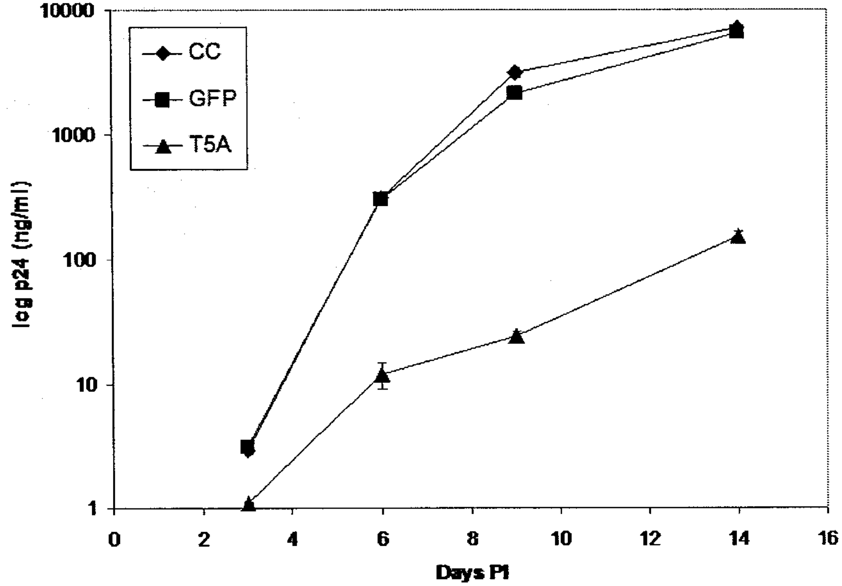

Figure 3. HIV-1 challenge of TRIM5α_{rh}-HA transduced Magi-CXCR4 cells: Vector transduced cells were challenged with X4-tropic HIV-1 NL4-3 at an m.o.i of 0.01. Culture supernatants were collected from non-transduced (CC) (♦), GFP-alone (■), and T5A (▲) cultures at different days post challenge. p24 antigen levels were assayed by ELISA. Data presented is from triplicate experiments.

Clearly from these results, TRIM5α_{rh} transduced Magi-CXCR4 cells were highly resistant to HIV-1 infection. Since it has been reported that TRIM5α restriction is saturable and could be overcome by increased viral input, additional challenge experiments with increased m.o.i. were also used to evaluate resistance levels. In these experiments, we utilized a replication competent reporter virus, HIV-NL4-3, that expresses a murine CD24 heat stable antigen (HSA). Upon productive infection, infected

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cells will express HSA on their surface which can be detected by surface staining and FACS analysis. At low m.o.i. of 0.01, nearly all TRIM5α_{rh} transgenic cells were resistant to HIV-1 challenge even on day 7 post-infection (Fig. 4B) during which time most of the control cells were infected and positive for CD24 expression (Fig. 4A and 4B). On the contrary, at a ten fold higher m.o.i., the number of infected cells increased substantially on day 7 but the overall infection levels were still lower than in control cells (Fig. 4D). These data confirm that a portion of TRIM5α_{rh} resistance could be overcome with increased viral input.

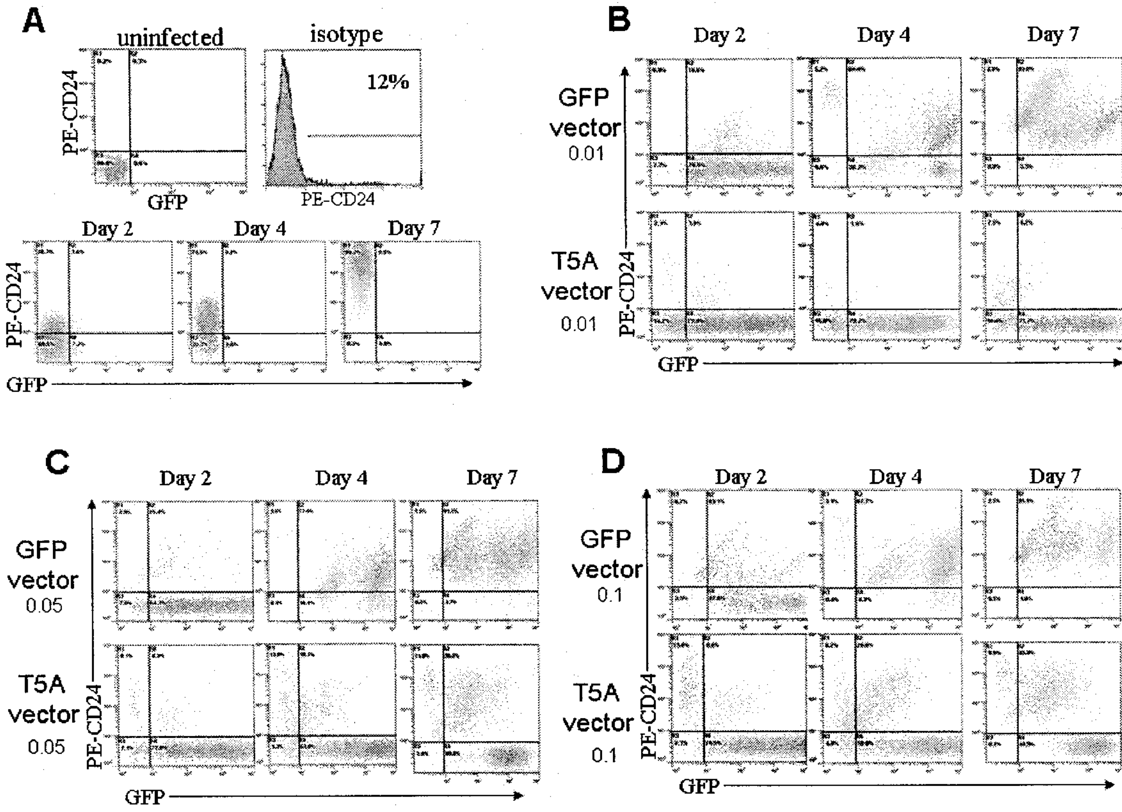

Figure 4. Evaluation of HIV-1 resistance in TRIM5α_{rh}-HA transgenic Magi-CXCR4 cells with increasing viral challenge doses: Transduced Magi-CXCR4 cells were challenged with various m.o.i. with an HIV-1 NL4-3 strain that expresses the murine CD24 heat stable antigen (HSA). Cells were subsequently analyzed by FACS. A) Control non-transduced Magi-CXCR4 cells challenged with NL43-HSA at an m.o.i. of 0.01. Unstained and isotype control stained non-transduced cells are also shown. Panels B-D: GFP-alone and T5A vector transduced cells were challenged with various m.o.i and analyzed at different days post infection. M.o.i levels: B) 0.01, C) 0.05, and D) 0.1. On days 2, 4, and 7 post-infection, cells were stained with a PE conjugated anti-murine CD24 antibody and analyzed by FACS. These data represent triplicate experiments.

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Derivation of TRIM5α_{rh} transgenic macrophages from lentiviral vector transduced CD34+ hematopoietic progenitor cells:

For gene therapy strategies utilizing restriction factors to succeed long-term, it is necessary that hematopoietic progenitor cells be transduced to give rise to virally resistant macrophages and helper T cells. As a first step towards determining the feasibility of using TRIM5α_{rh} in a stem cell setting, we transduced CD34+ cells with the T5A vector to derive transgenic macrophages *in vitro*. When transduced CD34+ cells were cultured in cytokine media with appropriate cytokines, macrophage development was found to be normal. No differences were observed in the levels of macrophages obtained when compared with the non-transduced cultures or cultures that were transduced with the control vector.

To determine whether TRIM5α_{rh} expressing macrophages were phenotypically normal, FACS analysis was performed to analyze the expression of normal macrophage surface markers. Both control cells and TRIM5α_{rh} transduced cells were CD14 positive, confirming their differentiation into mature macrophages (Fig. 5A). HIV-1 receptor and coreceptor expression as determined by FACS analysis for CD4, CCR5, and CXCR4 expression was comparable between nontransduced, GFP-alone vector transduced, and T5A vector transduced cells (Fig. 5B-D). Antigen presenting cell surface marker expression was also analyzed by FACS for HLA-DR (MHCII) and B7.1. The levels of MHCII markers were also comparable between all cell types analyzed (Fig. 5E).

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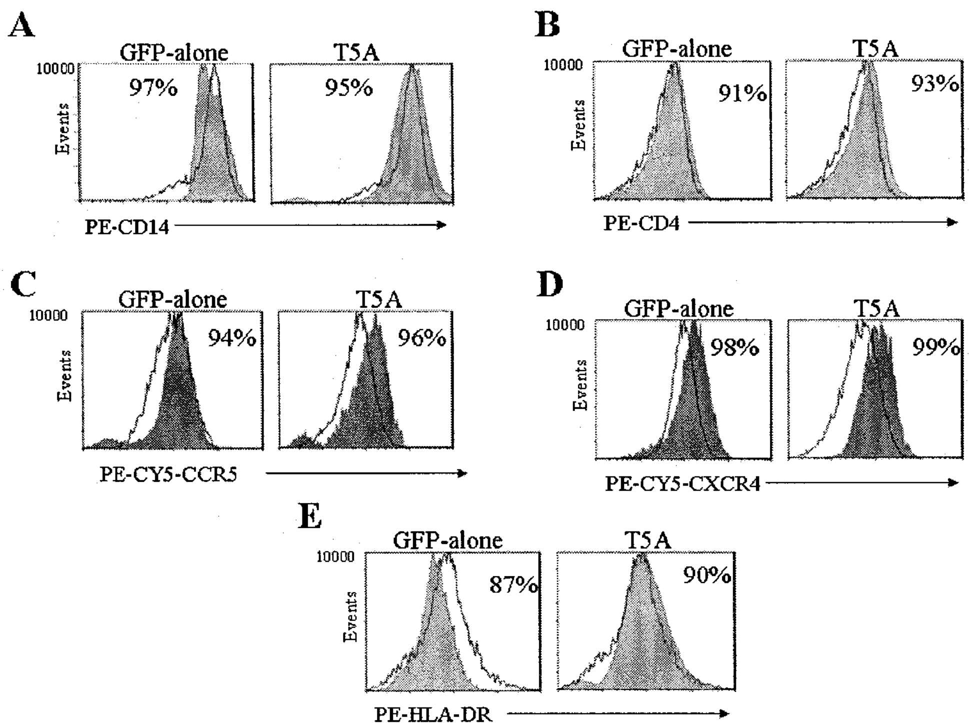

Figure 5. Immunophenotypic analysis of TRIM5α_{rh}-HA transduced macrophages: Transgenic macrophages were analyzed by FACS to determine the levels of macrophage cell surface markers. Cells were stained with the conjugated antibodies A) PE-CD14, B) PE-CY5 CD4, C) PE-CY5 CCR5, D) PE-CY5 CXCR4, and E) PE-HLA-DR. Levels of cell surface markers of non-transduced cells are superimposed.

Activated macrophages up-regulate the expression of B7.1 co-stimulatory molecules and present antigen to memory and effector T cells. Macrophages normally express low levels of B7.1. However, upon activation with various stimuli, B7.1 is up-regulated on the cell surface. We wanted to determine if the transgenic macrophages are functionally normal in up-regulating the B7.1 co-stimulatory molecule in response to LPS stimulation. Our results showed that B7.1 up-regulation is normal in TRIM5α_{rh} transgenic macrophages when compared with control non-tranduced and control GFP-alone vector transduced macrophages (Fig. 6). Thus phenotypically and functionally normal macrophages could be obtained from TRIM5α_{rh} transduced CD34+ cells.

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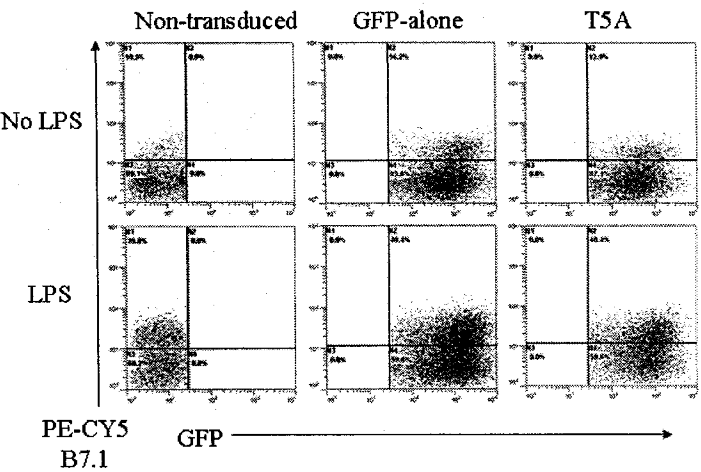

Figure 6. Costimulatory molecule B7.1 upregulation in LPS stimulated transgenic macrophages: Non-transduced, GFP-alone, and T5A vector transduced macrophages were stimulated with LPS. Twenty-four hours post stimulation cells were stained with PE-CY5 conjugated anti-B7.1 antibody and analyzed by FACS. These data are representative of triplicate experiments.

TRIM5α_{rh} restricts HIV-1 in CD34+ cell derived macrophages:

To determine if TRIM5α_{rh} transduced *in vitro* derived macrophages were resistant to HIV-1 infection, cells were challenged with either X4 or R5-tropic strains of HIV-1. As seen in Fig. 7A, over a 2 log difference in viral antigen levels were seen in TRIM5α_{rh} transduced macrophages compared to control cells when challenged with NL4-3. Over a 1.5 log difference in antigen levels was seen in TRIM5α_{rh} transduced cells when challenged with BaL-1 (Fig. 7B).

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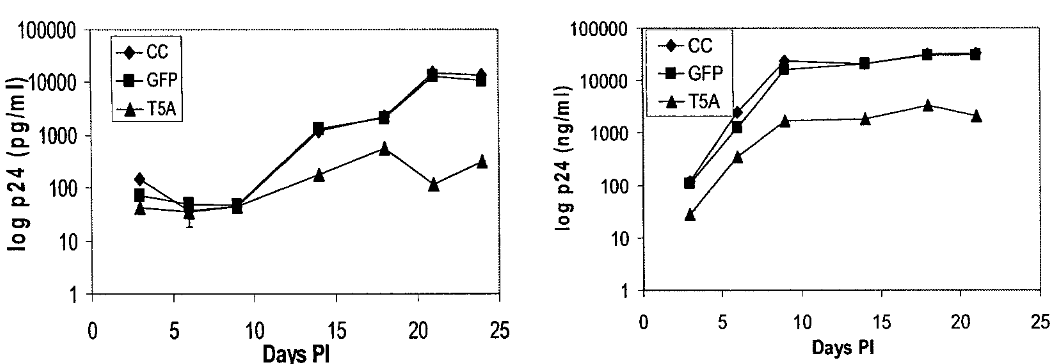

Figure 7. HIV-1 challenge of TRIM5α_{rh}-HA transduced CD34+ cell derived macrophages: Vector transduced cells were challenged with either A) X4 or B) R5-tropic strains of HIV-1 at an m.o.i. of 0.01. Viral supernatants were collected from non-transduced (CC) (♦), GFP-alone (■), and T5A (▲) cultures at different days post challenge and p24 antigen was assayed by ELISA. Data presented is from triplicate experiments.

Due to the low levels of infection and viral replication of T cell adapted strains of HIV-1 in macrophages, NL4-3 infections are more difficult to establish. This can be seen with a 3.5 log difference in infections of NL4-3 compared to BaL-1 in control macrophages. Therefore, with a strong restriction due to the expression of TRIM5α_{rh}, no productive infections were seen when challenged with NL4-3. These results established that the expression of TRIM5α_{rh}, in a stem cell gene therapy setting can confer resistance to HIV-1 infection in CD34+ derived macrophages.

DISCUSSION

Harnessing species specific innate resistance to control HIV-1 infection is a potentially rewarding approach as it paves the way for new avenues of viral inhibition using naturally occurring host cell molecules. Several new studies have established the remarkable potency of TRIM5α_{rh} in restricting HIV-1 replication *in vitro* and highlighted its potential as a protective tool for gene therapy application[29,31-33,36-39]. To translate this promising new discovery into the clinic, a number of important issues need to be

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addressed and evaluated. These involve stable introduction of the TRIM5α_{rh} transgene into hematopoietic stem cells to derive mature macrophages and T cells which are the primary viral targets *in vivo* and expression of the transgene to the levels that confer viral resistance.

In these studies, we introduced the rhesus macaque version of TRIM5α into a third generation lentiviral vector and tested its efficacy in human cells. We showed here, for first time, that stably lentiviral vector transduced hematopoietic stem cells resist HIV-1 infection. Initial experiments with the TRIM5α_{rh} lentiviral vector were first performed in the Magi-CXCR4 cell line followed by testing in hematopoietic stem cells. The presence of an EGFP reporter gene in the vector permitted enrichment by FACS sorting and monitoring of cells. The stably transduced cells were viable and continued to express the transgene when tested at each passage of cells in culture thus indicating no apparent toxicity of TRIM5α_{rh}. The presence of the influenza HA epitope tag in the TRIM5α_{rh} protein allowed for immunodetection in transduced cells. Expression levels of TRIM5α_{rh} in transduced cell populations were robust and comparable to the levels of cellular actin (Fig. 2C). When challenged with T tropic NL4-3 or M tropic BaL-1, transgenic cells showed remarkable viral resistance thus providing the proof of principle that TRIM5α_{rh} could be exploited in a gene therapy setting using lentiviral vectors.

Restriction, mediated by TRIM5α_{rh}, is believed to be due to its destructive interaction with the viral capsid protein. It was also previously shown that TRIM5α_{rh} restriction could be saturable by increasing the viral dose[25-27]. To evaluate if this is the case in vector transduced cells, we infected transgenic Magi-CXCR4 cells with increasing levels of HIV-1, using a replication competent T tropic NL4-3 virus that contained a murine CD24 reporter gene which can be analyzed by FACS (Fig. 4). At low m.o.i, as seen on day 7, nearly all TRIM5α_{rh} transgenic cells were resistant to HIV-1 challenge, whereas, in contrast, at a ten fold higher dose challenge, a substantially higher number of cells were infected although the overall infection levels were still significantly suppressed. These results showed that TRIM5α_{rh} resistance could be partly overcome with higher viral load.

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In these experiments using increasing doses of challenge virus, other interesting features of lentiviral vector transduction/expression and TRIM5α_{rh} restriction came into focus. In the TRIM5α_{rh} vector transduced cell population, a gradient of vector expressing cells could be seen as determined by their varying levels of EGFP expression. For example, in the FACS plots seen in Fig. 4B-D, based on fluorescence intensity, both low and high EGFP expressing cells separated by medium expressing cells could be seen. High expression is most likely due to multiple vector integrants and/or high transcription levels of the transgenes. In experiments with higher m.o.i., the high GFP expressing cells were resistant to HIV-1 challenge even on day 7 of infection whereas the low GFP expressing cells became infected with the virus by day 7 during which time the m.o.i. would have increased significantly higher due to new virus released from the ongoing virus infection in the culture. These data, in addition to substantiating that increased viral dose could overcome innate resistance conferred by lower levels of TRIM5α_{rh}, also established that high expressor cells were completely resistant to viral challenge at the various m.o.i. tested. Based on these observations it is encouraging to find that when TRIM5α_{rh} is expressed at higher levels, cells become fully refractory to viral infection. Furthermore, it is apparent that high TRIM5α_{rh} expressors will have selective survival advantage and will outgrow the susceptible cell population in the face of viral infection as would be expected *in vivo*.

The above data presented with transgenic Magi-CXCR4 cells provides the proof of concept that lentiviral vectors could be used to transfer species specific innate resistance to otherwise susceptible cells to HIV-1 infection. For TRIM5α_{rh} gene therapy strategies to succeed long-term, it is essential that the transgene is sustained and expressed *in vivo* for a prolonged period of time. Therefore, an obvious approach is to introduce the TRIM5α_{rh} gene into hematopoietic stem cells (HSC). These cells are ideal having the capability to self-renew and differentiate into lymphocytes, macrophages and dendritic cells, which are the cellular targets for HIV. Accordingly, we transduced CD34+ hematopoietic progenitor cells with the T5A vector to determine the feasibility of using TRIM5α_{rh} in a stem cell setting. In these experiments, our objectives were to determine if TRIM5α_{rh} expression has any detrimental effects or toxicity during lineage specific differentiation of stem cells, if the terminally differentiated cells are

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phenotypically normal, and finally if the end stage differentiated cells were resistant to HIV-1 infection. Our results have shown that transgenic macrophages could be differentiated with no apparent adverse effects as similar numbers of mature cells were obtained when compared to control cells. The transgenic macrophages were also phenotypically normal as they displayed the characteristic cell surface markers CD14, CD4, CCR5, CXCR4, and MHC class II molecules. Activated macrophages up-regulate the expression of B7.1 co-stimulatory molecules and present antigen to memory and effector T cells. Functional assays that evaluated the up-regulation of B7.1 in response to LPS stimulation showed that its up-regulation is normal in transgenic macrophages when compared with control non-transduced and control GFP-alone vector transduced macrophages. Thus, TRIM5α_{rh} expression in differentiated primary cells has no adverse effects on these HIV-1 target cells. Our results have also shown that the TRIM5α_{rh} transgenic macrophages resist HIV-1 infection establishing for the first time that innate resistance conferred by species specific restriction factors could be genetically transferred into hematopoietic stem cells to derive HIV-1 resistant progeny cells.

A common feature of many anti-HIV-1 intracellular immunization strategies is that they are designed to act on various stages of virus replication in a cell, which is already infected, but would not prevent infection of a naive susceptible cell. The present strategy of conferring innate resistance via TRIM5α_{rh} expression has several advantages in addition to preventing viral infection at a very early stage before reverse transcription. First, it uses a naturally occurring molecule. Second, since this innate resistance has been evolutionary selected for, the chances of generating viral escape mutants are low when compared to using viral molecules as targets. Third, strain specific differences in the infecting HIV-1 genome will not be an issue since the effect of TRIM5α_{rh} in restricting viral infection is believed to be innate and of broad range. Therefore infection of both T tropic and M tropic viruses could be prevented using this strategy.

A new trend in HIV-1 gene therapy strategies is to combine several anti-viral effector molecules in a single gene delivery vector. For example, recent studies have shown that an anti-CCR5 ribozyme, a tar decoy, and an anti tat/rev siRNA could be engineered into a single lentiviral vector for stable transduction of human primary cells[11]. Similarly, two siRNAs targeted to both the HIV coreceptors CCR5 and CXCR4 were

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engineered into a single lentiviral vector for gene delivery[13]. These approaches have targeted both cell receptors and viral specific regulatory molecules. Addition of TRIM5α_{rh} to such combinatorial constructs to provide innate viral restriction is likely to increase the effectiveness of such combinatorial constructs and decrease the possibility of generating viral escape mutants during therapy.

Our results have shown that HIV-1 resistant macrophages could be derived from TRIM5α_{rh} transduced CD34+ cells. Additional experiments are required to show that such a strategy will also work in deriving HIV-1 resistant T cells. The SCID-hu mouse animal model that harbors a functional human thymus in which gene transduced stem cells could be differentiated into T cells will be an ideal preclinical model to test whether transgenic helper lymphocytes could be obtained[20]. Recent data had shown that a single mutation in the human TRIM5α could render this human protein a potent restriction factor against HIV-1[37,39]. By introducing specific point mutations, the human TRIM5α isoform was engineered to restrict HIV-1 infections. Use of this human derivative may have an added advantage for long range *in vivo* application since it is less likely to be immunogenic compared to the simian version. In summary, our studies have shown that the lentiviral restriction factor, TRIM5α_{rh}, can be used in a stem cell gene therapy setting to inhibit HIV-1 infections and that this strategy has promising clinical application potential.

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CHAPTER 8

INHIBITION OF HIV-1 BY A LENTIVIRAL VECTOR EXPRESSING A COMBINATION OF REV/TAT shRNA, TAR DECOY, AND CCR5 RIBOZYME

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ABSTRACT

Combinatorial therapies for the treatment of HIV infections have proven to be highly effective in prolonging life and slowing the progression of AIDS. However, due to the emergence of drug resistant mutants, the high cost of therapy, and toxic side effects, new therapeutic approaches need to be developed. A multitude of RNA based therapies have been developed for use in HIV gene therapy including siRNAs, decoys, and ribozymes. Since HIV is highly prone to mutation, it is necessary to target multiple stages of the viral life cycle. Based on this consideration, in these studies, a combination of highly potent constructs have been inserted into a third generation lentiviral vector for transduction of target cells. The combinatorial construct, named Triple, contains an shRNA targeting rev and tat, a TAR decoy, and a CCR5 ribozyme. CD34+ hematopoietic progenitor cells were transduced with this lentiviral vector and derived into thymocytes in SCID-hu mouse thymic grafts. The transgenic thymocytes were resistant to HIV-1 replication upon *in vitro* challenge and seem to be phenotypically normal as determined by FACS analysis. The data presented here demonstrates the efficacy of this combinatorial vector for the treatment of HIV in a stem cell gene therapy setting.

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INTRODUCTION

Combinatorial therapies to treat HIV-1 infections have proven effective in controlling disease progression and reducing viral load in the body. However, with the discovery of viral escape mutants and the eventual progression towards disease, the necessity for alternative therapeutic approaches has come into focus. Intracellular immunization using gene therapy to treat HIV-1 infections has offered promising results in various *in vitro* experiments performed. Some of these RNA-based therapies include decoys, ribozymes, transdominant proteins, and siRNAs.[1-2] Of the many possibilities, siRNAs have shown to be the most potent. However, as this mechanism is highly sequence specific, the potential for the generation of escape mutants is greater compared to other molecules.[1] By combining multiple anti-HIV genes targeted to various stages of HIV's life cycle, escape mutants may be avoided.

For long term gene therapy to succeed, anti-HIV transgenes need to be expressed constitutively in cells targeted for HIV-1 infection. For this to occur, lentiviral vectors offer the ability to efficiently and stably transduce nondividing as well as dividing cells.[2] Transducing these anti-HIV genes into hematopoietic progenitor stem cells (HSCs) offers a number of advantages for gene therapies. They are able to self renew, persist throughout an individual's lifetime, and reconstitute the entire hematopoietic system of an individual.[1] Therefore, the genetic manipulation of HSCs can have long lasting effects for all blood cells, including those susceptible to HIV infection.

The genome of HIV is complex and involves a multitude of splicing events and transcriptional regulation. The two main regulatory proteins, rev and tat, play critical roles in viral replication. Rev is necessary for unspliced viral transcripts to be exported

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into the cytoplasm for translation. Tat is a powerful transactivator of transcription for proviral gene expression. This action is mediated through the interaction with the viral stem loop TAR region located at the 5' end of transcripts. Without this interaction, only very short transcripts are synthesized. Various numbers of cellular molecules are also critical for HIV replication, one of these being the essential chemokine receptor, CCR5, used by R5-tropic strains of HIV to infect macrophages.[3] In a segment of the human population, a 32bp deletion or a premature stop codon in the CCR5 ORF abolishes its cell surface expression and function.[4-6] Homozygous individuals carrying this allele show resistance to infection. Also, if infection does occur, there is a significantly longer progression to AIDS. By targeting multiple stages of HIV's life cycle with therapeutic genes, greater resistance and decreased possibilities of generating escape mutants can be achieved.

To better evaluate potential anti-HIV genes for use in a gene therapy setting, relevant animal models are needed to test vectors before they can be used in the clinic. The SCID-hu mouse model which harbors an intact human thymus implanted under the mouse kidney capsule, provides an ideal environment for thymopoiesis.[7] To generate transgenic thymocytes, transduced CD34+ hematopoietic cells can be directly injected into thymic grafts. After allowing 4-6 weeks for thymopoiesis to occur, transgenic thymocytes can be obtained from these grafts.[7-10]

A combinatorial vector containing three anti-HIV transgenes, a TAR decoy, a CCR5 ribozyme, and an shRNA targeting an overlapping ORF of both rev and tat was generated to attack multiple stages of HIV's life cycle. This vector, named Triple, has been previously shown to confer resistance to HIV infection in cultured cell lines, CD34+

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cells, and CD34+ derived monocytes[11]. The generation of escape mutants was also tested using this vector. No viral resistance or generation of escape mutants could be detected.[11] The shRNA component targeting an overlapping ORF of both rev and tat was previously shown to be efficacious in inhibiting HIV-infection in thymocytes derived from CD34+ stem cells engrafted in a SCID-hu mouse model.[8] However, further analysis of single anti-HIV siRNA constructs showed that escape mutants could arise over time.[12-13] Therefore, multiple stages of HIV's life cycle need to be targeted in order to circumvent the problem of escape mutants.

Here I describe the effectiveness of this Triple vector in inhibiting HIV-1 infection in thymocytes derived from transduced CD34+ cells in a SCID-hu mouse model. As introducing foreign genes has the possibility of causing undesireable effects in transgenic cells, phenotypic analyses of characteristic cell surface markers was also performed. The data presented here supports the use of this vector for HIV gene therapy.

MATERIALS AND METHODS

Lentiviral vector construction and transduction of stem cells:

A third generation lentiviral vector, as described above, was used for this study. The transfer vector designated Triple was designed to contain a combination of three anti-HIV genes as described previously.[11] The shRNA targeting rev/tat is under the control of a U6 promoter, the TAR decoy is embedded in the U16 snoRNA backbone for nucleolar localization and also driven by a U6 promoter, and the CCR5 ribozyme is under the control of an adenoviral VA1 promoter (Fig 1). Lentiviral vectors were generated in 293T cells as described above and concentrated by high speed centrifugation. CD34+

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cells were isolated and transduced with both GFP-alone control vector and the Triple vector at an m.o.i. of 30 with 4ug/ml polybrene. Transductions were done on two consecutive days. Transduction efficiencies ranged from 58-73% for Triple and GFP-alone , respectively.

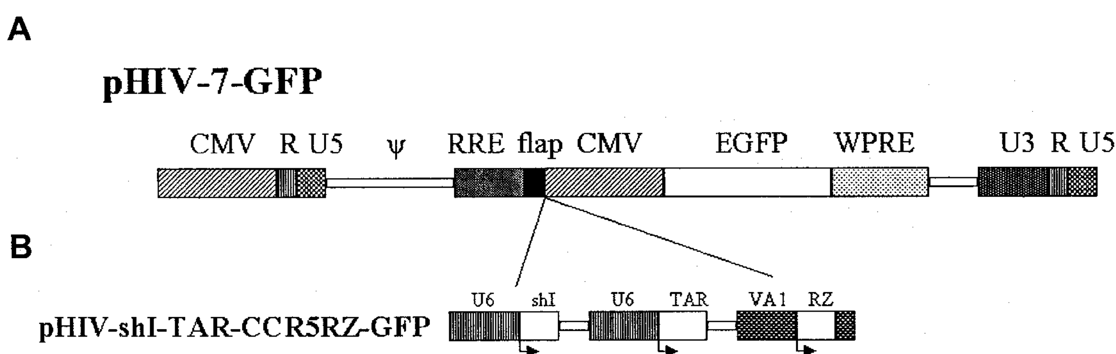

Fig 1. Triple lentiviral vector. (A) A third generation lentiviral vector pHIV-7-GFP was used as the backbone for construction of the Triple vector and contains an EGFP reporter gene under the control of the CMV immediate early promoter. (B) The Triple vector was constructed by inserting a U6 promoter driven shRNA targeting rev/tat, a nucleolar localizing TAR decoy under the control of the U6 promoter, and an adenoviral VA1 promoter driven CCR5 ribozyme.

Reconstitution of human thymic grafts of SCID-hu mice with transduced CD34+ cells:

To evaluate the capabilities of shRNA/TAR decoy/CCR5 ribozyme transduced CD34+ to develop into thymocytes, a SCID-hu mouse model, that supports thymopoiesis was used.[7-10] Transduced CD34+ cells, 3×10^6 cells per mouse, were injected directly into the thymic grafts. Forty-nine days post-injection, cells were biopsied out of the grafts and analyzed for GFP expression by FACS to evaluate reconstitution levels.

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Ex vivo HIV challenge of transgenic thymocytes:

To determine whether transgenic thymocytes were resistant to HIV-1, challenge assays were performed with an X4-tropic HIV-1 strain, NL4-3. Thymocytes derived from thymic grafts were sorted based on CD3 and GFP expression and subsequently stimulated with IL-2 (10ng/ml) and PHA (1ug/ml). Three days post-stimulation, cells were challenged with NL4-3 HIV-1 at an m.o.i. of 0.01. For infections, 10^5 cells were challenged in triplicate from three different mice. On various days post-infection, cell culture supernatants were taken and assayed using a p24 antigen ELISA kit (Beckman Coulter, Fullerton, CA).

Multi-parametric FACS analysis of transgenic thymocytes for phenotypic markers:

With the introduction of foreign transgenes into cells, undesirable effects may occur. To determine whether transgenic thymocytes obtained from the SCID-hu thymic grafts were phenotypically normal, various cell surface markers were analyzed: CD3, a normal T cell marker, CD4 and CD8 to detect subpopulations of T helper, T cytotoxic, and immature double positive cells, CCR7 and CD45RA to detect naive thymocytes, CD28, a costimulatory molecule needed for antigen presenting cell stimulation, and CXCR4, a critical coreceptor used by HIV for attachment and fusion. The following antibodies were used for analysis: Texas Red-CD3, Alexa Fluor 405-CD8 (Caltag, Burlingame, CA), APCCY7-CD4, PECY7-CCR7, APC-CD45RA, PE-CD28, and PECY5-CXCR4 (BD Biosciences, San Jose, CA). Cells were analyzed on a Beckman

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Coulter ARIA. For analysis of transgenic thymocytes, all gating was performed from EGFP expressing cells.

RESULTS

Triple vector transduced CD34+ cells can reconstitute SCID-hu mouse thymic grafts and generate HIV-1 resistant thymocytes:

SCID-hu thymic grafts offer an ideal environment for thymopoiesis and can be reconstituted with transduced CD34+ hematopoietic progenitor cells through injection.[7] Reconstitution levels of GFP-alone and Triple transduced CD34+ cells varied from graft to graft. The levels of reconstitution obtained as determined by EGFP expression ranged from 2.4-14.8% for GFP-alone and 1.7-5.3% for Triple transgenic thymocytes. To determine the levels of viral inhibition conferred by Triple transduced CD34+ derived thymocytes, cells were sorted based on EGFP expression and challenged with X4-tropic NL4-3 HIV-1 at an m.o.i. of 0.01. As shown in Fig. 2, complete resistance to productive infections was observed with triple transgenic thymocytes compared to nontransduced and GFP-alone vector transduced thymocytes. Greater than a 1.2 log inhibition was observed.

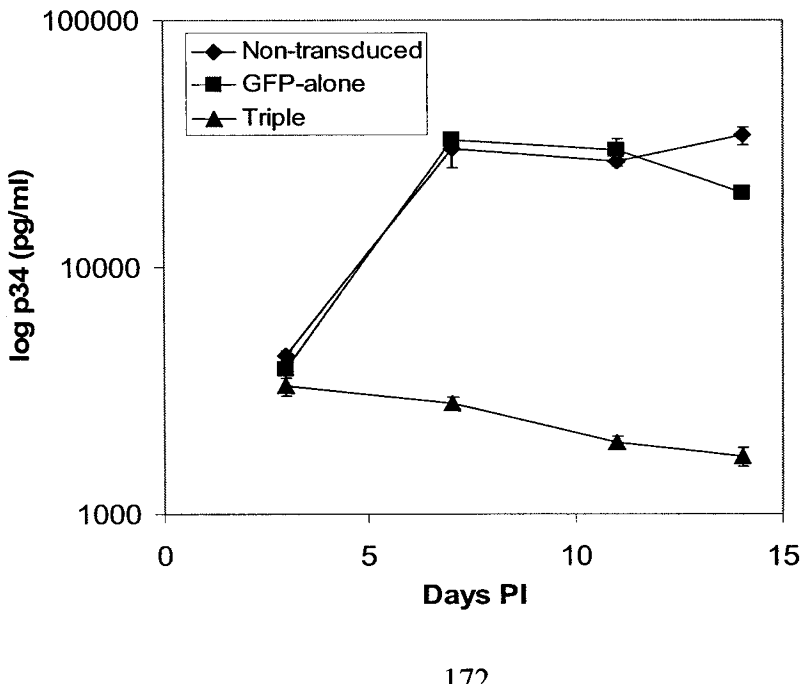

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Fig 2. Inhibition of HIV-1 replication in Triple transgenic thymocytes. Thymocytes derived from trnsduced CD34+ cells injected into SCID-hu thymic grafts were biopsied and challenged ex vivo. Infections were performed with an X4-tropic NL4-3 strain of HIV-1 at an m.o.i. of 0.01. Data represents challenges performed in triplicate from three different mice for each cell subset.

***Phenotypic analysis of transgenic thymocytes*:**

As introducing foreign transgenes into target cells can cause undesirable effects, evaluating transgenic cells to determine if they are physiologically normal is essential. Phenotypic analyses were performed to detect the normal expression of various thymocyte cell surface markers. The data shown are representative figures from all replicates analyzed. As shown in Fig. 3A, the majority of cells analyzed were positive for CD3: control cells 82%, GFP-alone transduced 92%, and Triple transduced 93%. Further analyses were then performed by gating on these cell populations to assure only T cells were evaluated. All three populations of CD4+, CD8+, and CD4+/CD8+ cells were seen in nontransduced, GFP-alone, and Triple transduced thymocyte sets (Fig 3B).

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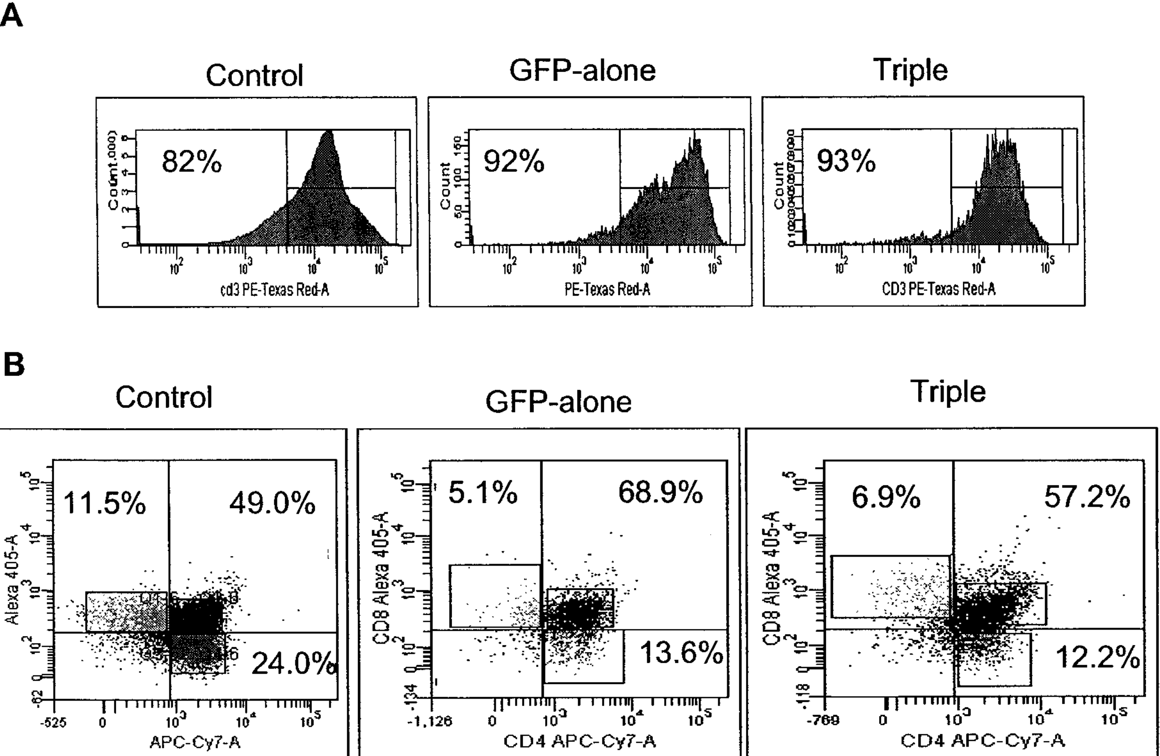

Fig 3. FACS profiles of normal thymocyte cell surface markers. To determine the levels of normal thymocyte markers, cells biopsied from SCID-hu thymic grafts, control, GFP-alone, and Triple were stained for the normal T cell marker CD3 (A) and subsequently analyzed for the thymocyte subsets CD4+, CD8+, and CD4+/CD8 (B). Data represents analyses from replicate mice.

From these three subset populations, further analyses were performed to detect the T cell activation markers CCR7 and CD45RA. When T cells are in a naive state, they express both of these cell surface markers. Upon stimulation, the expression of these molecules decreases. Similar profiles were observed in all three cell groups for expression of CCR7 and CD45RA from each of the three thymocyte subset populations of CD4+, CD8+, and CD4+/CD8+ cells (Fig 4).

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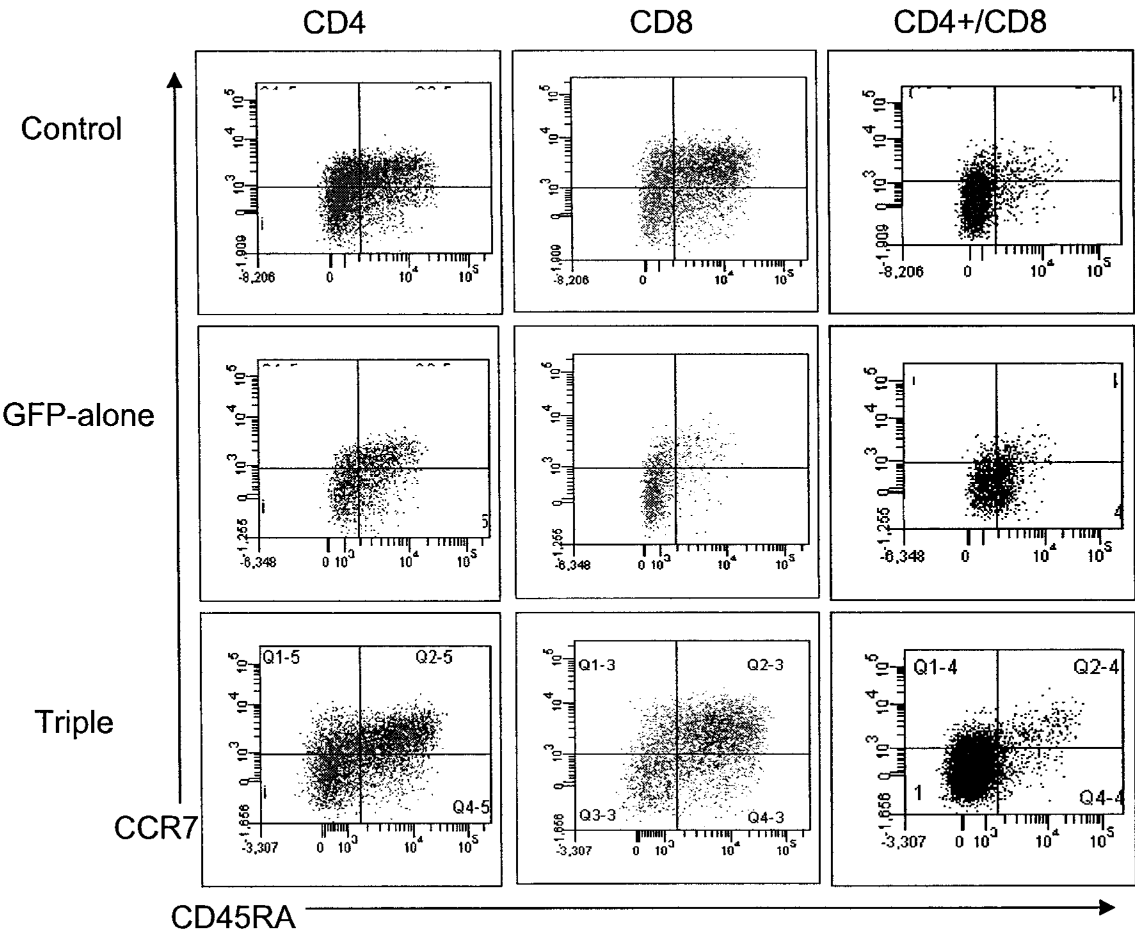

Fig 4. FACS analysis of naive T cell markers CCR7 and CD45RA. Thymocytes biopsied from control, GFP-alone, and Triple SCID-hu thymic grafts and analyzed for CCR7 and CD45RA expression. FACS plots are based on the three thymocyte subsets, CD4+, CD8+, and CD4+/CD8+ from Fig 3. Data represents analyses from replicate mice.

CD28 and CXCR4, two normal thymocyte markers, were also analyzed for expression. The levels of CD28 and CXCR4 were shown to be similar for Triple thymocytes compared to nontransduced and GFP-alone transduced thymocytes (Table 1). The levels of these surface molecules are shown as mean fluorescence intensity (MFI).

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CD28 and CXCR4 Expression on SCID-hu Derived Thymocytes

		CD28[a]			CXCR4[a]	
	CD4+	CD8+	CD4+/CD8+	CD4+	CD8+	CD4+/CD8+
Control	6487	2308	2703	2526	4952	4578
GFP	6700	2576	2862	2024	4476	3891
Triple	5948	2672	2265	2564	4521	3241

[a] Cells biopsied from SCID-hu thymic grafts were analyzed for the thymocyte markers CD28 and CXCR4. Values are expressed as mean fluorescence intensity (MFI) and analyzed from the three thymocyte subsets CD4+, CD8+, and CD4+/CD8+.

These data demonstrate that transgenic thymocytes do not show any aberration in expression of normal T cell markers. The expression of the three anti-HIV transgenes did not seem to affect the phenotypic profile of thymocytes as all normal cell populations were observed.

DISCUSSION

The diverse mechanisms of pathogenesis of HIV used in disease progression needs to be assessed when developing effective therapeutics. Some of these include viral integration, a high mutation rate, viral latency, and the ability to evade the immune system in immunologically privileged sites.[1-2] Due to these characteristics of infection, multiple stages of HIV's life cycle need to be targeted to avoid generating escape

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mutants. A combination of constructs targeting both pre-entry and post-integration steps are more attractive than monotherapies that only target one step of the viral life cycle.

The development of drug resistant mutants to current therapies and the toxic side effects make it necessary to develop alternative treatments. The potential for gene therapeutics offers a novel avenue for treating HIV infections. Ribozymes, decoys, and shRNAs are just a few RNA based therapies curently being evaluated in an HIV-1 gene therapy setting. Lentiviral vectors are ideal to deliver these anti-HIV constructs into target cells as they are able to transduce nondividing cells, including primary T cells and hematopoietic stem cells which can be derived into HIV-1 susceptible cells.[1] Also, lentiviral transduced genes were found to be refractory to gene silencing during differentiation of stem cells.[1] A number of recent studies have demonstrated the ability of RNAi and other HIV RNA inhibitory molecules to resist viral replication *in vitro*.[8,11,14-31] Translating these findings into practical *in vivo* applications needs to be addressed before clinical use can begin.

Based on this concept, a Triple lentiviral vector construct was designed to target multiple stages of HIV's life cycle. The critical coreceptor CCR5 needed for entry of HIV into target cells was targeted by a ribozyme under the control of an adenoviral VA1 promoter. The two essential regulatory proteins, rev and tat were also targeted. An shRNA under the control of a U6 Pol-III promoter was designed to target a segment of an overlapping ORF of both rev and tat transcripts and was previously shown to be hghly effective as a single construct. The tat protein was also targeted by a TAR decoy that acts to sequester tat and decrease transactivation of viral transcripts. Each of these expression cassettes were expressed under seperate promoters and were previously shown to

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accumulate in high quantities. Using a combination of these anti-HIV transgenes in a single lentiviral vector will offer additional protection from the generation of possible viral mutants that may arise during the course of therapy.

In the present study, the ability of this Triple lentiviral vector in transducing hematopoietic CD34+ stem cells was tested. High efficient transduction of these cells was seen with transduction efficiencies seen at >50% with only two rounds of transductions. Upon injection into SCID-hu thymic grafts which provide an ideal environment for thymopoiesis, cells were allowed to develop into thymocytes. Subsequent analysis of transgenic cells showed that normal cell populations of thymocytes could be obtained in both control and Triple transgenic cells. The three subpopulations of thymocytes, CD4+, CD8+, and CD4/CD8+, cells were seen as well as other phenotypic markers. Naive T cells express the cell sruface markers CD45RA and CCR7. Upon analysis of thymocytes, normal expression of these markers was seen on Triple thymocytes compared to control and GFP-alone transduced cells. The normal co-stimulatory molecule CD28 and the HIV coreceptor CXCR4 were also observed at normal levels in comparison with control, GFP-alone, and Triple thymocytes. To determine whether transduction of these three anti-HIV-1 transgenes conferred HIV-1 resistance, reconstituted cells were obtained and sorted for GFP expression. Cells were then challenged with T cell tropic NL4-3 HIV-1.

For stem cell gene therapy to succeed, anti-HIV transgenes need to be constitutively expressed throughout development from progenitor to end stage cells. In these studies, I demonstrate that hematopoietic progenitor cells transduced by a lentiviral vector containing a combination of an anti-rev/tat shRNA, a TAR decoy, and a CCR5

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ribozyme can be derived into normal HIV-1 resistant thymocytes *in vivo*. These studies have shown that high level transductions can be achieved in CD34+ stem cells (>50%) using these lentiviral vectors. The expression of the introduced transgenes was stable as seen from EGFP expression from thymic graft thymocytes. The lower levels observed for reconstitution, 2.4-14.8% for GFP-alone and 1.7-5.3% for Triple transgenic thymocytes, as compared to transduction efficiencies may be due to dilution of injected cells with current thymocytes already present in the grafts. Also, the lower levels of EGFP positive cells biopsied from the grafts may be due to a decreased number of true stem cells injected whereby their fate was already committed.

The main goal of anti-HIV gene therapy is to engineer cells to express anti-HIV transgenes and become resistant to HIV replication. *Ex vivo* challenge of Triple transgenic thymocytes resulted in a significant resistance in viral replication compared to control cells. These results demonstrated for the first time that thymocytes expressing an anti-rev/tat shRNA, a TAR decoy, and a CCR5 ribozyme can be derived from CD34+ stem cells in SCID-hu thymic grafts and resist HIV-1 replication. Transgenic thymocytes were also shown to develop into all thymocyte populations as shown by FACS analysis of cell surface markers.

For gene therapy to succeed long term, transgenic cells need to be continuously generated and differentiate into normal functioning end stage cells. As the introduction of foreign transgenes has the propensity for deleterious effects on transgenic cells, phenotypic and functional abilities need to be assessed. Here I have shown that the introduction of these three anti-HIV transgenes did not disrupt normal thymopoiesis and that transgenic thymocytes seem to be phenotypically normal developing into all cell

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populations. To further assess any minor effects caused by the introduced transgenes, future analyses using microarray technology should be performed. This vector is currently being prepared for a clinical trial using hematopoietic stem cells obtained from AIDS patients for bone marrow transplantation.

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CHAPTER 9

SUMMARY

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The spread of HIV/AIDS continues to rise at an alarming rate with 4.9 million new infections and 3.1 million deaths in 2004 alone. With the onset of combination HAART therapy, disease progression has slowed and patient's lives have been extended. However with the relatively high cost of this treatment, toxic side effects, and the discovery of escape mutants, new and innovative alternative therapies need to be developed.[1-6] The idea of intracellular immunization using RNA and protein based therapies has proven to be highly efficacious in a laboratory setting.[7-8] Translating these findings for clinical use requires the evaluation of constructs in a stem cell gene therapy context. Stem cell therapy is an optimal choice for long range gene therapy based on the fact that these cells will continuously renew and generate virus resistant progeny.[8] Hematopoietic cells are the progenitors for all blood cells including macrophages and T cells which are the main cellular targets of HIV. To introduce anti-HIV transgenes into CD34+ hematopoietic progenitor cells, viral vectors are excellent candidates for gene transfer. Earlier work focused on using retroviral vectors.[8] However, these vectors can only integrate in dividing cells and pose a risk as integration sites are found to be mostly in promoter regions. Many of these vectors are also derived from oncogenic viruses such as MLV.[8] Lentiviral vectors offer some advantages over retroviral vectors in that they can transduce both dividing and nondividing cells. They are also not found to integrate in promoter regions.[8] The lentiviral vector used in my current studies is a third generation self-inactivating vector derived from HIV.[9-10]

The areas of HIV inhibition that I wanted to focus on surrounded pre-entry and post-entry/pre-integration steps of HIV's life cycle. Targeting these steps will prevent the virus from stably integrating and remaining latent inside an infected cell. Once

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integration occurs, there is a greater chance of generating escape mutants due to the high mutation rate of the viral reverse transcriptase. With the discovery of the highly potent and specific mechanism of RNAi, another tool was added to the arsenal of anti-HIV therapeutics. As siRNAs have been shown to be more potent in targeted gene silencing compared to other mechanisms, such as ribozymes and transdominant proteins, they were used for my studies.

HIV's life cycle is complex and involves a multitude of both viral as well as cellular proteins to undergo a complete replication cycle. The first cellular molecules it encounters are the major receptor CD4 and a critical coreceptor, the two major ones being CXCR4 and CCR5.[11] These coreceptors are critical for viral entry and infection. CCR5 was shown to be dispensable for normal human physiology as a segment of the population has a 32bp deletion in their CCR5 gene which renders the protein defective.[12-14] Therefore CCR5 is an excellent target for gene knockdown. However, selection may occur for X4-tropic strains of HIV if those variants are still able to infect cells. Therefore, both coreceptors need to be targeted. Using siRNAs to down regulate the expression of CXCR4 and CCR5 offers great promise towards inhibiting HIV infections.

Many different siRNAs can be designed against a single transcript, however, only a select few are highly potent for target mRNA degradation. SiRNAs need to be first evaluated by transfection methods to identify potent molecules capable of down-regulating sufficient amounts of target transcripts. To design the siRNA targeting CXCR4, nucleotides 3-23 were chosen on the basis of the design of an anti-CCR5 ribozyme that targeted the 5' end of the CCR5 transcript.[15-17] Fortunately, after testing just one CXCR4 siRNA, a potent one was found that corresponded to a 79% knockdown

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in cultured Magi-CXCR4 cells. This knockdown conferred a high level of viral resistance to X4-tropic HIV-1 compared to control transfected cells. Numerous CCR5 siRNAs, in collaboration with Dharmacon, were tested for efficacy in CCR5 gene knockdown. Three siRNAs, one that was already published (#B)[18], were shown to be highly effective in mRNA degradation working at greater than 97% as detected by quantitative real time PCR. These siRNAs, #B, #5, and #6, tested as both 19mer sense/antisense hybrids or as 28mer shRNAs with a stem loop structure, were effective at inhibiting R5-tropic BaL-1 HIV-1. To deliver both a CXCR4 and a CCR5 shRNA into a single cell at the same time, a double stem loop shRNA with an 8nt spacer connecting the two shRNAs was designed. Transfecting this bispecific molecule followed by FACS analysis showed that both coreceptors were effectively targeted and down regulated. The down-regulation showed by shRNA transfection conferred viral resistance of both X4 and R5-tropic strains of HIV-1. Another double stem loop shRNA targeting CXCR4 and CD4, used as proof-of-concept, was also effective in down regulating its targets. When CXCR4-CD4 shRNA transfected cells were challenged with HIV-1, even greater protection was seen. This result showed that by targeting multiple molecules needed for HIV infection/replication, greater protection can be achieved. These results helped to identify potent shRNAs capable of down regulating the critical HIV coreceptors, CXCR4 and CCR5, thus conferring viral resistance. As the effect of transfected shRNAs are only transient in nature, constitutive expression is needed and can be achieved by harnessing the abilities of viral vectors.

To constitutively express these shRNAs in HIV target cells, expression cassettes utilizing Pol-III promoters were designed and inserted into a lentiviral vector backbone.

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I designed and generated a lentiviral vector containing two shRNA expression cassettes in tandem: a CXCR4 shRNA expression cassette under the control of the human U6 promoter followed downstream by a CCR5 shRNA expression cassette under the control of the human H1 promoter. This vector was named XHR. The lentiviral vector backbone used, pHIV-7-GFP, is a third generation self-inactivating vector that can be generated in high titer and has been shown to efficiently and stably transduce a variety of cell types including CD34+ hematopoietic stem cells.[9-10,19] It contains an EGFP reporter gene and two cis-acting elements as described above.[9]

Upon transduction of cultured cell lines, stable knockdown was achieved for both CXCR4 and CCR5 expression. This knockdown conferred viral resistance to both X4 and R5-tropic strains of HIV-1. As certain shRNAs have been shown to induce the interferon response, western blot analysis was performed to detect the up-regulation of protein kinase R (PKR). No increase in PKR expression was detected. Intracellular expression of the respective CXCR4 and CCR5 shRNAs were also detected by northern blot analysis using an RNase protection assay. These data showed the efficacy of the bispecific lentiviral vector, XHR, to stably and constitutively down regulate the expression of CXCR4 and CCR5 thus conferring HIV-1 resistance to both X4 and R5-tropic strains of HIV-1. To determine the efficacy of anti-HIV constructs in a stem cell gene therapy setting, transduction of CD34+ hematopoietic stem cells and evaluation of transgenic progenitor cells was performed.

On this note, the bispecific vector, XHR, was used to transduce CD34+ stem cells and derive transgenic macrophages. Upon subsequent analysis, a potent down regulation of both CXCR4 and CCR5 cell surface expression was observed by FACS analysis. This

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down regulation conferred viral resistance to both X4 and R5-tropic strains of HIV-1. Subsequent analyses of phenotypic and functional aspects of mature macrophages were performed to determine if the expression of these shRNAs and the down regulation of CXCR4 and CCR5 had any adverse effects. Major functions of macrophages including phagocytosis, cytokine secretion, and the up-regulation of the co-stimulatory molecule B7.1 were analyzed. Transgenic macrophages showed no significant defect in their abilities to perform any of these functions. The phenotypic analyses including the major macrophage cell surface markers CD14, CD4, and MHCII were also shown to be normal on transgenic macrophages compared to non-transduced and GFP-alone transduced cells. If CXCR4 and CCR5 shRNA vectors are to be used in future gene therapy settings, more work needs to be done to further test the efficacy of these constructs. Targeting CCR5 should not be a problem long range since it was shown that people carrying the Δ32 deletion allele being physiologically normal.[12-14] However, as certain shRNAs may cause off target effects, microarrays should be performed on transgenic cells to determine if any deleterious effects occur with expression of these shRNAs. As for targeting CXCR4, this approach may cause defects in thymopoiesis and stem cell homing.[20] If expression can be controlled by tissue specific promoters or by drug-inducible promoters, a more regulated control of CXCR4 knockdown could be achieved. These approaches along with finding a more potent CXCR4 shRNA and microarray analyses of transduced cells may permit the use of CXCR4 knockdown for HIV gene therapy.

It has long been known that species-specific restriction factors exist that confer resistance to retroviral and lentiviral infections. Therefore the restriction factors that block viral infection could also be exploited for HIV-1 gene therapy. In a cDNA screen

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of rhesus monkey lung fibroblasts, a gene encoding TRIM5α was shown to inhibit HIV-1 infections when introduced and expressed in susceptible cells.[21] The rhesus macaque isoform of TRIM5α was shown to restrict productive HIV-1 infections through direct interaction with the incoming viral capsid disrupting pre-integration complex stability.[22] To translate this discovery into a stem cell gene therapy setting, I subsequently cloned the CMV-TRIM5α_{rh} expression cassette into pHIV-7-GFP and transduced both an HIV-1 susceptible Magi-CXCR4 cell culture line and CD34+ hematopoietic stem cells. Upon transduction, CD34+ cells were sorted based on GFP expression and derived into mature macrophages. When challenged with HIV-1, both Magi-CXCR4 cells and the CD34+ derived macrophages were resistant to viral infection. As seen from the above data, TRIM5α_{rh} is highly potent in restricting HIV-1 infections as seen from the Magi-CXCR4 challenge experiments. When challenged with a 5 and 10-fold higher m.o.i., transgenic Magi-CXCR4 cells were still resistant to infection. Harnessing this evolutionarily selected mechanism to inhibit infections holds promise for use in a stem cell gene therapy setting. Future work, ongoing, will focus on using an engineered human isoform of TRIM5α that contains a 13-aa patch in the SPRY domain of rhesus macaque TRIM5α that was shown to be a major determinant for viral resistance.[23] By engineering the human isoform of TRIM5α to contain the viral restriction determinants, a more wild-type form of TRIM5α can be used with minimal chance of immunological rejection.

As HIV will always have the ability to mutate at a rapid rate, targeting only one stage of the virus life cycle will not suffice for long range therapy. Even with current therapies, escape mutants have been isolated that resist current HAART treatment. Therefore, when using RNA or protein based therapies for stem cell gene therapy, the

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approach of multi-stage targeting should also be considered. In collaboration with our colleagues at the City of Hope Medical Center in Duarte, California, we tested an anti-HIV Triple vector in a SCID-hu mouse model. The Triple vector, containing an anti-rev/tat shRNA, a TAR decoy, and a CCR5 ribozyme was used to derive transgenic thymocytes from SCID-hu thymic graft injected CD34+ cells. Upon recovery of transgenic thymocytes, both HIV-1 challenge and multi-color phenotypic analyses were performed. Triple transgenic thymocytes were completely resistant to HIV-1 replication showing a 1.2 log difference in viral p24 antigen levels compared to non-transduced and GFP-alone transduced cells. Phenotypic analyses revealed that no adverse effects were seen with Triple transgenic thymocytes compared to control cells. By staining for various T cell markers, transgenic thymocytes seemed to be phenotypically normal as all cell populations were identified. Variations, however, were seen from mouse to mouse in relation to exact percentages of distinct cell populations and cell surface markers. This variation needs to be further investigated along with microarray data for transgenic thymocytes and macrophages. This work is currently ongoing.

As HIV continues to ravage the globe with increasing infections and deaths every year, new and innovative approaches need to be developed to combat any further spread. The use of anti-HIV RNA and protein molecules in a stem cell gene therapy setting offers the advantage of constitutive expression of these genes in self-renewing cell populations. With the recent advances in knowledge of HIV's life cycle and the discovery of RNA interference, new and more potent technologies can be harnessed to inhibit infections. By combining multiple potent anti-HIV molecules in a single vector construct, such as a CCR5 shRNA, TRIM5α, and a TAR decoy, potent suppression of HIV infection and

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replication can occur. Targeting the initial stages of HIV's life cycle, i.e. attachment/entry and integration will allow for better protection against escape mutants by inhibiting proviral formation. Further testing and analysis of current constructs, however, still needs to be performed to determine complete efficacy of these molecules.

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