Published in final edited form as:

J Acquir Immune Defic Syndr. 2009 October 1; 52(2): 152–161. doi:10.1097/QAI.0b013e3181b010a0.

Specific Transduction of HIV-Susceptible Cells for CCR5 Knockdown and Resistance to HIV Infection: A Novel Method for Targeted Gene Therapy and Intracellular Immunization

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Summary

HIV-1 gene therapy offers a promising alternative to small molecule antiretroviral treatments and current vaccination strategies by transferring, into HIV-1-susceptible cells, the genetic ability to resist infection. The need for novel and innovative strategies to prevent and treat HIV-1 infection is critical due to devastating effects of the virus in developing countries, high cost, toxicity, generation of escape mutants from antiretroviral therapies, and the failure of past and current vaccination efforts. As a first step toward achieving this goal, an HIV-1-susceptible cell-specific targeting vector was evaluated to selectively transfer, into CCR5-positive target cells, an anti-HIV CCR5 shRNA gene for subsequent knockdown of CCR5 expression and protection from HIV-1 infection. Using a ZZ domain/monoclonal antibody-conjugated Sindbis virus glycoprotein pseudotyped lentiviral vector, here we demonstrate the utility of this strategy for HIV-1 gene therapy by specifically targeting HIV-1-susceptible cells and engineering these cells to resist HIV-1 infection. CCR5-positive human cells were successfully and specifically targeted in vitro and in vivo for transduction by a lentiviral vector expressing a highly potent CCR5 shRNA which conferred resistance to HIV-1 infection. Here we report the initial evaluation of this targeting vector for HIV-1 gene therapy in a preexposure prophylactic setting.

Keywords

CCR5 shRNA; HIV gene therapy; HIV preexposure prophylaxis; lentiviral vector; targeting vector

INTRODUCTION

Current strategies to treat HIV infection including small molecule antiretroviral therapies have been successful in suppressing viral replication and reducing morbidity and mortality from HIV, however, after prolonged use, toxicity can occur and viral escape mutants can arise from a continued low level of viral replication.¹⁻⁵ Vaccine strategies including live attenuated, whole inactivated virus, protein subunits, DNA vaccines, and viral expression vectors encoding HIV proteins have failed due to the broad diversity of HIV-1 strains and the ability of the virus to evade immune responses.⁶ Gene therapy provides an alternative approach to current therapies and prophylactic strategies by offering the potential of long-

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Authors contributions: This study was developed, designed, and performed by J.S.A. The article was written by J.S.A. The retro-orbital injections were performed by J.W. The principal investigators in this study are J.A.N. and G.B. who edited the article.

This work will be presented at the American Society of Gene Therapy Conference, May 28, 2009, San Diego, CA.

term and constitutive protection from HIV infection and spread. Numerous anti-HIV RNA molecules have been developed and used to inhibit HIV infection and replication in gene therapy protocols including antisense RNAs, RNA decoys, ribozymes, and small interfering RNAs (siRNAs).⁷⁻¹⁸ Many of these anti-HIV genes have proven to be highly potent HIV inhibitors both in vitro and in vivo with a select few proceeding to clinical trials.¹⁹⁻²³

RNA interference utilizes an innate cellular mechanism to silence gene expression by degradation of mRNA.²⁴ RNA interference is highly effective in targeted gene knockdown for both gene discovery and the development of therapeutics by using 19–28 base pair (bp) siRNAs to seek out homologous mRNA for destruction.²⁵ In numerous studies, this technology has been harnessed to inhibit HIV-1 infection by targeting viral genes.^{7,10,17,18,22} Cellular genes necessary for HIV-1 attachment and fusion to target cells, such as the major receptor CD4 and the 2 main coreceptors CCR5 and CXCR4, have also been previously targeted by siRNAs.^{7,11,13-17} By blocking the attachment and fusion of HIV with the target cell membrane, viral entry and productive infection can be inhibited. Due to a naturally occurring 32-bp deletion in the CCR5 gene, both homozygous and heterozygous individuals harboring this mutant allele are highly resistant to HIV-1 infection and are physiologically normal; thus designating CCR5 as an excellent candidate for knockdown by siRNA for HIV gene therapy.²⁶⁻²⁸ Recently, long-term control of HIV-1 replication was observed in an infected individual who received a stem cell transplant for acute myeloid leukemia with donor stem cells from an individual homozygous for the CCR5 32-bp mutant allele.²⁹ The results provided from this study demonstrated the importance of developing anti-HIV molecules to block the availability of CCR5, thus preventing HIV-1 infection.

Current gene therapy protocols rely on ex vivo transduction of hematopoietic stem cells or peripheral blood mononuclear cells (PBMCs) using retroviral or lentiviral vectors pseudotyped with amphotropic or pantropic envelopes. These procedures require either apheresis of PBMCs, mobilization of peripheral blood stem cells, or bone marrow aspirations. Clinical grade methods to isolate the target cells, tissue culture methods to introduce the vector, and finally readministration of the gene modified cells into the patient are also required. The development of cell-specific targeting vectors capable of selectively transducing cells of interest upon direct injection, in vivo, would greatly simplify and enhance HIV gene therapy applications by bringing them to areas where sophisticated laboratories and clinics are not available.

Several vector-targeting approaches have focused on generating chimeric proteins with receptor ligands or single-chain antibodies fused to membrane-spanning molecules such as amphotropic retroviral glycoproteins and the influenza hemagglutinin protein.³⁰⁻⁴¹ Another novel method utilizes the ability of Sindbis virus glycoproteins to pseudotype lentiviral vectors. By modifying the Sindbis virus envelope through insertion of the ZZ domain from the *Staphylococcus aureus* protein A into the receptor-binding region of the glycoprotein E2 gene, pseudotyped vectors acquired the ability to bind purified antibodies.^{42,43} The ZZ domain encodes an immunoglobulin binding domain allowing for a direct conjugation between the selected monoclonal antibody (mAb) and the vector particle. These antibody-modified envelopes can then direct the lentiviral vectors to specific cells of interest for targeted transduction. These engineered vectors have been shown to transduce specific cell types such as CD34⁺ cord blood stem cells, metastatic melanoma cells, tumor cell lines, and cells expressing CD4 or the human leukocyte antigen.⁴²⁻⁴⁷

For improved in vivo HIV gene therapy to prevent the spread of viral infection and disease, innovative strategies need to be evaluated to provide preexposure protection for patients in both developed and developing countries. Here we evaluated the capacity of the Sindbis-ZZ

envelope design to protect HIV-1-susceptible cells in a gene therapy setting by delivering an anti-HIV CCR5 shRNA specifically to cells expressing CCR5 on the cell surface. Targeted transduction was achieved in mixed cell populations with both cultured and primary cells. Potent knockdown of CCR5 expression (>93%) was observed, thus conferring HIV-1 resistance in CCR5 shRNA vector-transduced cells. This report provides an initial assessment of this vector system for targeted lentiviral vector transduction and transfer of an anti-HIV gene into HIV-1-susceptible cells in a preexposure prophylactic setting.

METHODS

Lentiviral Vector Design and Production

A third-generation HIV-derived lentiviral vector containing an enhanced green fluorescent protein (EGFP) reporter gene was used in this study, pCCLc-x-PGK-EGFP (Fig. 1). The CCR5 shRNA gene driven by the human polymerase-III U6 small RNA promoter was generated, as described previously, and inserted upstream of the PGK-EGFP reporter gene cassette.⁴⁸ Sequencing of clones was confirmed by Laragen Inc, Los Angeles, CA.

Lentiviral vectors were generated in human embryonic kidney (HEK)-293T cells by lipofection with 25 µg of the packaging construct, p 8.9 (packaging plasmid containing the *gag* and *pol* genes), 25 µg of pCCLc-x-PGK-EGFP (control empty vector) or the CCR5 shRNA construct pCCLc-CCR5shRNA-PGK-EGFP (transfer vector), and 12 µg of pSindbis-ZZ (envelope). Vector supernatants were collected at 72 hours posttransfection and concentrated by ultracentrifugation at 20,000 rpm.

Lentiviral vectors pseudotyped with the Sindbis-ZZ envelope were incubated with a purified CCR5 mAb, clone 2D7, (BD Biosciences, San Jose, CA) on ice for 1 hour. Vectors were titrated on Ghost-R5-X4-R3 cells which express CCR5 on the cell surface. These cells were obtained from the AIDS Reference and Reagent Program and cultured in complete Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and supplemented with hygromycin, puromycin, and G418 according to the supplier's protocol. CCR5-targeting vectors were incubated on Ghost-R5-X4-R3 cells for 2 hours at 37°C with 8 µg/mL protamine sulfate. Complete DMEM containing 10% FBS was then added to the transduced cells. Forty-eight hours posttransduction, cells were analyzed by fluorescence activated cell sorting (FACS) for EGFP expression. Vector titers $\sim 1.0 \times 10^7$ TU/mL were routinely achieved.

Targeted Transduction of Mixed Cell Populations

A mixed population of cultured cells including HEK-293T and Ghost-R5-X4-R3 cells were plated in complete DMEM including 10% FBS. Cells were transduced with the CCR5-targeting vectors, either EGFP-alone or the CCR5 shRNA vector (multiplicity of infection [MOI] 10) for 2 hours at 37°C with 8 µg/mL protamine sulfate. PBMCs were isolated from whole blood by Ficoll-Paque (GE Healthcare, Piscataway, NJ). Total white blood cells were cultured in complete RPMI media containing 10% FBS and supplemented with 10 ng/mL IL-2. Cells were either left unstimulated or were stimulated with 1 µg/mL phytohemagglutinin (PHA) for 4 days before transduction. Both unstimulated and stimulated PBMCs were transduced with the CCR5-targeting vectors, either EGFP-alone or the CCR5 shRNA vector (MOI 10) for 2 hours at 37°C with 8 µg/mL protamine sulfate. Four days posttransduction, both cultured cells and primary human PBMCs were analyzed by flow cytometry for EGFP expression and downregulation of CCR5 expression.

Flow Cytometry Analysis and Quantitative Real-Time Polymerase Chain Reaction

To determine the cell-specific targeted transduction and the subsequent CCR5 downregulation conferred by the CCR5 shRNA, transduced cell populations were analyzed by FACS. Four days posttransduction, nontransduced, EGFP-alone, and CCR5 shRNA lentiviral vector-transduced cells, both cultured cells and PBMCs, were analyzed by FACS for EGFP expression. Cultured HEK-293T/Ghost-R5-X4-R3 mixed cells were stained with antihuman phycoerythrin (PE)-conjugated CD4 and CCR5 antibodies (BD Biosciences, San Jose, CA) to determine the specificity of transduction and also the downregulation of CCR5 expression in the CCR5 shRNA vector-transduced cells. PBMCs were stained with antihuman CD3-allophycocyanin (APC) (T cells), CD19-PE (B cells), CD14-PE (monocyte/macrophage), and CCR5-PE (BD Biosciences, San Jose, CA) to determine cell-specific targeted transduction and CCR5 downregulation. All FACS data was obtained on a Beckman Coulter Cytomics FC500 flow cytometer and analyzed using CXP analysis software.

To more accurately quantitate the downregulation of CCR5 in CCR5 shRNA-transduced cells, quantitative real-time polymerase chain reaction (QRT-PCR) was performed on total RNA extracted from transduced cells using RNA-STAT-60 (Tel-Test, Friendswood, TX). First-strand cDNA synthesis was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). QRT-PCR was then performed using the SYBR Green PCR Master Mix Kit (Applied Biosystems) using the primer set, 5'-ACTGCAAAAGGCTGAAGAGC-3' and 5'-AGCATAGTGAGCCCAGAAGG-3'. GAPDH was used as an internal control.

HIV-1 Challenge of Transduced Cells

To determine whether the CCR5-targeted transduction and the successive downregulation of CCR5 expression could confer HIV-1 resistance, transduced cells were challenged with BaL-1, an R5-tropic strain of HIV-1. Transduced cells, both HEK-293T/Ghost-R5-X4-R3 mixed cells (MOI 0.05) and PBMCs (MOI 0.01), were challenged with BaL-1 for 2 hours at 37°C with 8 µg/mL polybrene. On various days post-infection, cell culture supernatants were sampled for use in p24 antigen enzyme-linked immunosorbent assay (ELISA) and infectious virus assays. Supernatant aliquots were quantified for p24 by ELISA according to the manufacturer's protocol (Zeptometrix Corp, Buffalo, NY). Challenged cell culture supernatants were also analyzed for infectious virus by the Ghost Cell assay. Briefly, 75 µL of challenge culture supernatant was added to uninfected Ghost-R5-X4-R3 cells (1.0×10^6) with 8 µg/mL polybrene for 2 hours. Forty-eight hours postinfection, infected Ghost-R5-X4-R3 cells were analyzed by flow cytometry for EGFP expression to determine levels of infectious virus particles.

In vivo CCR5 Cell-Specific Targeting

To determine the in vivo targeting ability of the CCR5 cell-specific vector, the immunodeficient non-obese diabetic severe combined immunodeficient (NOD/SCID)-IL2r-knockout mouse model was used to evaluate targeted transduction. Adult mice, at least 4 months old, were sublethally irradiated with 300 rads total body irradiation. Freshly isolated primary human PBMCs were injected retro-orbitally (RO) with 1×10^7 cells. Cells were allowed to engraft for 2 weeks and were followed by RO injection with 1×10^6 TU of the EGFP-alone CCR5-targeting vector. Five days postinjection of vector, single-cell suspensions from mouse organs were analyzed for cell-specific transduction of engrafted human cells using the PBMC antibody staining panel described above. FACS data were collected on a Beckman Coulter Cytomics FC500 flow cytometer and analyzed with CXP analysis software.

RESULTS

CCR5 Cell-Specific Transduction and CCR5 Downregulation

Lentiviral vectors, both the CCLc-EGFP alone and CCLc-CCR5shRNA, were pseudotyped with the Sindbis virus envelope containing the ZZ domain from protein A of *S. aureus* and were evaluated for their ability to specifically transduce CCR5-positive cells when conjugated with a CCR5 mAb. A mixed population of cultured cells including HEK-293T cells (CD4 and CCR5 negative) and Ghost-R5-X4-R3 cells (CD4 and CCR5 positive) were transduced with the CCR5-targeting vectors. Cells were analyzed 4 days post-transduction for EGFP expression to determine cell-specific targeted transduction. Ghost-R5-X4-R3 cells express CCR5 and should be selectively transduced in the mixed cell culture. Another surface molecule, CD4, expressed in the Ghost-R5-X4-R3 cells but not in the HEK-293T cells, was used as an additional cell detector to analyze targeting specificity using flow cytometry. Only cells expressing CCR5 were transduced as displayed by the double positive staining for CD4 and EGFP expression (Fig. 2). Ghost-R5-X4-R3 cells were selectively transduced by both the EGFP-alone and CCR5-shRNA CCR5-targeting vectors as compared with nontransduced cells in the same cultures which were negative for CD4 and EGFP expression. Primary PBMCs, both unstimulated and PHA stimulated, were transduced with the CCR5-targeting vector as described above. Both T-cell (CD3⁺) and monocyte/macrophage (CD14⁺) cell populations were selectively transduced because these cell populations express CCR5 in contrast to B cells (CD19⁺), which were used as an internal negative control (Fig. 3). Targeted transduction was observed for both the EGFP-ZZ and CCR5shRNA-ZZ vectors as compared with cells transduced with Sindbis-ZZ pseudotyped vector which was not conjugated to the CCR5 mAb. Both unstimulated and stimulated PBMCs displayed similar transduction patterns in both transduction efficiency and specificity (unstimulated cell data not shown). These data confirm the ability of this vector to specifically transduce CCR5-expressing cells, both cultured and primary PBMCs.

The transduced mixed cultures of Ghost-R5-X4-R3 and HEK-293T cells and also the PBMCs were stained for CCR5 cell surface expression and analyzed by flow cytometry to determine the levels of CCR5 downregulation conferred by the CCR5 shRNA vector (Fig. 4A). The Ghost-R5-X4-R3 cells transduced with the EGFP-ZZ vector were selectively transduced as displayed by double positive staining for CCR5 and EGFP expression. The CCR5-positive cells were also specifically transduced with the CCR5shRNA-ZZ vector, however, due to the expression of the CCR5 shRNA, a subsequent knockdown of CCR5 expression was observed by negative staining for CCR5 within the EGFP-positive transduced population. A knockdown level of >93% was observed as compared with nontransduced and EGFP-alone vector-transduced cells. To further quantitate the level of CCR5 knockdown in shRNA-transduced Ghost-R5-X4-R3 cells, QRT-PCR was performed to analyze the intracellular levels of CCR5 mRNA. Intracellular CCR5 mRNA levels were decreased in CCR5 shRNA-transduced cells, >93%, as compared with control nontransduced and EGFP-alone-transduced cells (Fig. 4B). These results correlated with the CCR5 flow cytometry data (Fig. 4A). CCR5 downregulation, >91%, was also observed in primary PBMC cultures transduced with the CCR5shRNA-ZZ vector (Fig. 4C). The above results established that CCR5-expressing cells can be specifically targeted by the CCR5 antibody-conjugated vector and that these cells, when transduced with the CCR5shRNA-ZZ vector, have decreased levels of CCR5 expression.

HIV-1 Inhibition of CCR5 shRNA-Transduced Cells

To determine whether CCR5-targeted CCR5 shRNA vector-transduced cells were capable of resisting HIV-1 infection upon downregulation of cell surface CCR5 expression, cells were challenged with BaL-1, an R5-tropic strain of HIV-1. Challenge experiments were

performed at an MOI of 0.05 to evaluate the level of protection afforded by the potent knockdown of cell surface CCR5. Strong inhibition of viral infection was observed in cultured Ghost-R5-X4-R3 cells transduced with the CCR5 shRNA vector (>1.5 log inhibition) as compared with EGFP-alone-transduced cells as measured by p24 antigen ELISA (Fig. 5A) and quantitation of infectious virus (Fig. 5B). Upon challenge of CCR5 vector-targeted PBMCs (MOI 0.01), viral inhibition was observed in CCR5 shRNA-transduced cells as compared with nontransduced and EGFP-alone vector-transduced cells. Strong protection levels (>1 log) were observed at the peak of viral replication (day 14 postinfection) as measured by p24 antigen ELISA (Fig. 5C) and quantitation of infectious virus (Fig. 5D). These data confirm that targeted transduction of HIV-1-susceptible cells can confer preexposure protection to HIV-1 infection by the selective downregulation of CCR5 expression in successfully transduced CCR5-positive cells.

In vivo Targeted Transduction of CCR5-Expressing Cells

The above data confirmed the CCR5-targeting ability of this vector system to selectively transduce cells that are positive for CCR5 expression in vitro. To further evaluate the potential of this targeting vector, the in vivo efficacy was assessed in a NOD/SCID-IL2r-knockout mouse model engrafted with freshly isolated human PBMCs. Sublethally irradiated adult mice were injected RO with 1×10^7 unstimulated PBMCs. Two weeks after cell injection, to allow for human cell engraftment, 1×10^6 transducing units of the CCR5-targeting vector were injected RO. Five days postinjection with the vector, single-cell suspensions from mouse peripheral blood and lymphoid organs were analyzed by FACS to evaluate the in vivo targeted transduction efficiency. Human T cells (CD3⁺) in the lymph node and spleen (Fig. 6A) and macrophages (CD14⁺) in the bone marrow (Fig. 6D) were successfully transduced with the CCR5-targeting vector in vivo as displayed by an increase in EGFP expression as compared with engrafted mice not injected with vector. B cells (CD19⁺) in the spleen and bone marrow (Fig. 6C), however, were not transduced because they do not express CCR5. FACS analysis for CCR5-positive cells was also performed to determine targeting efficiency. Only those cells that were positive for CCR5 expression in the lymph node and peripheral blood were successfully transduced in vivo (Fig. 6B). These results confirm that this novel targeting vector has predictable and reproducible efficacy to target and transduce CCR5-expressing human cells in vivo.

DISCUSSION

Current ex vivo cellular transduction protocols for clinical gene therapy, which include bone marrow harvesting, apheresis of PBMCs, and manipulation in a clean room setting, are not feasible in developing countries where sophisticated laboratories and equipment necessary to perform these procedures are unavailable. Therefore, new and innovative ways to transduce and protect HIV-1-susceptible cells, in vivo, need to be developed. Cell-specific targeting vectors capable of selectively transducing a particular population of cells of interest, after direct injection into the body, have the potential to protect cells and can provide an “off the shelf” therapy. Numerous strategies have been evaluated for their ability to specifically target cells in vitro and in vivo.³⁰⁻⁴¹ One approach utilizes the ability of the Sindbis virus envelope to pseudotype lentiviral vectors. By inserting the immunoglobulin-binding ZZ domain of protein A from *S. aureus* into the binding region of the Sindbis virus E2 glycoprotein, these envelopes are then able to bind mAbs.^{42,43} This strategy is simple yet ingenious because only 1 type of vector needs to be manufactured in large scale, certified, and stored. Depending on which cell population will be targeted, 1 specific, well characterized mAb can be selected and conjugated to a small portion of the vector to render a cell-specific vector preparation which is then suitable for in vivo targeting and transduction.

Mainly, R5-tropic strains of HIV-1 which utilize CCR5 as a coreceptor are primarily responsible for viral transmission and early stage infection.⁴⁹ These strains establish a primary infection and viral reservoir in an infected individual. Targeting CCR5 through therapeutic approaches utilizing either small molecule HIV-1 entry inhibitors or gene silencing by siRNAs in a gene therapy setting offers a promising way to inhibit and possibly prevent initial HIV-1 infection. As observed with the natural 32-bp deletion mutant allele occurring in individuals resistant to HIV-1 infection, a targeted approach to knockdown expression of CCR5 in the HIV-susceptible population could provide protection from HIV infection. We therefore applied the Sindbis-ZZ strategy to construct a CCR5-positive cell-specific targeting vector for inhibition of initial HIV-1 infection by intracellular immunization with a CCR5 shRNA.

Upon incubation with the CCR5-targeting vector, only those cells expressing CCR5 were selectively transduced with a lentiviral vector expressing a highly potent CCR5 shRNA capable of potent knockdown of CCR5 gene expression. This approach was successful in mixed cell populations of both cultured and primary human cells. Transduction of freshly isolated PBMCs with the CCR5-targeting vector demonstrated transduction of only T cells and monocyte/macrophage populations which express CCR5 on the cell surface and are the major cell types normally infected by HIV-1. B cells in the culture remained nontransduced because they do not express CCR5, demonstrating specificity. Transduction was also shown to be dependent on vector conjugation with a mAb. When cells were incubated with vector not conjugated to any antibody, no transduction was observed (Fig. 3). These data confirm the necessity for a specific mAb for the vector to elicit an effect and also verifies the lack of nonspecific transduction by unconjugated vector particles. Upon stimulation, the levels of CCR5 expression in total PBMC cultures increases, however, these cultures left unstimulated or stimulated with PHA displayed similar levels of transduction efficiency with the EGFP-ZZ and CCR5shRNA-ZZ vectors. This suggests that the targeting efficacy and transduction ability of the CCR5-targeting vector is independent of cell stimulation and is highly efficient in targeting cells with low levels of CCR5 expression. After transduction with the CCR5 antibody-conjugated vector, PBMC cultures also retained viability and the number of total cells remained constant as observed by cell counting before HIV-1 challenge experiments. This demonstrated that no adverse effects were associated with the transduction of primary cells with the CCR5 antibody-targeting vector. Targeted transduction of CCR5-expressing cells with the CCR5 shRNA vector and the subsequent downregulation of CCR5 expression conferred protection from HIV-1 infection as measured by p24 antigen ELISA and by quantitating infectious virus (Fig. 5). A slight rise in viral replication was observed in the CCR5 shRNA-transduced population due to infected nontransduced cells; however, a subsequent decrease in detection of virus followed after the infected cells were killed. This observation highlights the selective survival advantage of CCR5 shRNA-transduced cells which were able to resist infection during the course of the virus challenge and to eventually take over the culture.

The efficacy of this targeting strategy was further evaluated, *in vivo*, in a NOD/SCID-IL2r-knockout mouse model engrafted with freshly isolated human PBMCs. Upon irradiation, injection of cells, and engraftment of the PBMCs, the mice were injected with the CCR5-targeting lentiviral vector. Cell-specific transduction was observed in both the T-cell and monocyte/macrophage cell populations in a similar manner as seen in the *in vitro* PBMC transductions. Specific cell targeting could be demonstrated *in vivo* by the fact that none of the human B cells were transduced, as measured by EGFP expression (Fig. 6C). This lack of B-cell transduction could also be observed in the *in vitro* experiments and is due to the absence of the CCR5 receptor on B cells. Upon retro-orbital injection, the CCR5-targeting vector will first drain into the lymphatics and selectively transduce cells in the lymph nodes followed by transduction of cells in other lymphoid organs and in the peripheral blood. The

flow of vector particles from the retro-orbital capillary beds to the lymphatics was confirmed by the high T-cell transduction efficiency observed in the lymph nodes (Fig. 6A).

The use of an integrating lentiviral vector as an HIV therapeutic may pose potential risks associated with target cell chromosomal integration. Unlike retroviral vectors, the preferred integration sites of lentiviral vectors are not within or near transcriptional start regions of genes. Also, current lentiviral vectors used are self-inactivating vectors where the long terminal repeats (LTRs) are rendered incapable of promoting transcription. Our group recently published the results of a 10-year study in more than 600 mice demonstrating that retroviral and lentiviral vector-transduced CD34⁺ cells did not cause cancers originating from the integrated vectors.⁵⁰ These observations give strong evidence for the use of this vector strategy as an HIV therapeutic. Another potential drawback to the current anti-HIV strategy is that down-regulating CCR5 expression on immune system cells has been implemented in an increased risk of West Nile disease.⁵¹ This must be kept in mind when developing anti-HIV strategies which abrogate either complete or near complete expression of CCR5.

The results presented here offer an encouraging and alternative method of therapeutic intervention for HIV-1 infection by utilizing an HIV-1-susceptible cell-specific targeting strategy via direct injection of vector particles. This innovative strategy could potentially be applied as a therapeutic strategy for highly susceptible human populations, even in areas where sophisticated laboratories are not available. Upon injection with the CCR5 cell-specific targeting vector and after transduction of a sufficient number of CCR5-expressing cells, the viral load of infected individuals could potentially decrease due to expression of the CCR5 shRNA to silence CCR5 and thus block HIV-1 entry. Gene therapy could therefore aid in transferring the genetic ability to resist HIV-1 infection to susceptible individuals by silencing the expression of CCR5, mimicking the natural ability of individuals harboring the 32-bp mutant allele to resist infection. Extensive preclinical in vivo safety and efficacy testing of our strategy toward a potential clinical application is currently ongoing.

Acknowledgments

The Sindbis virus glycoprotein and ZZ domain plasmids were generously provided by Brian Geiss (Colorado State University). We would like to acknowledge John Javien for his technical support. The National Institute of Health AIDS Research and Reference Reagent Program provided many reagents and cell lines used in this work. We have filed a provisional patent for the CCR5 cell-specific targeting CCR5 shRNA lentiviral vector.

Supported by the University of California-Davis Health System start-up funds from the Dean's office for the Stem Cell Program and by the James B. Pendleton Charitable Trust.

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CCLc-U6-CCR5shRNA-PGK-EGFP **FIGURE 1.**

CCR5 shRNA lentiviral vector: A third-generation lentiviral vector, CCLc-x-PGK-EGFP, containing an EGFP reporter gene was used to generate the CCR5 shRNA construct. The CCR5 shRNA was expressed under the control of the human polymerase-III U6 small RNA promoter and was inserted upstream of the EGFP reporter gene.

FIGURE 2.

CCR5-positive cell-specific targeting of Ghost-R5-X4-R3 cultured cells: A mixed culture of Ghost-R5-X4-R3 and HEK-293T cells was transduced with the CCR5-targeting vectors, EGFP-ZZ and CCR5shRNA-ZZ. Seventy-two hours posttransduction, the cells were stained with a PE-conjugated anti-human CD4 antibody. Cells were analyzed by FACS for CD4 and EGFP expression.

FIGURE 3.

CCR5-positive cell-specific targeting of human primary PBMCs: Freshly isolated PBMCs were transduced with the CCR5-targeting vectors. Seventy-two hours posttransduction, cells were stained with either an APC-conjugated antihuman CD3 (T cells), PE-conjugated antihuman CD14 monocyte/macrophages), or a PE-conjugated antihuman CD19 (B cells) antibody and analyzed by FACS.

FIGURE 4.

Downregulation of CCR5 protein and mRNA levels in transduced Ghost-R5-X4-R3 cells and PBMCs: A mixed culture of Ghost-R5-X4-R3 and HEK-293T cells was transduced with the CCR5 targeting vectors, EGFP-ZZ and CCR5shRNA-ZZ. A, Seventy-two hours posttransduction, the cells were stained with a PE-conjugated antihuman CCR5 antibody and analyzed by FACS for CCR5 and EGFP expression. B, Ghost-R5-X4-R3 cells were also analyzed by QRT-PCR for intracellular CCR5 mRNA. C, Human PBMCs were transduced with the CCR5-targeting vectors, EGFP-ZZ and CCR5shRNA-ZZ, and analyzed by FACS for CCR5 and EGFP expression.

FIGURE 5.

HIV-1 challenge of CCR5 shRNA vector-transduced Ghost-R5-X4-R3 cells and PBMCs: Mixed culture Ghost-R5-X4-R3/HEK-293T cells transduced with the EGFP-ZZ (○) or CCR5shRNA-ZZ (■) vector were challenged with an R5-tropic BaL-1 strain of HIV-1, MOI 0.05, and analyzed for (A) p24 antigen by ELISA and (B) quantitated for infectious virus by a Ghost cell assay. PBMCs, non-transduced (△), EGFP-ZZ (○), or CCR5shRNA-ZZ (■) vector-transduced were challenged with HIV-1 BaL-1 at an MOI of 0.01. Cell culture supernatants were assayed for (C) p24 antigen by ELISA and (D) quantitated for infectious virus by a Ghost cell assay.

FIGURE 6.

In vivo CCR5-positive cell-specific targeting: Adult NOD-SCID-IL2r⁻ knockout mice were injected RO with fresh PBMCs after 2 weeks later by injection with the CCR5-targeting vector. Cells from various lymphoid organs were analyzed for cell transduction by staining for (A) T cells, (B) CCR5, (C) B cells, (D) macrophages and EGFP by FACS.