Chemistry & Biology
Article

Targeted Disruption of the *CCR5* Gene in Human Hematopoietic Stem Cells Stimulated by Peptide Nucleic Acids

Erica B. Schleifman,[1] Ranjit Bindra,[1] Jean Leif,[2] Jacob del Campo,[1] Faye A. Rogers,[1] Pradeep Uchil,[3] Olaf Kutsch,[4] Leonard D. Shultz,[5] Priti Kumar,[6] Dale L. Greiner,[2] and Peter M. Glazer[1,*]
[1]Departments of Therapeutic Radiology and Genetics, Yale University School of Medicine, New Haven, CT 06510, USA
[2]Division of Diabetes, Department of Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
[3]Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT 06536, USA
[4]Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA
[5]The Jackson Laboratory, Bar Harbor, ME 04609, USA
[6]Section of Infectious Disease, Department of Medicine, Yale University School of Medicine, New Haven, CT 06510, USA
*Correspondence: peter.glazer@yale.edu
DOI 10.1016/j.chembiol.2011.07.010

SUMMARY

Peptide nucleic acids (PNAs) bind duplex DNA in a sequence-specific manner, creating triplex structures that can provoke DNA repair and produce genome modification. *CCR5* encodes a chemokine receptor required for HIV-1 entry into human cells, and individuals carrying mutations in this gene are resistant to HIV-1 infection. Transfection of human cells with PNAs targeted to the *CCR5* gene, plus donor DNAs designed to introduce stop codons mimicking the naturally occurring *CCR5*-delta32 mutation, produced 2.46% targeted gene modification. *CCR5* modification was confirmed at the DNA, RNA, and protein levels and was shown to confer resistance to infection with HIV-1. Targeting of *CCR5* was achieved in human CD34$^+$ hematopoietic stem cells (HSCs) with subsequent engraftment into mice and persistence of the gene modification more than four months posttransplantation. This work suggests a therapeutic strategy for *CCR5* knockout in HSCs from HIV-1-infected individuals, rendering cells resistant to HIV-1 and preserving immune system function.

INTRODUCTION

R5-tropic HIV-1 infection requires cell surface expression of both the primary receptor, CD4, and CCR5, a seven transmembrane G protein-coupled coreceptor, for viral entry. Hence, the CCR5 coreceptor has become an attractive target for development of small-molecule entry inhibitors as well as for gene therapy (Holt et al., 2010; Perez et al., 2008; Tsibris and Kuritzkes, 2007). A naturally occurring 32 bp deletion (*CCR5*-delta32) produces a truncated protein that fails to localize to the cell surface (Liu et al., 1996), providing individuals homozygous for this mutation with immunity to R5 tropic HIV-1 (Samson et al., 1996). In a recent report, an HIV-1-positive patient with acute myeloid leukemia received an allogeneic stem cell transplant with cells that were homozygous for the *CCR5*-delta32 mutation (Hütter et al., 2009). Posttransplantation, the recipient had no detectable virus and had increased CD4 counts, demonstrating reconstitution of the immune system.

The formation of triplex DNA structures by oligonucleotides can stimulate recombination in a site-specific manner in human cells (Knauert et al., 2006). Peptide nucleic acids (PNAs) represent a class of synthetic oligonucleotides capable of triple helix formation with high affinity and specificity for duplex DNA. PNAs consist of purine and pyrimidine bases attached to a polyamide backbone, maintaining a spacing similar to DNA but yielding an achiral, neutrally charged molecule. Highly stable PNA/DNA/PNA triplexes can be formed with two PNA strands and the homopurine DNA strand of the duplex. One PNA strand binds by strand invasion to the duplex via Watson-Crick pairing in an antiparallel orientation, whereas the other can bind in the major groove of the DNA/PNA duplex via Hoogsteen base pairs (in a parallel orientation), forming a highly stable PNA/DNA/PNA triplex, with displacement of the remaining DNA strand (Egholm et al., 1993). If connected by a linker of sufficient flexibility, the two PNA strands can be contained in a single molecule, yielding a clamp structure upon DNA binding (PNA clamp).

To mimic the *CCR5*-delta32 mutation, we sought to permanently alter the sequence of *CCR5* in human cells using site-specific, triplex-induced homologous recombination to insert a stop codon in the vicinity of the delta32 mutation site. It was expected that such a strategically placed stop codon would result in a truncated protein, rendering modified human cells resistant to R5-tropic HIV-1 entry.

Using a newly optimized design and the inherent ability of triplex-forming PNAs to bind DNA and catalyze genomic modification, we report here the generation of site-specific targeted modification of the *CCR5* gene in human cells, rendering them permanently resistant to HIV-1 infection. This work provides the basis for a new therapeutic strategy for AIDS and represents a novel form of genomic medicine, whereby the *CCR5* gene in hematopoietic stem cells is permanently altered, allowing for the proliferation of a reservoir of CCR5-negative, HIV-1-resistant cell lineages, which can preserve immune function in infected individuals.

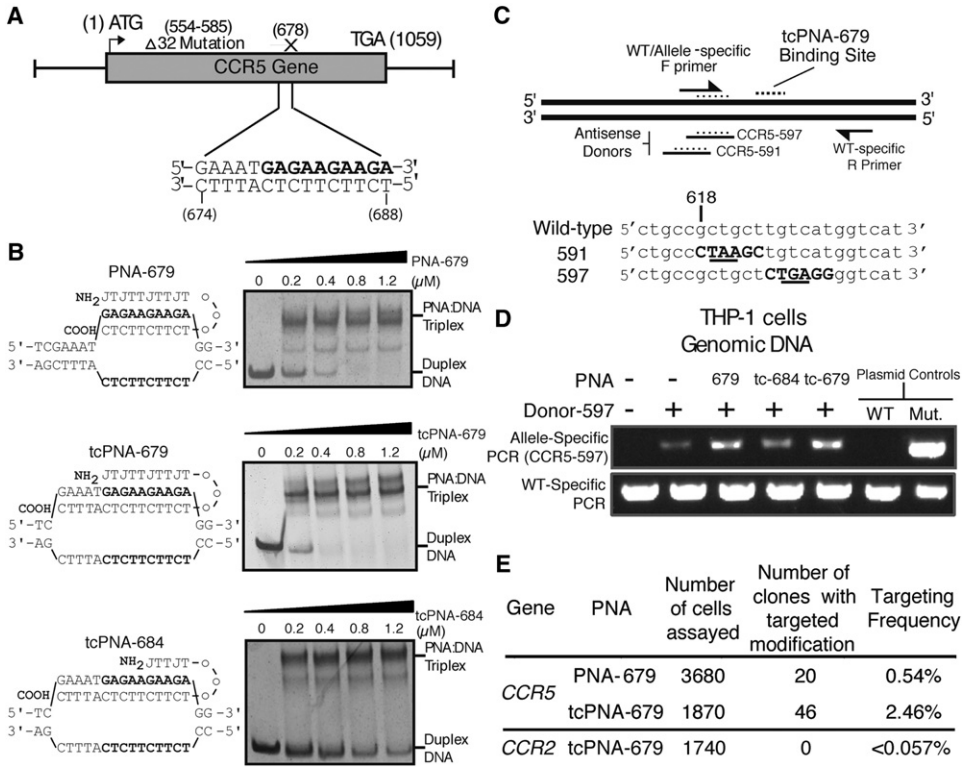

Figure 1. PNA-Stimulated Modification of the *CCR5* Gene in Human THP-1 Cells

(A) Depiction of the *CCR5* gene indicating the PNA binding site at bp 674-688 (purine stretch in bold) and the position of the delta32 deletion creating a stop codon at 678.

(B) Diagram and gel mobility shift assays demonstrating binding of PNA-679, tcPNA-679, and tcPNA-684 to the polypurine target site in *CCR5*. J indicates pseudoisocytosine substituted for C to allow pH-independent triplex formation. All PNAs have three lysine residues conjugated to each end and 8-amino-2,6-dioxaoctanoic acid linkers were used as the flexible linker "O."

(C) AS-PCR assay in which the 3′ end of the AS forward primer contains either the specific 6 bp mutation or the WT sequence. PNA binding site and relative position of the 60 nt donor DNAs; either donor 597 or donor 591 are indicated. Sequences of the 6 bp mutations to be introduced in *CCR5* by the donor DNAs. Donor sequences are the reverse complement of those partially shown. Mutations are in bold, with resulting stop codons underlined.

(D) AS-PCR demonstrates targeted modification of the *CCR5* gene in human THP-1 cells. THP-1 cells were transfected with donor 597, donor 597 and PNA-679, donor 579 and tcPNA-679, donor 597 and tcPNA-684, or buffer alone. 48 hr posttransfection, genomic DNA was isolated, and AS-PCR was performed on genomic DNA and controls as indicated.

(E) Quantification of PNA-induced *CCR5* gene targeting by enumeration of *CCR5*-modified single-cell clones. THP-1 cells were transfected with either tcPNA-679 or PNA-679, plus donor DNA(s). 48 hr later the treated cell populations were diluted into multiwell plates to allow for isolation and analysis of single-cell clones by AS-PCR of genomic DNA. The *CCR2* gene was analyzed for modification by direct sequencing of 1740 clones.

See also Figure S1.

RESULTS

Design of Molecules to Target *CCR5*

We designed a series of triplex-forming PNAs to bind to the *CCR5* gene at a polypurine stretch encompassing positions 679-688 (Figure 1A). PNA-679 forms a PNA clamp with Watson-Crick and Hoogsteen (Egholm et al., 1993) binding domains of equal length (Figure 1B), similar to PNA clamp designs used in our prior work (Chin et al., 2008). To extend the length of the recognition site beyond the homopurine sequence, we tested a series of "tail-clamp" PNAs (tcPNAs) (Bentin et al., 2003). tcPNA-679 binds to form a PNA/DNA/PNA triple helix "clamp" within the polypurine stretch at positions 679-688 of the *CCR5* gene and also includes an additional 5 bp "tail," forming a PNA/DNA duplex at positions 674-678 (Figure 1B). This molecule therefore mediates a mode of PNA binding to DNA that encompasses both triplex and duplex formation (Bentin et al., 2003; Kaihatsu et al., 2003) and in doing so targets a unique 15 bp sequence in the *CCR5* gene. In this complex, the PNA/DNA/PNA triple helix and the PNA/DNA duplex both produce displacement of the pyrimidine-rich strand, creating an altered helical structure that has been shown to provoke the nucleotide excision repair (NER) pathway and activate the site for recombination with a donor DNA molecule. PNA/DNA/PNA triplexes have been shown to stimulate NER on a plasmid substrate even more than direct UV damage to the plasmid DNA (Rogers et al., 2002). We also tested another tail-clamp PNA with an extended tail but a shorter Hoogsteen binding domain (tcPNA-684; Figure 1B). Based on an in vitro gel shift assay, all three PNAs were found to bind to their specific *CCR5* target sites in a plasmid substrate at physiological pH, although tcPNA-679 clearly shows the greatest binding (Figure 1B).

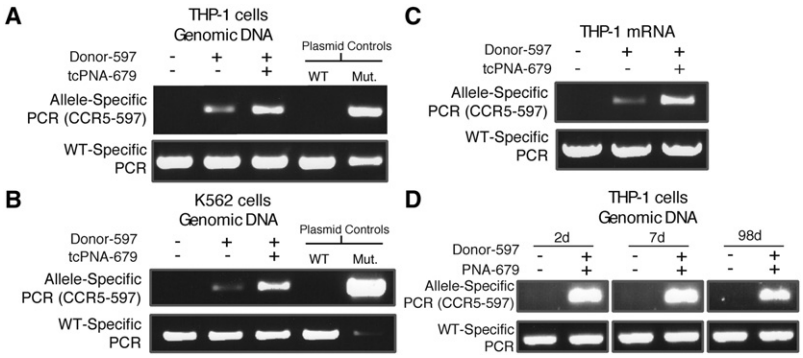

Figure 2. PNA-Induced Modification of *CCR5* Is Reproducible, Can Be Detected at the mRNA Level, and Persists in Culture for More than Three Months

(A) THP-1 cells were transfected with donor 597, donor 597 and tcPNA-679, or buffer alone and analyzed 48 hr posttransfection by AS-PCR.

(B) Targeted modification of the *CCR5* gene in K562 cells, treated and analyzed as above.

(C) Demonstration of targeted *CCR5* modification at the mRNA level. Total mRNA was isolated from THP-1 cells 48 hr after transfection with donor 597, donor 597 and tcPNA-679, or buffer alone and subjected to AS-RT-PCR.

(D) Long-term persistence of the targeted 597 mutation in PNA and donor DNA-treated THP-1 cells after up to 98 days in culture as indicated, based on AS-PCR of genomic DNA.

In the strategy for PNA-induced recombination (Figure 1C), PNA binding is expected to stimulate recombination between the chromosomal *CCR5* gene and a cotransfected donor DNA. For this, two single-stranded antisense-oriented 60 nt donor DNAs, 591 and 597, were designed to be homologous to a portion of the *CCR5* gene except for a central 6 bp segment intended to introduce, via recombination, an in-frame stop codon (Figure 1C) and create a sufficient sequence change to be easily detectable by an allele-specific PCR (AS-PCR) assay (Figure 1C).

CCR5 Targeting and Quantification in Human Cells

THP-1 cells, a human acute monocytic leukemia cell line that expresses CCR5 and can be infected with HIV-1, were used as an initial model to assay for targeted modification of *CCR5* (Konopka and Düzgüneş, 2002). THP-1 cells were mock-transfected, transfected with donor 597 alone, or with donor 597 and one of the three PNAs. After 48 hr, genomic DNA was prepared and analyzed by AS-PCR (Figure 1C). Introduction of the targeted mutation into the *CCR5* gene was produced at a low level by the donor DNA alone but was substantially induced by cotransfection of either PNA-679 or tcPNA-679 with the donor molecule (Figure 1D), whereas tcPNA-684 was less effective. As an additional control to validate the assay, genomic DNA from untreated cells was spiked in vitro with donor DNA, and then the AS-PCR assay was performed. No signal was observed under these conditions (see Figure S1A available online), eliminating the possibility that any false-positive results could arise from persistence of the donor DNAs.

To better quantify the PNA-induced recombination and to establish the generation of cells with long-term, heritable genomic modification, THP-1 cells were treated with either PNA-679 or tcPNA-679 (in both cases plus donor DNA) and then were diluted out into multiwell dishes. Single-cell clones were obtained and individually assayed for *CCR5* gene modification (Figure 1E). PNA-679 gave a targeted modification frequency of 0.54%, consistent with the frequencies of induced recombination by PNAs of this design previously reported for the *beta-globin* gene target (Chin et al., 2008). However, tcPNA-679, which showed superior target site binding in vitro (Figure 1B), yielded 46 positive clones out of 1870 cell clones, for an overall frequency of 2.46%. This increased activity may be attributed to both increased binding affinity and greater disruption of the underlying duplex target, thereby better provoking DNA repair and recombination, but this remains to be tested. These results demonstrate the targeting for one allele. To test for the possibility of double knockout of both alleles simultaneously, we treated cells with tcPNA-679 and two different donors (591 and 597) combined. 1870 cells were analyzed individually but a doubly targeted clone was not found, giving an upper limit for the double allele targeting frequency of less than or equal to 0.05%.

As a determination of potential off-target effects, the gene with the highest homology to *CCR5*, the *CCR2* gene, was directly sequenced in clones isolated by limiting dilution from tcPNA-679 and donor DNA-treated THP-1 cells. Analysis of the *CCR2* sequence in 1740 cells revealed the presence of only wild-type (WT) sequences, indicating no off-target changes, giving an upper limit to the off-target frequency of less than 0.057% (Figure 1E). This is not surprising because the binding of tcPNA-679 to the partially homologous region in *CCR2* is reduced by at least 4-fold compared with *CCR5* (Figure S1B). Prior work has established that even a small number of mismatches can drastically reduce target site binding affinity and thereby substantially diminish the ability of triplex-forming molecules to induce recombination (Knauert et al., 2005). In work by Perez et al. (2008) using *CCR5*-targeted zinc finger nucleases, off-target modification of the *CCR2* gene was determined to occur at a frequency of 5.39%, only 6-fold less than their reported frequency of *CCR5* modification (35.6%). In contrast, our off-target frequency is at least 43-fold lower than our frequency to target *CCR5* (2.46%) and at least two orders of magnitude less than the off-target frequency reported by Perez for zinc-finger nucleases, showing the greater specificity of the PNA-mediated gene-targeting strategy. In addition, the region of the *CCR5* gene containing the PNA binding site was also directly sequenced in 960 cell clones from tcPNA-679- and donor DNA-treated cells, and no sequence changes were found at or around the PNA binding site (data not shown).

PNA-Induced Modification of *CCR5* and Its Persistence in Culture

Reproducible *CCR5* gene targeting was seen not only in THP-1 cells but also in another human cell line, K562 (Figures 2A and 2B). Treated cells were also analyzed at the mRNA level using allele-specific reverse transcriptase polymerase chain reaction (AS-RT-PCR), confirming that the targeted sequence change is present in the mRNA expressed from the *CCR5* gene (Figure 2C). In addition, samples were taken intermittently from serially

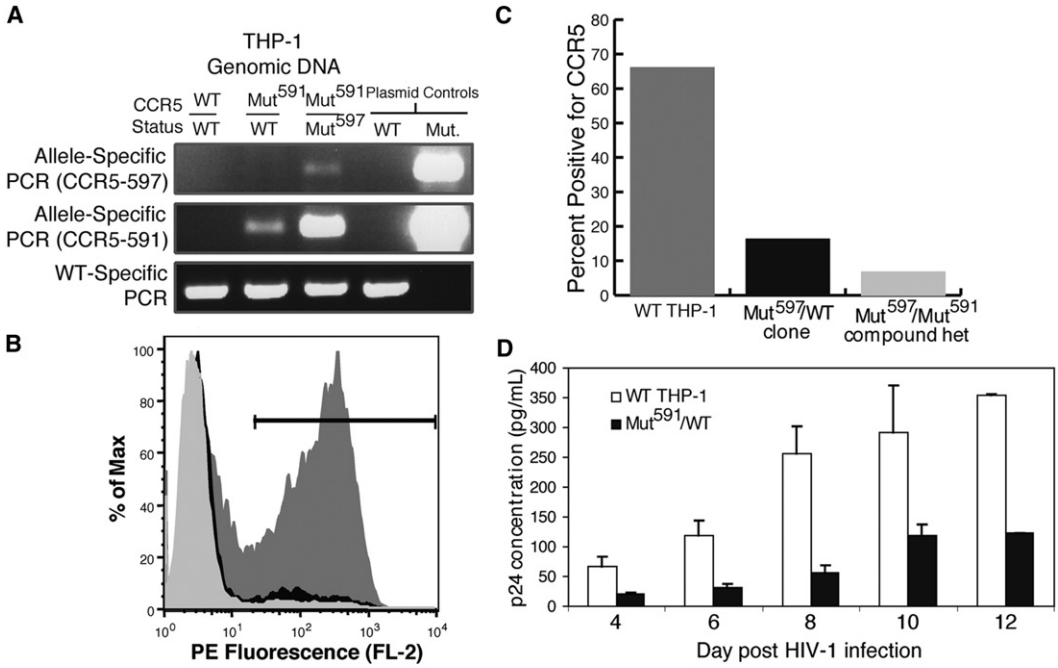

Figure 3. Targeted Modification of the *CCR5* Gene in THP-1 Cells Yields Decreased CCR5 Protein Cell Surface Expression and Provides Resistance to R5-tropic HIV-1 Infection

(A) AS-PCR to identify individual THP-1 cell clones carrying the 597 mutation or both the 597 and 591 mutations. Allele-specific primers were used to amplify the 597, 591, or WT *CCR5* sequences from genomic DNA as indicated. WT and the respective mutant plasmids were used as PCR controls.

(B) Flow cytometry assessment of CCR5 cell surface expression in parental THP-1 cells versus CCR5-modified clones. THP-1 cells were treated with either buffer or PMA (50 ng/ml) for 48 hr. Cells were collected and stained with a PE-conjugated CCR5 antibody and assayed by FACS to determine CCR5 protein expression levels. Parental THP-1 cells, Mut^{591}/WT heterozygous cells, and doubly modified Mut^{591}/Mut^{597} THP-1 cells are represented by dark gray, black, and light gray, respectively.

(C) Quantification of decreased cell surface CCR5 expression in parental and *CCR5*-targeted THP-1 cells, as above.

(D) Cell culture supernatant p24 levels were measured by ELISA at the indicated times after viral challenge. Parental THP-1 cells and Mut^{591}/WT heterozygous cells are in white and black, respectively. Results are graphed as the mean and standard deviation (SD) of duplicate determinations from two wells.

See also Figure S2.

passaged cell populations that had been treated with PNA-679 and donor 597. AS-PCR of these cell populations confirmed that cells containing the targeted 597 mutation persisted for at least 98 days in culture (Figure 2D), establishing that a heritable sequence change had been generated and that the modified cells are viable, further eliminating concern regarding any potential PCR artifacts that could arise from transient persistence of the donor DNA. Similarly, five clones isolated in the work above were maintained in culture by serial passage for 13 months, and all five were confirmed to retain the targeted modification in *CCR5*.

Isolation and Characterization of Single Cell-Modified Clones: Effect on CCR5 Protein Expression and HIV-1 Infectibility

To generate a cell population with both *CCR5* alleles modified, one clone carrying the 597 modification (confirmed not only by allele-specific PCR but also by genomic DNA sequencing to have the modified allele) (Figure S2A) was expanded and transfected with tcPNA-679 and DNA donor 591. From this treated population, doubly mutant clones with both alleles modified were identified by AS-PCR (Figure 3A). The frequency that such clones were induced from the heterozygous cells was 0.98% (18/1840 clones). Note that in Figure 3A, the WT allele seems to be present even in the compound heterozygous clone because the two targeted modifications are not exactly in the same location in the gene. Hence, the 3′ end of what we refer to as the "WT forward primer" that is used to amplify the control band—when using the 591 donor—ends at the last 6bp of the 591 modification and does not overlap with the 597 mutation. Therefore, this primer can still amplify off of the 597 modified allele without any effect on the strength of the PCR product. This PCR product is only presented in the figure as a control to show that our samples contain genomic DNA with the human *CCR5* sequence.

The parental THP-1 cells, a heterozygous mutant (Mut^{597}/WT) clone, and a double mutant (Mut^{597}/Mut^{591}) clone were then tested for targeted *CCR5* modification at the protein level. For this, we took advantage of the ability of phorbol myristate acetate (PMA) to promote differentiation of THP-1 cells into a macrophage-like state (Figure S2B), thereby yielding high levels of CCR5 cell surface expression (Jagodzinski et al., 1999; Konopka and Düzgüneş, 2002). In the parental THP-1 cells, PMA treatment increased *CCR5* mRNA expression approximately 5-fold, as assessed by a Taqman RT-PCR assay (Figure S2C), and increased CCR5 protein cell surface expression 17-fold

as determined by antibody staining and flow cytometry (Figure S2D). The Mut^{597}/WT heterozygous clone was treated with PMA and determined by flow cytometry to have a ~50% reduction in cell surface expression of CCR5 compared with WT/WT cells (Figure 3B). The double mutant clone, Mut^{597}/Mut^{591}, had minimal cell surface staining for CCR5 (essentially at the background level of the isotype staining in the assay), even after PMA treatment (Figure 3B). These results, quantified in Figure 3C, show at the protein level that PNA plus donor DNA treatment of THP-1 cells produces functional disruption of the *CCR5* gene, leading to reduced cell surface expression. This is consistent with the introduction of stop codons by donors 591 and 597 to yield truncated proteins that fail to properly localize to the cell membrane, thereby mimicking the effect of the naturally occurring *CCR5*-delta32 mutation.

Because the goal of *CCR5* gene targeting is to create a cell population that is resistant to HIV-1 entry, we tested the *CCR5*-modified THP-1 cells for infectibility by R5-tropic HIV-1 infection. After a single treatment of THP-1 cells with tcPNA-679 and DNA donor 591, as described before, we isolated Mut^{591}/WT heterozygous clones. One randomly selected clone was expanded and was seeded into multiwell plates at 2 × 10^5 cells per well in multiple replicates. In parallel, parental THP-1 cells, WT at the *CCR5* locus, were seeded in the same manner. The cells were treated with PMA for 48 hr to induce CCR5 cell surface expression and then were challenged by addition of live HIV-1$_{BaL}$ at a multiplicity of infection (moi) of 1. At 4, 6, 9, 10, and 12 days after infection, supernatants were harvested and frozen. Upon completion of the time course, the supernatants were thawed and analyzed by enzyme-linked immunosorbent assay (ELISA) for core protein p24 antigen levels as a measure of viral infection of the cells (Allain et al., 1987). The ELISA results indicate a substantial decrease in HIV-1 infection of the cells modified at the *CCR5* locus by treatment with tcPNA-679 and DNA donor 591 (Figure 3D), demonstrating functional knockout of the CCR5 coreceptor (correlating further with the *CCR5* DNA, RNA, and protein expression analyses) and establishing the endpoint of reduced HIV-infectibility of PNA-modified human cells.

Modification of Human HSCs and Their Engraftment Capability

For clinical application, ex vivo modification of HSCs from HIV-1-infected patients could offer a method to create a renewable source of virus-resistant immune cells. Importantly, human CD34$^+$ HSCs have been shown to remain uninfected even in HIV-1-infected patients (Shen et al., 1999). Hence, modification of such cells would provide a source of HIV-1-resistant cell lineages. We tested the ability of tcPNA-679 plus donor DNA 597 to modify the *CCR5* gene in primary human CD34$^+$ cells. The cells were transfected by nucleofection with tcPNA-679 and donor 597, with donor 597 alone, or with buffer alone; and 24 or 48 hr posttransfection cells were harvested for analysis of genomic DNA and mRNA. The 597 mutation was detected in the CD34$^+$ cells treated with donor alone, but in the population transfected with tcPNA-679 and donor 597, the level of modification at the target site was substantially higher (Figure 4A). The stimulation of gene modification by tcPNA-679 was determined to be approximately 12-fold as quantified by real-time AS-PCR (Figure 4B). To further quantify the targeting frequencies, a standard curve was established by mixing genomic DNA from *CCR5* heterozygous and WT cells in defined ratios. Real-time AS-PCR was performed on the DNAs from the experimental samples, and the results were quantified based on the standard curve. CD34$^+$ cells transfected with donor DNA alone were calculated to have a modification frequency of 0.03%, whereas the PNA-treated CD34$^+$ cells were estimated to have a modification frequency of 2.8% (Figure S3), a more than 90-fold increase owing to the effect of the PNA. Importantly, targeted modification in the CD34$^+$ cells was also confirmed at the mRNA level by AS-RT-PCR (Figure 4C), indicating that the successfully modified CD34$^+$ HSCs express mutant *CCR5* mRNA. These results support the feasibility of PNA-induced genomic modification in human primary cells.

It is important to note that the relative level of PNA-induced gene modification of *CCR5* was comparable or even higher in CD34$^+$ cells than in THP-1 cells. These results show not only that primary human HSCs are susceptible to targeted gene modification mediated by triplex-forming PNAs, but also that effective gene modification in primary HSCs is highly dependent on the stimulatory effect of the PNAs. Although some studies have reported genome modification in transformed human and rodent cell lines using single-stranded oligonucleotide donor DNAs alone (Pierce et al., 2003), our results suggest that primary HSCs are much less susceptible to this strategy and indicate that the use of triplex-forming PNAs to stimulate recombination is needed. There is, however, evidence that human stem cells can be successfully modified by the use of donor DNA fragments in the range of 500–600 bp (Goncz et al., 2006), but this approach might be even better when combined with triplex-forming PNAs.

We next tested for the ability of PNA-modified CD34$^+$ HSCs to mediate human hematopoietic stem cell engraftment in immune-deficient mice. CD34$^+$ HSCs were transfected, as mentioned before, with tcPNA-679 plus both donor 591 and donor 597. Successful modification of the *CCR5* gene was first confirmed by AS-PCR after 24 hr in an aliquot of the treated cell population. The treated cells were then transplanted into newborn NOD-*scid IL2r*γ^{null} HLA A2.1 mice via intracardiac injection (Figure 4D). At four months posttransplant, peripheral blood cells from the engrafted mice were harvested and fluorescence-activated cell sorter (FACS) was used to confirm engraftment of the human HSCs in the mice by the identification of cells carrying human cell surface markers, including CD3, CD4, CD8, CD20, and CD45 (Figure 4E). AS-PCR on genomic DNA isolated from the mouse spleen was then performed to test for the presence of the targeted modification of the human *CCR5* gene in donor human cells in the engrafted mice. The results show the presence of the targeted 591 and 597 mutations in the human *CCR5* gene persisting more than four months posttransplant in the spleen of the mice transplanted with PNA-targeted human CD34$^+$ cells (Figure 4F), but not in the spleens of control mice. Representative results from one of two transplanted mice are shown; the results from the other mouse were similar. In addition, CD4$^+$ cells were isolated from the spleen of one of the transplanted mice at five months posttransplant, and these also showed the targeted modifications in *CCR5* (Figure 4G). These data indicate that modification of primary HSCs via PNA and donor DNA transfection does not disrupt their engraftment

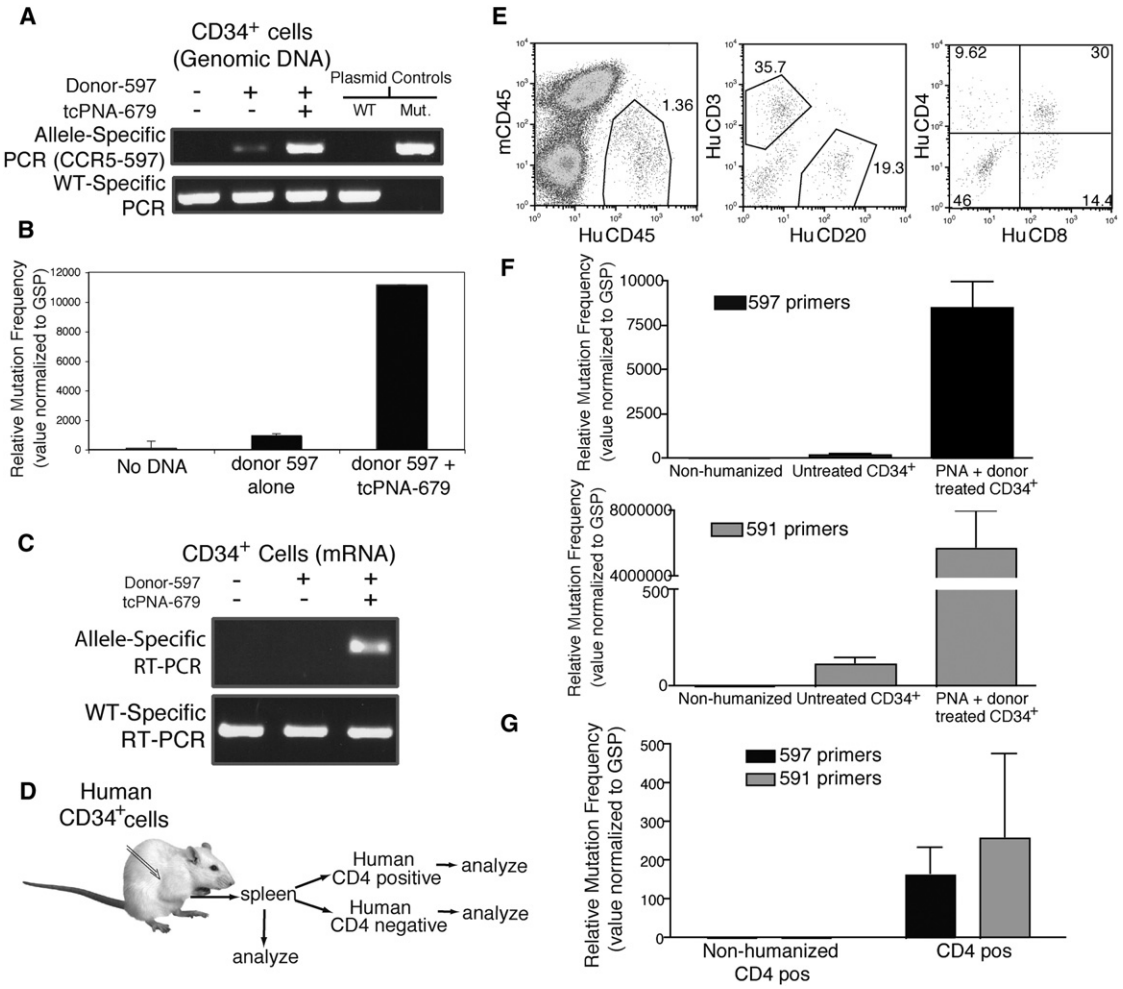

Figure 4. Targeted Modification of the *CCR5* Gene in Human CD34⁺ HSCs and Engraftment in Mice

(A) CD34⁺ HSCs were transfected with either donor 597 alone or with donor 597 and tcPNA-679, or they were mock-transfected as indicated. One day later, genomic DNA was isolated to detect targeted modification of the *CCR5* gene by AS-PCR.

(B) Quantification of *CCR5* gene modification by quantitative real-time AS-PCR. Mean results from two replicates are shown, with error bars (expanded 1000x for visualization) indicating SD. All samples were normalized to gene-specific primers.

(C) Targeted modification of *CCR5* in CD34⁺ cells at the mRNA level by AS-RT-PCR.

(D) NOD-*scid IL2rγ*^{null} HLA A2.1 mice were injected with treated CD34⁺ HSCs and allowed to engraft for 12 weeks. CD4-positive cells were isolated from the spleen for analysis.

(E) FACS plots showing the engraftment of modified CD34⁺ cells in NOD-*scid IL2rγ*^{null} HLA A2.1 mice. Presence of cells expressing human CD45 and CD20 indicate engraftment. Representative results from one of two mice are shown.

(F) Identification of targeted modification of the *CCR5* gene in cells isolated from the spleen of mice that were injected with human CD34⁺ cells transfected with tcPNA-679 plus donor 597 and donor 591. Genomic DNA was harvested from spleen four months posttransplant. Quantitative AS-PCR was performed with both 591- and 597-specific primers. Samples include spleen from a control mouse, spleen from a mouse injected with unmodified CD34⁺ cells, and spleen from a mouse injected with CD34⁺ cells transfected with tcPNA-679 plus donor DNAs. Similar results were seen in other transplanted mice.

(G) Quantitative AS-PCR was performed with both 591- and 597-specific primers (gray and black, respectively) on genomic DNA prepared from CD4⁺ T cells isolated from a transplanted mouse and a control mouse.

See also Figure S3.

capability and still allows for multilineage repopulation, establishing that this approach to targeted gene modification could provide the basis for a clinical therapy.

DISCUSSION

The results presented here show that PNAs capable of forming site-specific PNA/DNA/PNA triple helices can stimulate recombination in the endogenous *CCR5* locus in human cells. Treatment of cells with a *CCR5*-targeted tcPNA plus 60 nt donor DNA molecules introduced stop codons into the *CCR5* gene, mimicking the naturally occurring *CCR5*-delta32 mutation. Experiments in THP-1 cells confirmed targeted modification at the DNA, RNA, and protein levels and showed persistence of the targeted mutation in pooled cell populations for more than three months in culture. By isolating individual clones from

treated cell populations, we were able to directly quantify the frequency of genomic modification, obtaining a targeting frequency of approximately 2.46%. Direct sequencing of a closely related gene, the *CCR2* gene, showed no modifications in the 1740 cells analyzed, yielding an upper limit for the off-target frequency in this highly homologous gene of less than 0.057%. Functionally, we found that targeted modification of the *CCR5* gene in THP-1 cells led not only to decreased cell surface expression of the protein but also to a substantial decrease in infectibility by R5-tropic HIV-1.

The application of tcPNAs as reported here represents their first successful use for targeted gene modification in human cells. Prior studies of tcPNAs highlighted their potential but were focused on in vitro binding properties (Bentin et al., 2003). Our results with the lysine-conjugated tcPNAs used here demonstrate that this specialized PNA design can have a robust effect leading to increased specificity, binding, and targeting frequency. We had initially sought to use DNA-based triple-forming oligonucleotide (TFOs) for this work, because more than 10 years ago Belousov et al. reported the use of a chlorambucil-conjugated DNA TFO for targeted binding to the *CCR5* gene (Belousov et al., 1998). However, this was accomplished only in detergent permeabilized and consequently dead cells. We followed up on this work, but we were not able to develop any DNA or RNA-based TFOs to bind to the short polypurine region in *CCR5* in live cells even with the incorporation of several strategic base substitutions. The tcPNAs therefore represent a major step forward in triplex technology for gene targeting in human cells.

The targeting frequency of 2.46% in a single treatment may already be sufficient for therapeutic application because CCR5-deficient cells are known to have a selective advantage in the face of HIV infection (Hütter et al., 2009; Kumar et al., 2008; Perez et al., 2008). However, we expect that further increases in modification frequency may be possible. We achieved a 5-fold increase by use of the tail-clamp PNAs and we will continue to optimize PNA design to determine whether further increases can be achieved. In addition, we are testing treatment of the cells with chloroquine as a lysosome-disrupting agent to enhance PNA bioavailabilty, SAHA (suberoylanilide hydroxamic acid) as a histone deacetylase inhibitor to improve chromosome accessibility, and encapsulation of PNAs and DNAs in PLGA nanoparticles to increase transfection efficiency. Early results suggest that these treatments may have positive effects (Chin et al., 2008; Lonkar et al., 2009).

In addition, we have shown that PNA and donor DNA transfection can induce targeted gene modification in primary human $CD34^+$ HSCs and that this modification is increased 90-fold with the addition of PNA to the donor DNA. Modified HSCs are also capable of engrafting in NOD-*scid IL2r*γ^{null} HLA A2.1 mice and upon engraftment can produce multiple lineages of hematopoietic cells, including $CD4^+$ cells, which have the targeted modification. These experiments establish proof of principle for the use of triplex-mediated gene targeting as a method to create modified human hematopoietic progenitor cells as a potential renewable source of HIV-1-resistant blood cell lineages.

Other strategies for modifying immune cells to resist HIV-1 include *CCR5*-directed ribozymes or siRNAs directed against HIV-1-encoded RNAs. These are promising approaches but they require continual long-term expression for activity (Akkina et al., 2003; Amado et al., 2004; Anderson and Akkina, 2005). The use of zinc-finger nucleases to induce mutagenic double-strand breaks in the *CCR5* gene in $CD4^+$ T cells has been reported (Perez et al., 2008). However, expression of zinc-finger nucleases requires complex viral vectors to introduce large plasmid constructs, and the safety profile of such nucleases is unknown. Importantly, in the work of Perez et al. (Perez et al., 2008), using *CCR5*-targeted zinc finger nucleases, off-target modification the *CCR2* gene was determined to occur at a frequency of 5.39%, only 6-fold less than their reported frequency of *CCR5* modification (35.6%). Our off-target frequency is at least 43-fold less than our frequency to target *CCR5* (2.46%), showing the superior specificity of the PNA-mediated gene targeting strategy. In a direct comparison, the off-target frequency in *CCR2* in our PNA-mediated approach, with an upper limit of 0.057%, is at least two orders of magnitude less than that produced by zinc-finger nucleases (Perez et al., 2008). In fact, the use of triplex-forming DNA or PNA oligonucleotides has not yielded detectable mutagenesis in off-target reporter genes such as *cII* or *HPRT* in analyses of more than one million alleles (Rogers et al., 2004; Vasquez et al., 2000), suggesting that the off-target effects are probably substantially lower than the 0.057% upper limit calculated here. We expect that this value will likely decrease as we sequence more alleles. Hence, although PNA mediated targeting is about 10-fold less efficient than by use of zinc-fingers, the off-target effects are greatly reduced. Because zinc-finger nuclease-induced double-strand breaks, leading to an unpredictable mixture of mutations and chromosomal translocations, there is a risk of induced leukemias and other deleterious events. PNA-mediated approaches may be advantageous in this regard and may ultimately carry much less risk of untoward side effects in patients.

Furthermore, the lack of off-target effects in *CCR2* in spite of partial homology to the tcPNA-679 binding site likely reflects the mechanism of action of triplex-forming molecules for gene modification. The capacity of triplexes to stimulate recombination comes from the ability of such altered helical structures to provoke DNA repair via the nucleotide excision repair pathway (Chin et al., 2008; Datta et al., 2001; Rogers et al., 2002; Wang et al., 1996). To stimulate DNA repair, the PNA or DNA oligonucleotides must bind extremely tightly to the DNA. Mismatches can reduce the binding enough so that the ability to induce recombination is greatly diminished (Knauert et al., 2005). With reduced affinity, such loose binding oligomers—even if they do bind—can be easily removed from the DNA by helicases. Importantly, the PNAs have no intrinsic nuclease activity themselves (unlike zinc finger nucleases), and so they must bind tightly to their target to have any effect by triggering DNA repair. In light of this mechanism, it is therefore not surprising that there are very low off-target effects at mismatched sites, even if they are only partially mismatched.

The PNA-based approach described here uses chemically synthesized oligonucleotides, a class of agents that has been extensively studied in clinical trials with an overall favorable safety profile (Vasquez et al., 2000; Vasquez et al., 1999). Plus, the PNA-induced modification in *CCR5* is templated by the donor DNAs, leading to a precisely prescribed sequence change. As such, the work reported here establishes proof of

principle that PNA-induced, sequence-specific modification of *CCR5* can be achieved in primary human CD34$^+$ HSCs as a means to provide a long-term source of cell lineages, constituting a potential therapy for HIV-1 infection. The work presented here could also be used for other genetic diseases, allowing for precise genomic modifications to correct or introduce mutations in targeted genes for both research and gene therapy.

SIGNIFICANCE

CCR5 encodes a chemokine receptor required for HIV-1 entry into human cells, and individuals carrying a naturally occurring 32bp deletion mutation in this gene are resistant to HIV-1 infection, thus making _CCR5_ an attractive target for gene therapy. Bifunctional triplex-forming peptide nucleic acids (PNAs) that consist of linked Hoogsteen and Watson-Crick binding domains can form PNA/DNA/PNA triplexes. These triplexes can induce DNA repair and stimulate recombination of target genes with donor DNAs in human cells. _CCR5_-targeted PNAs were optimized to contain a "tail" on the Watson-Crick domain to provide increased duplex invasion and greater binding specificity, yielding a substantial increase in targeting efficiency in human cells. Site-specific disruption of the _CCR5_ gene was achieved in 2.46% of human cells in a single treatment, a frequency estimated to be clinically relevant. The targeted modification was designed to mimic the naturally occurring _CCR5_-delta32 mutation and thus render the cells resistant to HIV-1 infection. This was confirmed by direct challenge of PNA-modified human cells with live virus, thereby directly establishing that this strategy inhibits HIV-1 infection of human cells.

This work describes an advancement in triplex technology for intracellular gene targeting and presents the basis for its use as a therapeutic strategy for AIDS: triplex-mediated, permanent knockout, by sequence-specific genomic modification, of the _CCR5_ gene in hematopoietic stem cells (HSCs) to create a reservoir of _CCR5_-mutated HSCs. Such cells can give rise to multiple HIV-1-resistant cell lineages and thereby preserve immune system function in HIV-1-infected individuals.

This work may also lead to greater adoption of this approach as a platform technology for site-specific manipulation of the genome for both research and therapeutic applications.

EXPERIMENTAL PROCEDURES

PNAs and Oligonucleotides

tcPNA-679 (N- Lys-Lys-Lys-JTJTTJTTJT-OOO-TCTTCTTCTCATTTC-Lys-Lys-Lys-C) was synthesized by Bio-Synthesis Inc. (Lewisville, TX) and purified by reverse phase-high performance liquid chromatography (RP-HPLC). Three lysines were conjugated to both the N- and C-terminal ends of the PNA for increased bioactivity and 8-amino-2,6-dioxaoctanoic acid linkers were used as the flexible linker "O." DNA oligonucleotides were synthesized by the Midland Certified Reagent Company Inc. (Midland, TX) and purified by RP-HPLC. The sequences of the DNA oligonucleotides are (mutation sites in bold) donor 591: 5′ AT TCC CGA GTA GCA GAT GAC CAT GAC AG**C TTA G**GG CAG GAC CAG CCC CAA GAT GAC TAT C 3′ and donor 597: 5′ TT TAG GAT TCC CGA GTA GCA GAT GAC C**CC TCA G**AG CAG CGG CAG GAC CAG CCC CAA GAT G 3′. All oligonucleotides were 5′ and 3′ end-protected with three phosphorothioate internucleoside linkages.

Electrophoretic Mobility Shift Assays

Binding of PNAs to the *CCR5* target site was determined by gel mobility shift assays using a plasmid containing the *CCR5* or the *CCR2* target site as a substrate, as described (Kim et al., 2006).

Cell Culture

THP-1 and K562 cells were maintained in RPMI medium supplemented with 10% FBS and L-Glutamine (GIBCO; Invitrogen, Carlsbad, CA). Human CD34$^+$ stem cells were isolated from apheresis of granulocyte colony stimulating-factor (G-CSF)-mobilized peripheral blood from healthy donors and then selected using a Baxter 300i Isolex Device and cryopreserved (Yale Center of Excellence in Molecular Hematology). Cells were thawed and maintained in StemSpan Serum-Free Expansion Media supplemented with StemSpan CC110 cytokine mixture (100 ng/ml rh Flt-3 Ligand, 100 ng/ml rh Stem Cell Factor, 20 ng/ml rh IL-3, 20 ng/ml rh IL-6, StemCell Technologies Inc., Vancouver, BC, Canada). THP-1 differentiation was induced by treatment with phorbol 12-myristate 13-acetate (PMA) (Sigma, St. Louis, MO) at 50 ng/ml.

Electroporation of Molecules

THP-1 and K562 cells were electroporated at 350V, 12 ms, 1 pulse using a BTX Electo Square Porator ECM 830 (Harvard Apparatus, Holliston, MA) in 100 μl phosphate-buffered saline (PBS) at indicated concentrations. After electroporation, cells were transferred to plates containing RPMI medium with 10% FBS and L-Glutamine. Human CD34$^+$ stem cells were thawed in media 24 hr before transfection. Nucleofection was performed using an Amaxa Human CD34 Cell Nucleofector Kit according to the manufacturer's protocol (Lonza Group, Basel, Switzerland). Cells were then plated in media, and 24 or 48 hr postnucleofection, cells were collected by centrifugation for genomic DNA or RNA isolation.

Genomic DNA/RNA Isolation

Genomic DNA was isolated from tissue culture samples using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI); from THP-1 cells in 96-well plates, the Wizard SV 96 Genomic DNA Purification System was used. RNA was isolated using the Absolutely RNA Miniprep Kit (Stratagene; Agilent Technologies, Santa Clara, CA). Synthesis of cDNA from total RNA samples was performed using the SuperScript First-Strand Synthesis System (Invitrogen).

Allele-Specific PCR

Primers were designed to amplify a 400 bp region in *CCR5*. The allele-specific forward primer was designed to contain the specific 6 bp mutations at the 3′ end, while the WT forward primer contained the WT *CCR5* sequence at the same position. Primers were synthesized by the W.M. Keck Facility at Yale University. Primer sequences and PCR conditions are available upon request. PCR products were separated on a 1% agarose gel and visualized using a gel imager. WT forward primers paired with the universal reverse primer were used as a loading control. 3.2 μM donor 597 was added to an aliquot of untreated THP-1 genomic DNA with or without 6μM tcPNA-679. AS-PCR was performed using untreated genomic DNA with or without PNA and donor to test the ability of the donor DNA to act as a primer or a template in the reaction.

Infectibility

THP-1 cells were induced to differentiate into adherent macrophage-like cells by treatment with PMA. Cells were plated at 2 × 10^5 cells/well in 96 well plates and treated with 50 ng/ml PMA for 48 hr at 37°C and washed thoroughly. Seven days after differentiation, PMA-treated THP-1 (THP-1/PMA) cells were exposed to HIV-1$_{BaL}$ at an MOI of 1. After a 4 hr incubation at 37°C with the virus, cells were washed three times and cultured in 10% RPMI medium. Infection was monitored by the viral p24 level in harvested culture supernatants (harvested on days 4, 6, 8, 10, and 12 postinfection) using ELISA plates obtained from PerkinElmer Life Sciences (Waltham, MA). Results are expressed as the mean and standard deviation (SD) of duplicate determinations from two wells. To calculate moi, HIV$_{BaL}$ was titrated on 1 × 10^5 primary

human monocyte–derived macrophages seeded in 96 well plates, and intracellular p24 antigen was measured by flow cytometry. Based on this, 100 ng p24 gag antigen equivalents per well was determined to be the $TCID_{50}$ (moi = 0.5).

Mouse Transplantation

All animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School and The Jackson Laboratory, and conformed to the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources et al., 1996). Targeted or untreated human $CD34^+$ cells were resuspended in RPMI medium supplemented with 1% fetal calf serum. NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/Sz Tg(HLA-A2/H2-D/B2m)1Dvs/Sz (NOD-*scid IL2r*γ^{null} HLA-A2.1) mice have been described previously and were obtained from the research colony maintained by L.D.S. at The Jackson Laboratory (Jaiswal et al., 2009). 4×10^5 $CD34^+$ cells in 50 μl were injected into two newborn NOD-*scid IL2r*γ^{null} HLA A2.1 mice by intracardiac injection. Twelve weeks postinjection mice were bled and analyzed by FACS to determine engraftment of human hematopoietic cells. Mice were sacrificed, and various tissues were harvested and flash frozen. CD4-positive cells were isolated from one fresh spleen sample from a PNA plus donors-treated mouse using BD IMag anti-human CD4 particles (BD Biosciences, Franklin Lakes, NJ). Genomic DNA was isolated from tissues by phenol/chloroform extraction and analyzed by quantitative real-time AS-PCR.

Quantitative Real-Time AS-PCR

Quantitative PCR was performed using Brilliant SYBR Green qPCR reagents on the Mx3000p real-time PCR system (Stratagene). Fluorescence intensity was monitored in real time, and cycle threshold cycles (CTs) were calculated based on dRn fluorescence with an adaptive baseline using the software supplied with the MX3000p. Comparative quantification was performed by comparing the CTs obtained from amplification of *CCR5* to those observed using gene-specific primers (forward: 5′ ACCTTTGGGGTGGTGACAAGTGTG 3′; reverse: 5′ TCTCCCCGACAAAGGCATAGATGA 3′) as a normalizer. Relative mutant *CCR5* abundance was calculated using the -ΔΔCT method (Stratagene). All assays were preformed in duplicate.

Set ratios of genomic DNA from WT and 597 heterozygous cells were mixed to create a standard curve to quantify targeting frequencies in treated cells. Standard curve samples were run in duplicate along with genomic DNAs from treated cells of unknown targeting frequency. Using the -ΔΔCT method (Livak and Schmittgen, 2001), values were obtained and plotted to create a standard curve. The equation for this curve was then used to calculate the targeting frequency of the unknown samples.

AS-RT-PCR

Primers were designed to amplify a 667 bp region in *CCR5*. The AS reverse primer was designed to contain the specific 6 bp mutation at the 3′ end, whereas the WT reverse primer contained the WT *CCR5* sequence. Forward primers were designed to bind in exon 2 with the reverse primer binding within exon 3, allowing for specific identification of cDNA as opposed to genomic DNA-amplified products. Primer sequences and PCR conditions available are upon request. The PCR products were separated on a 1% agarose gel and visualized using a gel imager. The forward primer paired with the WT reverse primer was used as a loading control.

Taqman Assay for RNA Induction

To test *CCR5* mRNA expression, cDNA was prepared from total RNA samples from PMA induced and uninduced THP-1 cells. cDNA was used in PCR reactions containing Taqman Universal master mix, premixed Taqman probes, and reference dye (Applied Biosystems, Life Technologies, Carlsbad, CA) in a final volume of 25 μl. Data were analyzed as previously described (Bindra et al., 2005; Bindra and Glazer, 2007). The amplification scheme was: 50°C for 2 min, 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min for 45 cycles preformed using the Mx3000p RT-PCR system (Stratagene). Fluorescence intensity was monitored in real time, and CTs were calculated based on dRn fluorescence with an adaptive baseline using the software supplied with the MX3000p. Comparative quantification was performed by comparing the CTs obtained from amplification of *CCR5* with those observed for 18S rRNA as a normalizer. Relative mRNA abundance was calculated using the -ΔΔCT method (Stratagene). *CCR5* probes were FAM labeled, and the 18S probe was MGB-labeled (obtained from Applied Biosystems). All assays were performed in duplicate.

Cell Surface Staining

To test for the presence of CCR5 at the cell surface, 2×10^6 cells were incubated with PMA dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 50 ng/ml in culture medium, or mock treated with DMSO alone for 48 hr. Cells were then collected, washed in PBS, and then washed in CCR5 staining buffer (2% FBS-certified [GIBCO], 1% sodium azide [10% w/v solution], and PBS [GIBCO]). Cell pellets were then resuspended in CCR5 staining buffer and functional grade purified anti-mouse CD16/32 (eBioscience, Inc., San Diego, CA) was added to THP-1 cells for blocking. Cells were allowed to incubate at room temperature for 10 min before being split into fresh tubes containing either a PE-conjugated anti-hCCR5 or Mouse IgG_1 Isotype Control antibody (R&D Systems, Inc., Minneapolis, MN) and kept on ice for 20 min. Cells were then pelleted, and the supernatant was removed. 500 μl of CCR5 staining buffer was used to wash the cells before they were resuspended in PBS for FACS. Samples were analyzed on a FACSCalibur with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

Sequence Analysis

Gene-specific primers (forward: 5′ ACCTTTGGGGTGGTGACAAGTGTG 3′ and reverse: 5′ TCTCCCCGACAAAGGCATAGATGA3′) were used to amplify a 400 bp region of the *CCR5* locus. PCR products were gel-purified using the QIAquick gel purification kit (QIAGEN, Venlo, The Netherlands). Purified PCR products were sequenced by the W.M. Keck Facility at Yale University. A 420 bp region of the *CCR2* gene encompassing the most homologous region to *CCR5* (FASTA 4201-4621) was amplified by PCR (forward: 5′ CCTGGCTATTGTCCATGCTG 3′; reverse: 5′ CTTTCACAGTTACTCAGGCCG 3′). The resulting PCR products were sequenced by the W.M. Keck Facility at Yale University.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.chembiol.2011.07.010.

ACKNOWLEDGMENTS

We thank L. Cabral, B. Johnson, and D. Hegan for their help. This work was supported by National Institutes of Health Grant R01HL082655 (to P.M.G.) and by the George R. Pfeiffer Fellowship (to E.B.S.).

Received: November 29, 2010
Revised: July 6, 2011
Accepted: July 8, 2011
Published: September 22, 2011

REFERENCES

Akkina, R., Banerjea, A., Bai, J., Anderson, J., Li, M.J., and Rossi, J. (2003). siRNAs, ribozymes and RNA decoys in modeling stem cell-based gene therapy for HIV/AIDS. Anticancer Res. *23* (3A), 1997–2005.

Allain, J.P., Laurian, Y., Paul, D.A., Verroust, F., Leuther, M., Gazengel, C., Senn, D., Larrieu, M.J., and Bosser, C. (1987). Long-term evaluation of HIV antigen and antibodies to p24 and gp41 in patients with hemophilia. Potential clinical importance. N. Engl. J. Med. *317*, 1114–1121.

Amado, R.G., Mitsuyasu, R.T., Rosenblatt, J.D., Ngok, F.K., Bakker, A., Cole, S., Chorn, N., Lin, L.S., Bristol, G., Boyd, M.P., et al. (2004). Anti-human immunodeficiency virus hematopoietic progenitor cell-delivered ribozyme in a phase I study: myeloid and lymphoid reconstitution in human immunodeficiency virus type-1-infected patients. Hum. Gene Ther. *15*, 251–262.

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

Belousov, E.S., Afonina, I.A., Kutyavin, I.V., Gall, A.A., Reed, M.W., Gamper, H.B., Wydro, R.M., and Meyer, R.B. (1998). Triplex targeting of a native gene in permeabilized intact cells: covalent modification of the gene for the chemokine receptor CCR5. Nucleic Acids Res. *26*, 1324–1328.

Bentin, T., Larsen, H.J., and Nielsen, P.E. (2003). Combined triplex/duplex invasion of double-stranded DNA by "tail-clamp" peptide nucleic acid. Biochemistry *42*, 13987–13995.

Bindra, R.S., and Glazer, P.M. (2007). Co-repression of mismatch repair gene expression by hypoxia in cancer cells: role of the Myc/Max network. Cancer Lett. *252*, 93–103.

Bindra, R.S., Gibson, S.L., Meng, A., Westermark, U., Jasin, M., Pierce, A.J., Bristow, R.G., Classon, M.K., and Glazer, P.M. (2005). Hypoxia-induced down-regulation of BRCA1 expression by E2Fs. Cancer Res. *65*, 11597–11604.

Chin, J.Y., Kuan, J.Y., Lonkar, P.S., Krause, D.S., Seidman, M.M., Peterson, K.R., Nielsen, P.E., Kole, R., and Glazer, P.M. (2008). Correction of a splice-site mutation in the beta-globin gene stimulated by triplex-forming peptide nucleic acids. Proc. Natl. Acad. Sci. USA *105*, 13514–13519.

Datta, H.J., Chan, P.P., Vasquez, K.M., Gupta, R.C., and Glazer, P.M. (2001). Triplex-induced recombination in human cell-free extracts. Dependence on XPA and HsRad51. J. Biol. Chem. *276*, 18018–18023.

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S.M., Driver, D.A., Berg, R.H., Kim, S.K., Norden, B., and Nielsen, P.E. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature *365*, 566–568.

Goncz, K.K., Prokopishyn, N.L., Abdolmohammadi, A., Bedayat, B., Maurisse, R., Davis, B.R., and Gruenert, D.C. (2006). Small fragment homologous replacement-mediated modification of genomic beta-globin sequences in human hematopoietic stem/progenitor cells. Oligonucleotides *16*, 213–224.

Holt, N., Wang, J., Kim, K., Friedman, G., Wang, X., Taupin, V., Crooks, G.M., Kohn, D.B., Gregory, P.D., Holmes, M.C., and Cannon, P.M. (2010). Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted to CCR5 control HIV-1 in vivo. Nat. Biotechnol. *28*, 839–847.

Hütter, G., Nowak, D., Mossner, M., Ganepola, S., Müssig, A., Allers, K., Schneider, T., Hofmann, J., Kücherer, C., Blau, O., et al. (2009). Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. N. Engl. J. Med. *360*, 692–698.

Institute of Laboratory Animal Resources; Commission on Life Sciences; and National Research Council. (1996). Guide for the care and use of laboratory animals (Washington, D.C.: National Academy Press).

Jagodzinski, P.P., Wierzbicki, A., Wustner, J., Kaneko, Y., and Kozbor, D. (1999). Enhanced human immunodeficiency virus infection in macrophages by high-molecular-weight dextran sulfate is associated with conformational changes of gp120 and expression of the CCR5 receptor. Viral Immunol. *12*, 23–33.

Jaiswal, S., Pearson, T., Friberg, H., Shultz, L.D., Greiner, D.L., Rothman, A.L., and Mathew, A. (2009). Dengue virus infection and virus-specific HLA-A2 restricted immune responses in humanized NOD-*scid* IL2rgamma^{null} mice. PLoS ONE *4*, e7251.

Kaihatsu, K., Shah, R.H., Zhao, X., and Corey, D.R. (2003). Extending recognition by peptide nucleic acids (PNAs): binding to duplex DNA and inhibition of transcription by tail-clamp PNA-peptide conjugates. Biochemistry *42*, 13996–14003.

Kim, K.H., Nielsen, P.E., and Glazer, P.M. (2006). Site-specific gene modification by PNAs conjugated to psoralen. Biochemistry *45*, 314–323.

Knauert, M.P., Lloyd, J.A., Rogers, F.A., Datta, H.J., Bennett, M.L., Weeks, D.L., and Glazer, P.M. (2005). Distance and affinity dependence of triplex-induced recombination. Biochemistry *44*, 3856–3864.

Knauert, M.P., Kalish, J.M., Hegan, D.C., and Glazer, P.M. (2006). Triplex-stimulated intermolecular recombination at a single-copy genomic target. Mol. Ther. *14*, 392–400.

Konopka, K., and Düzgüneş, N. (2002). Expression of CD4 controls the susceptibility of THP-1 cells to infection by R5 and X4 HIV type 1 isolates. AIDS Res. Hum. Retroviruses *18*, 123–131.

Kumar, P., Ban, H.S., Kim, S.S., Wu, H., Pearson, T., Greiner, D.L., Laouar, A., Yao, J., Haridas, V., Habiro, K., et al. (2008). T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. Cell *134*, 577–586.

Liu, R., Paxton, W.A., Choe, S., Ceradini, D., Martin, S.R., Horuk, R., MacDonald, M.E., Stuhlmann, H., Koup, R.A., and Landau, N.R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell *86*, 367–377.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Lonkar, P., Kim, K.H., Kuan, J.Y., Chin, J.Y., Rogers, F.A., Knauert, M.P., Kole, R., Nielsen, P.E., and Glazer, P.M. (2009). Targeted correction of a thalassemia-associated beta-globin mutation induced by pseudo-complementary peptide nucleic acids. Nucleic Acids Res. *37*, 3635–3644.

Perez, E.E., Wang, J., Miller, J.C., Jouvenot, Y., Kim, K.A., Liu, O., Wang, N., Lee, G., Bartsevich, V.V., Lee, Y.L., et al. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat. Biotechnol. *26*, 808–816.

Pierce, E.A., Liu, Q., Igoucheva, O., Omarrudin, R., Ma, H., Diamond, S.L., and Yoon, K. (2003). Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells. Gene Ther. *10*, 24–33.

Rogers, F.A., Vasquez, K.M., Egholm, M., and Glazer, P.M. (2002). Site-directed recombination via bifunctional PNA-DNA conjugates. Proc. Natl. Acad. Sci. USA *99*, 16695–16700.

Rogers, F.A., Manoharan, M., Rabinovitch, P., Ward, D.C., and Glazer, P.M. (2004). Peptide conjugates for chromosomal gene targeting by triplex-forming oligonucleotides. Nucleic Acids Res. *32*, 6595–6604.

Samson, M., Libert, F., Doranz, B.J., Rucker, J., Liesnard, C., Farber, C.M., Saragosti, S., Lapoumeroulie, C., Cognaux, J., Forceille, C., et al. (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature *382*, 722–725.

Shen, H., Cheng, T., Preffer, F.I., Dombkowski, D., Tomasson, M.H., Golan, D.E., Yang, O., Hofmann, W., Sodroski, J.G., Luster, A.D., and Scadden, D.T. (1999). Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. J. Virol. *73*, 728–737.

Tsibris, A.M., and Kuritzkes, D.R. (2007). Chemokine antagonists as therapeutics: focus on HIV-1. Annu. Rev. Med. *58*, 445–459.

Vasquez, K.M., Wang, G., Havre, P.A., and Glazer, P.M. (1999). Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells. Nucleic Acids Res. *27*, 1176–1181.

Vasquez, K.M., Narayanan, L., and Glazer, P.M. (2000). Specific mutations induced by triplex-forming oligonucleotides in mice. Science *290*, 530–533.

Wang, G., Seidman, M.M., and Glazer, P.M. (1996). Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. Science *271*, 802–805.