CCR5 Gene Editing of Resting CD4+ T Cells by Transient ZFN Expression From HIV Envelope Pseudotyped Nonintegrating Lentivirus Confers HIV-1 Resistance in Humanized Mice

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CCR5 disruption by zinc finger nucleases (ZFNs) is a promising method for HIV-1 gene therapy. However, successful clinical translation of this strategy necessitates the development of a safe and effective method for delivery into relevant cells. We used non-integrating lentivirus (NILV) for transient expression of ZFNs and pseudotyped the virus with HIV-envelope for targeted delivery to CD4+ T cells. Both activated and resting primary CD4+ T cells transduced with CCR5-ZFNs NILV showed resistance to HIV-1 infection in vitro. Furthermore, NILV transduced resting CD4+ T cells from HIV-1 seronegative individuals were resistant to HIV-1 challenge when reconstituted into NOD-scid IL2rγc null (NSG) mice. Likewise, endogenous virus replication was suppressed in NSG mice reconstituted with CCR5-ZFN–transduced resting CD4+ T cells from treatment naïve as well as ART-treated HIV-1 seropositive patients. Taken together, NILV pseudotyped with HIV envelope provides a simple and clinically viable strategy for HIV-1 gene therapy.

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Subject Category: Therapeutic proof-of-concept Gene insertion, deletion & modification

Introduction

HIV-1 infection still remains a global epidemic with significant morbidity and mortality. Although anti-retroviral therapy (ART) has extended the lives of HIV-1-infected individuals, it has several limitations including the need for life long therapy, high cost and side effects associated with the drugs.1 Moreover, the virus is not completely eradicated with this approach and rebounds rapidly upon interruption of ART.2,3 Hence, there has been interest in pursuing alternative strategies that are aimed at rendering cells intrinsically resistant to HIV-1.

Zinc finger nucleases (ZFNs) have been widely used for genome editing in various types of cells and living organisms4–10 and is also a highly promising strategy for HIV-1 therapy aimed at disrupting specific host genes important for virus replication,11–18 or to eliminate provirus in latently infected cells.19 There has been a renewed interest in CCR5 disruption as a strategy for HIV-1 gene therapy because of the so-called “Berlin Patient”, who was completely cured of HIV-1 infection after receiving bone marrow stem cells transplanted from a donor homozygous for the Δ32 mutation (a 32-bp deletion in the single coding exon of the gene) in the CCR5 gene which leads to blockade of HIV-1 entry via this co-receptor.20,21 Unlike conventional gene knockdown by RNAi, which requires the continued presence of the effector siRNA moiety for silencing to be maintained, transient ZFN expression results in permanent and heritable gene editing so that the CCR5-modified cells can confer long-term HIV-1 resistance in patients. CCR5 disruption mediated by ZFNs has been tested in primary CD4+ T cells as well as in CD34+ stem cells.11–18 Reconstitution of NSG mice with activated CD4+ T cells transduced with adenovirus encoding CCR5-ZFNs resulted in resistance to HIV-1 challenge.22 Promising new data have been reported from one of the ongoing clinical trials in which CCR5 gene modified autologous CD4+ T cells were used for adoptive therapy in HIV infected subjects.23 The therapy was found to be safe and well tolerated. Moreover, increased levels of CD4+ T cells were seen following treatment interruption, which attest to the feasibility of the approach. However, large-scale expansion of gene-edited cells for personal therapy is labor intensive and requires a specialized laboratory set up. Alternative simplified regimens may facilitate the dissemination of the therapeutic approach for large-scale clinical application.

Lentiviral vectors are among the most efficient gene delivery vehicles for primary cells, including T cells, making them attractive vectors for ZFN delivery for HIV-1 therapy. The third generation vectors currently used in gene therapy trials have undergone extensive modifications to ensure safety.24–26 Nevertheless, insertional mutagenesis could pose a potential risk for gene therapy with integrating lentiviral vectors.27,28 On the other hand, non-integrating lentivirus (NILV) generated by packaging vectors with a mutated integrase gene (NILV) generated by packaging vectors with a mutated integrase gene minimizes vector integration into the host genome, which makes them ideal for gene editing applications, where only transient expression of the transgene is needed.29 Indeed, CCR5

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disruption using a non-integrating lentiviral vector in human stem cells has been attempted in vitro, but the applicability of the approach for selective delivery to primary CD4+ T cells for inducing HIV-1 resistance remains untested.

In this work, we tested transduction efficiency and CCR5 disruption frequency after ZFN delivery to cell lines and primary CD4+ T cells via a non-integrating lentiviral vector. In previous studies using adenoviral-based transduction, activated CD4+ T cells were gene-modified and expanded in vitro before transfection. However, in addition to being time-consuming and expensive, extended culture after T-cell activation may result in an altered cell surface phenotype and exhaustion of the cells, which may have an impact on the efficiency of gene therapy in the long-term. Although conventional adenoviral and lentiviral vectors efficiently transduce a multitude of cell types, including primary cells, they are inefficient in transducing resting T cells. Here, we used NILV pseudotyped with HIV-1 envelope to enable transduction of unstimulated CD4+ T cells from healthy or HIV-infected donors. This allowed selective transduction of the cells within unfractonated PBMCs, which could then be infected into NSG mice (Hu/PBL model) without further in vitro manipulation and subsequent reconstitution and resistance to HIV-1 infection. These efforts are anticipated to lead to the development of a simple approach for generating HIV-resistant cells for cellular therapy in HIV-1 infected patients.

RESULTS
Construction and characterization of non-integrating CCR5-ZFN encoding lentivirus
Non-integrating lentivirus has been demonstrated to successfully infect various types of cell lines, as well as stem cells without integration of viral DNA into the cellular genome. For nucleolytic activity, two arms of zinc finger nucleases are designed to bind to 9–18 nucleotides on the left and right hand side of the targeted gene on the sense and antisense strands respectively. To improve the disruption efficiency, we expressed the ZFN pair targeting a specific site within the CCR5 gene in a single construct by connecting them via a FMDV 2A sequence (Supplementary Figure S1a, for the ZFN protein sequence see Supplementary Figure S1c). The sequence was cloned into the lentiviral vector, pLVX-ZsGreen (a bicistronic IRES vector to allow simultaneous expression of ZFNs and ZsGreen from the same RNA transcript, enabling easy confirmation of ZFNs expression by monitoring ZsGreen expression) under the CMV promoter to produce a fusion protein containing both arms (Supplementary Figure S1b). However, this fusion protein is expected to be cleaved soon after translation, because of self-cleavage activity of the 2A sequence, yielding two functional units that simultaneously target their respective DNA binding sites. To derive the NILV, the ZFN vector was co-transfected with a packaging plasmid and an envelope (either VSV-G or HIV-1 env) encoding plasmid into 293 T cells and the supernatants collected after 48 hours of culture. In order to achieve transient ZFN expression, we used an intergrase-defective packaging plasmid, vTK939, to package the lentivirus. In vitro and in vivo studies have demonstrated that the resulting lentivirus shows much lower levels of illegitimate integration. We first tested the transduction efficiency of the VSV-G pseudotyped non-integrating vector compared with the integrating lentiviral vector in HEK 293 T cells. The results show that 48 hours after lentiviral infection, ZsGreen expression was comparable between the two vectors (Supplementary Figure S1d).

In silico genome-wide analysis with PROGNOS software for potential off target gene modification by the ZFN sequences used in this study (obtained from Sigma-Aldrich, St Louis, MO) did not reveal any off-target sites when two mismatches per ZFN half-site were allowed. However, deep sequencing data with K562 cells treated with the same ZFN pair by another group revealed no detectable off-target activity.

We next evaluated the efficiency of CCR5 disruption after NILV-mediated ZFN delivery in 293T cells. CCR5 disruption was quantified by PCR amplification across the CCR5 locus, heating and re-annealing of PCR products, and digestion with T7 endonuclease, which is able to cleave mismatched dsDNA caused by non-homologous end joining (NHEJ). At an MOI of 30 (60 ng of p24 for 1 x 10⁶ cells), the transduction efficiency was 63.8%, for HEK293T cells, with a corresponding CCR5 disruption frequency of 60% (Figure 1a).

HIV envelope pseudotyped NILV can deliver ZFNs to resting and activated CD4+ T cells to efficiently disrupt the CCR5 gene
Findings from multiple studies indicate poor transduction of unstimulated but not stimulated CD4+ T cells with VSV-G pseudotyped lentiviral particles. As CXCR-4 tropic HIV envelope pseudotyping has been shown to allow efficient lentiviral transduction of quiescent CD4+ T cells, we used this approach for NILV-mediated delivery of ZFNs to HIV-1-susceptible resting and activated CD4+ T cells. We first tested whether the HIV envelope pseudotyped NILV could deliver ZFNs to CD4 expressing Jurkat cells. As shown in Figure 1b, at an MOI of 30 (60 ng of p24 for 1 x 10⁶ cells), the transduction efficiency was 39.1% with a corresponding CCR5 disruption frequency of 34%.

We next compared NILV transduction in resting and activated primary T cells with conventional VSV-G or HIV env pseudotyped NILV, using ZsGreen to track expression. In agreement with previous data, we found equivalent ZsGreen expression with both HIV env and VSV-G in CD4+ T cells activated with phytohemagglutinin (PHA) prior to transduction. On the other hand, in unstimulated CD4+ T cells, there was negligible ZsGreen expression with VSV-G, whereas with HIV-env pseudotyped NILV the expression level was comparable to that in activated T cells (Supplementary Figure S4).
We also evaluated whether HIV env pseudotyping could be used for selective ZFN delivery to CD4+ T cells within a mixed PBMC population. PBMCs isolated from a normal donor were transduced with NILV at an MOI of 15. The frequency of ZFN-mediated CCR5 disruption in resting CD4+ T cells with or without activation after transduction was assessed by T7 nuclease assay. Purified resting CD4+ T cells were transduced with NILV at an MOI of 15. The frequency of CCR5 disruption assessed by T7 nuclease assay is indicated as percentage of total population, calculated by the formula: 100 × [1 − (1 − fraction of cleavage)1/2]. Representative data from experiments performed at least three times are shown.

**Figure 1** ZFN-expressing NILV-mediated disruption of CCR5 gene in cell lines and primary human CD4+ T cells. (a) Transduction efficiency and CCR5 disruption efficiency of HEK 293 cells. HEK 293 cells were transduced with CCR5/ZFNs NILV pseudotyped with VSV-G at an MOI of 30 and examined for ZsGreen expression by flow cytometry after 48 hours. The genomic DNA extracted from the transduced cells was tested for CCR5 disruption by T7 endonuclease assay using a Tris-Acetate PAGE gel. Empty vector (GFP only) was used as control. 
(b) Transduction efficiency and CCR5 disruption efficiency in Jurkat cells after ZFN delivery with HIV envelope pseudotyped NILV. 
(c) PHA-activated CD4+ T cells were transduced with CCR5/ZFN NILV pseudotyped with HIV envelope at an MOI of 30 and ZsGreen-positive cells were enumerated by flow cytometry. 
(d) Resting CD4+ T cells were transduced as in c, and then activated with PHA 4 hours after transduction. For both activated and resting CD4+ T cells transduction assays, the empty NILV (without CCR5/ZFN) was used as control. 
(e) The frequency of CCR5 disruption in activated and resting CD4+ T cells (activated 4 hours after transduction) was assessed by T7 endonuclease assay. 
(f) The frequency of ZFN-mediated CCR5 disruption in resting CD4+ T cells with or without activation after transduction was assessed by T7 nuclease assay. Purified resting CD4+ T cells were transduced with NILV at an MOI of 15. The frequency of CCR5 disruption assessed by T7 nuclease assay is indicated as percentage of total population, calculated by the formula: 100 × [1 − (1 − fraction of cleavage)1/2]. Representative data from experiments performed at least three times are shown.

CCR5 disruption protects primary CD4+ T cells from HIV-1 infection *in vitro*

To investigate whether CCR5 disruption by CCR5-ZFNs NILV can suppress HIV-1 infection, primary CD4+ T cells...
were transduced with HIV-pseudotyped NILV at an MOI of 30 (60 ng of p24 for 1 × 10⁶ cells) and then challenged with CCR5-tropic HIV-1 isolate HIVBaL at an MOI of 0.1 (200 pg p24 for 1 × 10⁶ cells). Activated cells were transduced with NILV and then challenged with HIV-1 48 hours after transduction, whereas resting CD4⁺ T cells were first transduced with NILV, activated with PHA for 2 days and then subjected to viral challenge. Supernatants were collected from the cultures on days 1, 4, 7, 10, and 14 for viral load determination by qRT-PCR. With mock and control vector transduction, the response pattern to HIV-1 challenge was very similar in both activated and resting CD4⁺ T cells in that the viral load peaked at day 7, and then dropped two- to fourfolds after day 14. We reason that this decrease in viral load is caused by the decreasing availability of CD4⁺ T cells for further de novo infection. By contrast, in both resting and activated CD4⁺ T cells transduced with CCR5-ZFNs, the viral load was substantially lower than either non-transduced or vector-transduced CD4⁺ T cells on day 7, and became nearly undetectable by 14 days after infection. These results suggest that CCR5 disruption protects against HIV-1 infection and thus provides survival advantage to the cells (Figure 2a.b).

**CCR5 disruption in resting CD4⁺ T cells from a seronegative donor confers HIV-1 resistance in Hu/PBL mice**

We used the Hu/PBL model to assess whether CCR5-ZFNs-transduced resting CD4⁺ T cells can be reconstituted in humanized mice, and whether the gene modified cells are able to resist HIV-1 infection in vivo. CD4⁺ T cells isolated from a HIV-1 seronegative donor, were transduced with HIV-pseudotyped NILV for 2 hours at an MOI of 10 (200 ng of p24 per 2 × 10⁶ cells) and injected into the animals (5 × 10⁶ CD4⁺ cells/mouse) along with 0.5 million unmanipulated PBMCs to facilitate engraftment. Non-transduced and empty NILV vector-transduced cells were also similarly injected in separate groups of mice as negative controls. All mice were challenged with 100 ng p24 of R5-tropic virus, HIVBaL 5 days after cell infusion. The CD4⁺ T cell profile (% CD3⁺CD4⁺) was monitored on days 7, 21, 34, and 42 (Figure 3a). The transduction efficiencies for vector and CCR5-ZFNs NILV were 10.1% and 16.7% respectively (Figure 3b). On day 7 after infusion, similar levels of CD4 reconstitution (around 50–70%) were observed in all groups. However, by day 21 after challenge, the control LV-transduced and non-transduced groups exhibited a dramatic decrease in CD3⁺CD4⁺ cells, compared to the CCR5-ZFN group, where five out of eight animals showed a significantly higher percentages of CD3⁺CD4⁺ cells. Furthermore, the CD4⁺ T cell levels were maintained over time in CCR5-ZFN group as assessed on days 21, 34, and 42, whereas the levels consistently remained low in both non-transduced group and vector-transduced groups (Figure 3c).

We also investigated whether transfer of ZFN modified CD4⁺ T cells could control plasma viremia by determining the p24 levels by ELISA in sera collected after sacrificing the animals 7 weeks after challenge. The results revealed that HIV-1 was at a very low level in most of the animals in the ZFN group (around 10 pg/ml in four mice and 50 pg/ml in one mouse), whereas the control groups showed a high viral load, ranging from 100–900 pg/ml (Figure 3d). The three animals in the ZFN group that failed to maintain their CD4 counts showed viral loads similar to the control mice. The result of p24 ELISA was also corroborated by analysis of HIV-1 cDNA late reverse transcripts (LRTs) by qRT-PCR. Even here, in the CCR5-ZFN transduced group, the viral loads were very low in the same five protected animals (1,000–2,500 copies/ml), whereas both non-transduced and vector transduced groups presented a significantly higher viral load (average around 10,000 copies/ml) (Figure 3e). Overall, these results suggest that transient ZFN expression in resting CD4⁺ T cells using HIV envelope pseudotyped NILV can generate HIV-1-resistant cells that efficiently engraft and expand in humanized mice.

The increase in the proportion of CD4⁺ T cells over time in the CCR5-ZFN group suggests that CCR5 disruption provides survival advantage to the gene modified relative to the non-transduced CD4⁺ T cells, which became infected and died. To confirm CCR5 disruption in this population, genomic DNA extracted from purified CD4⁺ T cells was subjected to T7 endonuclease assay. For many of the unprotected mice, the T7 endonuclease assay could not be carried out because of a paucity of CD4⁺ T cells after HIV-1 challenge. By contrast, the CCR5 ZFN-protected showed an average disruption frequency of 71.8%, confirming the enrichment of protected cells (Figure 3f).

**Figure 2 CCR5-ZFN-expressing NILV transduced activated and resting CD4⁺ T cells resist HIV infection in vitro.** (a) activated or (b) resting CD4⁺ T cells were challenged with HIVBaL 48 hours after transduction and the supernatants collected at different time points were tested by LRT qRT-PCR to determine the viral titers. PBMC and empty NILV were used as controls. All the samples for qRT-PCR were in triplicates and plotted as average copy numbers. One way analysis of variance was used to compare the differences between groups at different time points. Significance levels are shown in the figures as *P < 0.05; **P < 0.01; and ***P < 0.001.
Suppression of endogenous virus in Hu/PBL mice engrafted with CCR5 modified resting CD4+ T cells from HIV-1-seropositive subjects

Infusion of autologous CCR5 gene modified CD4+ T cells is being tried as a potential therapy for HIV-1 infection. Towards this end, we tested the protective efficacy of ZFN-mediated CCR5 disruption in Hu/PBL mice reconstituted with PBMCs from HIV-1+ donors. As HIV pseudotyped NILV is expected to selectively target CD4+ T cells, we directly transduced 3x10^6 PBMCs from the patients in the absence of CD4+ T-cell purification. PBMCs transduced at an MOI of 10 (300ng of p24 for 3x10^6 PBMCs) without prior activation were infused into NSG mice. The CD4+ T-cell levels were monitored over time and sera collected at the time of sacrifice were used for viral load analysis.

We first tested the strategy with PBMCs from two treatment-naive HIV-1+ subjects with high viral loads (for patient information see Supplementary Table S1). We used only four mice for each group (mock and ZFN transduced) because of limitation of the number of cells available. PBMCs from only one of the two subjects showed successful engraftment in NSG mice, with three out of four animals in both control and test groups showing adequate human cell reconstitution. The results showed that the CD4 count was maintained in the ZFN-treated group (CD3+CD4+ levels between 49–57%), whereas in the mock-transduced group, the CD4+ T cells dropped from a mean of 67% on day 7 to a mean of 49% on day 42 (Figure 4a). Protection was also assessed by measuring the viral load by p24 ELISA in sera collected on day 49 after transplantation. The viral load was very much reduced in the ZFN group (average 25 pg/ml blood, with undetectable virus in one animal) compared with the mock-treated group (average 1,500 copies/ml blood) (Figure 4b). Consistent with the p24 ELISA results, a qRT-PCR assay also revealed that the average viral load was around 1,500 copies/ml blood in CCR5 ZFN group, whereas in the mock treated group it was about fivefold higher (Figure 4c).

Next, we used the same Hu/PBL model to test HIV-1 resistance mediated by CCR5 disruption in resting PBMCs from 2 HIV-1+ subjects on ART, whose viral loads were undetectable.
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In both cases, good human cell engraftment was observed in all mice (Subject S24) or at very low level (Subject S30). In both cases, the CD4+ T-cell counts were slightly higher for subject S30 (30% for the vector-treated and 23% for ZFN-treated group) than for subject S24 (24% for the vector-treated and 16% for ZFN-treated group), which is consistent with the CD4+ T-cell counts in the input PBMCs (40% in S30 and 27% in S24). Although the CD4+ T-cell counts were slightly higher in the control group compared with the CCR5/ZFNs group on day 7, CD4+ T cells in this group gradually declined for both subjects (dropping from an average of 24% on day 7 to 12.7% on day 35 for S30 and from 23% to 3% for S24) (Figure 4a,e). By contrast, in the CCR5/ZFN group, the CD4+ T-cell levels increased with time (from ~23% to ~45% for S30, and from 16% to 50% for S24).

As the viral load was below detection by p24 ELISA in all groups (data not shown), we used the more sensitive qRT-PCR assay to evaluate plasma viremia. The results showed that the virus remained below the limit of detection (<60 copies/ml) in the sera of 6/7 mice (for S30) and 5/7 mice (for S24) in the CCR5/ZFN group, whereas for both subjects, a significantly higher viral load was detected in all the control mice (Figure 4g).

Taken together, these results demonstrate that direct transduction of PBMCs from HIV-1 seropositive subjects with HIV-1-pseudotyped NILV is a feasible approach for CCR5 gene editing; and that after infusion into Hu/PBL mice, the gene modified cells are protected from HIV-1 infection.

Discussion

Here, we used a non-integrating lentiviral system for transient expression of ZFNs to edit the CCR5 gene in primary CD4+...
T cells. More importantly, pseudotyping NILV with HIV envelope allowed targeting of resting CD4+ T cells, obviating the need for CD4+ T-cell isolation from PBMCs or prior activation. Using the Hu/PBL mouse model, we also showed that the gene modified resting CD4+ T cells from normal, as well as HIV-1+ donors, could engraft and provide protection from HIV-1 infection in vivo.

Recently, multiple studies have demonstrated the efficacy of ZFN-mediated CCR5 disruption as a therapeutic strategy to block HIV-1 infection. \(^\text{31–33,17,22}\) Adenoviral vector has been the preferred vehicle for ZFN delivery because of its ability to transduce hard to transfect primary cells. \(^\text{32}\) Despite a similar advantage, conventional lentiviral vectors have not been utilized because of their propensity to integrate into the host genome, which poses the obvious risk of insertional mutagenesis. In addition, long-term expression of ZFN from an integrated transgene can also increase the risks of off target effects. \(^\text{43–45}\) Tracking of ZsGreen expression revealed that NILV expression in cultured cells decreased to a nearly undetectable level by day 8, which is in agreement with published data showing that NILV expression through cell division reaches baseline levels by day 10. \(^\text{29}\) These properties of NILV make it well suited for transient ZFN expression to confer stable CCR5 gene modification and thereby induce HIV-1 resistance in susceptible cells.

Integrase defective vectors are known to induce only transient accumulation of episomal DNA in the nucleus as they retain a very weak integration capacity compared with WT vectors. \(^\text{46,47}\) NILVs lacks the integrase protein that facilitates integration, but background integrations due to illegitimate IN-independent insertions still occurs in transduced cells, the reported rate of which has varied considerably. \(^\text{48}\) With the D64E mutant used in this study, the residual integration was demonstrated to be ~0.2% in NILV-transduced 293T cells. \(^\text{36}\) Suwanmanee et al. have reported a 35-fold reduction in integration frequencies in liver tissue when the same D64E vector used in this study was injected in vivo to correct factor IX deficiency in a mouse model of hemophilia. \(^\text{49}\) Thus, although very low, some residual illegitimate integrations do occur with NILV, which poses a definite safety concern for clinical use of NILV. Recent studies have shown that the frequency of such residual integration can be reduced to minimal levels by using a PPT deleted vector. \(^\text{36}\) Another potential risk for use of NILV for HIV-1 therapy is the possibility that in HIV-1 infected cells NILVs could be complemented in trans by an active integrase of similar specificity. As suggested by Lombardo et al., incorporating cis-acting mutations in the integrase attachment sites of the vector may prevent such a rescue. \(^\text{51}\)

The ZFNs employed for CCR5 gene targeting in this study were obtained from a commercial source (Sigma-Aldrich) and differ in sequence from the one from Sangamo Biosciences that has been used in multiple studies for CCR5 gene editing in hematopoietic stem cells and primary T cells. \(^\text{12,13,19}\) In silico genome-wide analysis of the sequences targeted by the ZFN pair used in this study did not reveal any off-target sites. Although allowing two mismatches per ZFN half-site for in silico analysis identifies 32 potential off target sites, deep sequencing data published by Badia et al. found no off target effects in human K562 cells transected with the same ZFN pair. \(^\text{36}\) However, the recent demonstration that NILV can be captured at sites of double stranded breaks induced by ZFNs by nonhomologous end joining suggests that such illegitimate integrations could also contribute to off target toxicity. \(^\text{50,51}\) Thus, for actual clinical use, the genome wide impact of NILV-mediated ZFN delivery in primary T cells has to be understood. The potential for insertional mutagenesis is somewhat mitigated by studies showing that mature CD4+ T cells are relatively resistant to oncogenic transformation in vivo even after transduction with integrating retroviral vector expressing potent T-cell oncogenes. \(^\text{52}\)

Conventionally, VSV-G is used for pseudotyping lentiviruses because of its broad tropism for many different cell types. However, cells in the G0 stage of the cell cycle, such as resting CD4+ T cells are highly recalcitrant to transduction with VSV-G pseudotyped lentivirus. On the other hand, unlike VSV-G, CXCR4-tropic HIV envelope has been shown to mediate fusion of lentiviral particles in both unstimulated and stimulated CD4+ T cells. \(^\text{50}\) Our study also shows that lentivirus packaged with X4-tropic envelope from LAI can efficiently deliver ZFNs for CCR5 disruption into resting CD4+ T cells. This makes it possible to selectively target HIV-1 susceptible CD4+ T cells within unfractionated PBMC population. Furthermore, as ex vivo transduction is the only external manipulation required, after ZFN modification, the PBMCs can be immediately infused back into the patient, which would simplify ZFN-based therapy for wider clinical application.

CCR5 disruption has been the goal in most studies evaluating ZFNs for HIV-1 therapy. This is because the vast majority of newly transmitted HIV-1 strains use the co-receptor and CCR5 tropic viruses predominate during the early stages of infection. \(^\text{53–55}\) Nevertheless, over time, there is a significant increase in quasispecies that utilize CXCR4 as an alternative co-receptor for infection. \(^\text{55,56}\) Thus, in addition to CCR5, CXCR4 disruption may also be needed, particularly for the treatment of chronically infected patients.

Although a majority of animals transplanted with CCR5 ZFNs modified CD4 T cells were protected from HIV-1 challenge, we also observed that some animals (3 out of 8 animals receiving PBMCs from a normal donor and 2 out of 14 animals receiving patient PBMCs) failed to show protection. Although the reason for this is not clear, another study also found that a fraction of animals treated with CCR5 ZFN-modified T cells remained unprotected. \(^\text{12}\) As only a small number of xenoreactive cells are expected to expand after infusion of resting CD4+ T cells, it is possible that paucity of gene modified cells within this fraction in some of the animals could explain the lack of protection. Thus, increasing the infusion size may be necessary to show complete antiviral efficacy in humanized mice.

Taken together, our study demonstrates that NILV can serve as a potentially useful alternative vector for ZFN-mediated CCR5 alteration. HIV envelope pseudotyping of NILV allows unfractionated PBMCs from HIV-1 patients to be directly transduced ex vivo and reinfused back into the patients without extensive ex vivo manipulation. As no T-cell activation or large-scale expansion of gene modified cells are involved, the impact on immune homeostasis is likely to be minimal. The simplicity of the delivery approach used here is likely to broaden the scope of ZFNs-mediated CCR5 disruption for HIV-1 gene therapy.
Materials and Methods

Cells and cell culture. HEK 293T cells and TZM-bl cells were cultured in complete DMEM medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS), and penicillin-streptomycin-glutamine (PSG), 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine). Jurkat cells were cultured in complete RPMI medium (RPMI 1640 medium supplemented with 10% FBS and PSG). Human Peripheral blood mononuclear cells (PBMCs) were purified from normal human donor blood by Ficoll density gradient centrifugation. Primary CD4⁺ T cells were purified from either healthy donors or patients’ PBMCs by two rounds of negative selection using the EasySep Human CD4⁺ T-cell enrichment kit (Stemcell Technologies, Vancouver, BC, Canada) according to the manufacturer’s instructions.

Lentiviral vector. A CCR5-ZFN plasmid pair encoding the left and right ZFN arms was purchased from Sigma-Aldrich (St Louis, MO). Lentiviral vector pLVX-ZsGreen was purchased from Clontech Laboratory (Mountain View, CA). The following primer pairs were used to amplify ZFN1 and ZFN2 fragments from CCR5-ZFNs plasmids: zn1 forward: cgctgtgattccacctggactaacaagctgac (which includes a kozak sequence and the start codon) and zn1 reverse: gcccttcgagacctggaccaagctggcccctcttctgtaaagccagggcgagctgtggaagaaaaccccggtcctatgagatctgactacaaagaccatgac (which contains a 2A sequence from foot-and-mouth disease virus) and zn2 reverse: ccacagatcagagctgactgctccg. After PCR amplification of zfn fragments, the zfn1 fragment was digested with EcoR I/Xho I and zfn2 with Xho I/Xba I. pLVX-ZsGreen vector was digested with EcoR I/Xho I and the three digested fragments were ligated together at a molar ratio of 1:1:1 and transformed into E. coli DH5α. The resulting recombinant plasmid (pLVX-ZFN1-2A-ZFN2) which co-expresses both ZFN arms was used to transfect 293T cells to generate lentivirus.

Generation and titration of lentivirus. 1 x 10⁴ 293 T cells were co-transfected with 20 μg of vector plasmid (pLVX-ZFN1-2A-ZFN2, or pLVX as control), 16 μg of packaging plasmid and 10 μg of envelope plasmid using Polyethyleneimine (PEI, Polysciences, Warrington, PA).⁶⁰ For integrating lentivirus, pHRTV.9ΔVPR was used as packaging plasmid (which expresses functional integrase)⁴¹; while vTK939 (which expresses D64E-mutant integrase) was used in a similar manner as when making non-integrating lentivirus.⁶⁰ The plasmids pCMV-VSV-G or pHIV LAI (generously provided by Dr Una O’Doherty at the University of Pennsylvania) were used for pseudotyping the lentiviruses with VSV-G and HIV-1 envelopes respectively. After 30 minutes of transfection, DMEM supplemented with 10% FBS and PSG was added and the cells were incubated at 37 °C under 5% CO₂. Two days later, the supernatants were collected, filtered (low binding filter, PVDF membrane, Millipore) and concentrated by ultracentrifugation at 25,000g at 4 °C using an SW 40 Ti rotor for 2.5 hours and the lentivirus pellets were dissolved in PBS. To determine the viral titers, 293 T or TZM-bl cells were transduced with VSV-G or HIV-1 pseudotyped lentivirus respectively. Briefly, 1 x 10⁵ cells were spin-transduced with serially diluted lentivirus preparations in serum free DMEM in the presence of 7 μg/ml polybrevine (Sigma-Aldrich) at 1,200 g for 2 hours, and then incubated at 37 °C for another 2 hours. After changing medium to complete DMEM, the transduced cells were incubated at 37 °C for 48 hours. The cells were then fixed with 1% formaldehyde. The transduction efficiency was quantified by evaluating the infected cells for GFP expression by flow cytometry.

T7 endonuclease assay. Genomic DNA was extracted using the GenElute Mammalian Genomic DNA miniprep kit (Sigma-Aldrich) according to the manufacturer’s instructions. The frequency of gene modification by NHEJ was evaluated as described previously.¹⁶ Briefly, the purified genomic DNA was used as a template to amplify a fragment of the CCR5 gene using the specific primers (CCR5 forward: 5’-TGGTGGCCACATGTGGTCACTCTC-3’ and CCR5 reverse: 5’-TGTAGGGAGCCCAAGAGAAGA-3’), to yield a 346bp amplicon. To amplify the CCR5 gene fragment in CD4⁺ T cells from humanized mice the primer pairs were: CCR5 forward: 5’-GGTGCATCCCTCACTCCTGAT-3’ and CCR5 reverse: 5’-TGTAGGGAGCCCAAGAGAAGA-3’) which results in a 605bp amplicon. The PCR products were gel purified, denatured and re-annealed to form heteroduplex DNA, followed by treatment with T7 endonuclease. Since T7 endonuclease is mismatch-sensitive, the insertions and deletions caused by NHEJ could be detected by running a 7% Tris-Acetate PAGE gel and quantitated by densitometry using Quantity One 4.6.6 software. The disruption frequency was defined as 100 x (1-(1-fraction cleaved)⁰.⁵), wherein the fraction cleaved is the density sum of the cleaved bands divided by the density sum of the cleaved bands and un-cleaved band.

Viral load determination. HIV-1 viral loads in cell culture or in the sera were determined by HIV-1 CDNA Late Reverse Transcripts (LRTs) qRT-PCR and/or p24 ELISA. LRTs PCR was performed as described elsewhere but with a small modification. Briefly, for cell culture supernatants, the supernatants were treated with 10% Triton-100, then directly used for qRT-PCR. For mice sera, RNAs were isolated according to the manufacturer’s instructions (QiAamp viral RNA mini kit, QiaGen, Germantown, MD), and used as PCR templates. The PCR mixtures were formulated according to the manufacturer’s instructions (Tagman Fast Virus 1-Step Master Mix kit, Life Technologies, Grand Island, NY). The primers (Sigma-Aldrich) were as follows: MH531 (5’-TGTGGTGGCCACATGTGGTCACTCT-3’), MH532 (5’-GGTGCATCCCTCACTCCTGAT-3’), and fluorescent probe LRT-F (5’-FAM-CAGTGGCCGCCCAGACAGAGTATGAGAGC-3’). The PCR mixtures were incubated at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A sample quantitated by the 4th generation Roche Cobas Ampliprep/TaqMan method at the Laboratory of Clinical and Biological studies, Miller School of Medicine, University of Miami was used as standard. A standard curve generated using dilutions of this standard to calculate the absolute copy numbers of copies. The lower limit of detection of the assay was determined to be 50 copies/ml. The Alliance HIV-1 ELISA kit from PerkinElmer
(Waltham, MA) was used to perform p24 ELISA according to the manufacturer's instructions.

**Lentivirus transduction and HIV-1 challenge in vitro.** 293 T cells. Jurkat cells and primary CD4+ T cells were used for lentiviral transduction. CD4+ T cells were purified from PBMCs of HIV-1-seronegative donors by two rounds of negative selection. CD4+ T cells were transduced either directly (resting) or after activation with PHA (1 μg/ml) in the presence of 50 U/ml rIL-2 for 2 days. In brief, 1 × 10^6 cells were spin-transduced with VSV-G or HIV-pseudotyped lentivirus (ZFN or control virus) at indicated multiplicity of infection (MOI). Resting CD4+ T cells were activated with PHA following transduction for 2 days before HIV-1 challenge. Cells were then infected with the CCR5 tropic strain HIV longterm studies confirm the stability of the latent reservoir for HIV in resting CD4+ T cells. Nat Med 8: 727–728. 4. Mendoza, N and Wasney, G (2006). Targeted gene inactivation in zebrafish using engineered zinc-finger nucleases. Nat Biotechnol 24: 695–701. 5. Carroll, D (2006). Progress and prospects: zinc-finger nucleases as gene therapy agents. Gene Ther 13: 1453–1458. 6. Doyon, Y, McConnon, J, Miller, JC, Faraji, F, Ngo, C, Katibah, GE et al. (2008). Heritable targeted gene disruption in zebrafish using designed zinc-finger nucleases. Nat Biotechnol 26: 702–708. 7. Shukla, VK, Doyon, Y, Miller, JC, DeKelver, RC, Moehle, EA, Worden, SE et al. (2009). Precise genome modification in the crop species Zeya maya using zinc-finger nucleases. Nature 459: 437–441. 8. Townsend, JA, Wright, DA, Winfrey, RJ, Fu, P, Maeder, ML, Jonk, JK et al. (2009). High-frequency modification of plant genes using engineered zinc-finger nucleases. Nature 459: 442–445. 9. Jacob, HJ, Lazar, J, Dewnill, MR, Moreno, C and Guerts, AM (2010). Gene targeting in the rat: advances and opportunities. Trends Genet 26: 510–518. 10. Umoff, JD, Rebar, EJ, Holmes, MC, Zhang, HS and Gregory, PD (2010). Genome editing with engineered zinc-finger nucleases. Nat Rev Genet 11: 636–646. 11. Lombardi, A, Genovese, P, Beaupre, CM, Collee, S, Lee, YL, Kim, KA et al. (2007). Gene editing in human stem cells using zinc-finger nucleases and integrase-defective lentiviral vector delivery. Nat Biotechnol 25: 1398–1406. 12. Perez, EE, Wang, J, Miller, JC, Jouvenot, Y, Kim, KA, Lu, G et al. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. Nat Biotechnol 26: 810–816. 13. Holt, N, Wang, J, Kim, K, Friedman, G, Wang, X, Taunton, V et al. (2010). Human hematopoietic stem/progenitor cells modified by zinc-finger nucleases targeted for CCR5 control HIV-1 in vivo. Nat Biotechnol 28: 839–847. 14. Ilfen, CS, Wang, J, Tilton, JC, Miller, JC, Kim, KA, Rebar, EJ et al. (2011). Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases. PLoS Pathog 7: e1002020. 15. Yuan, J, Wang, J, Crain, K, Feiars, C, Kim, KA, Hua, KL et al. (2012). Zinc-finger nuclease editing of human CCR5 promotes HIV-1(CD4+) T cell resistance and enrichment. Mol Ther 20: 849–859. 16. Perez-Pinera, P, Ousterout, DG and Genbacher, CA (2012). Advances in targeted genome editing. Curr Opin Chem Biol 16: 268–277. 17. Stone, D, Kiem, HP and Jerome, KR (2013). Targeted gene disruption to cure HIV. Curr Opin HIV AIDS 8: 217–223. 18. Liu, L, Krymskaya, L, Wang, J, Janou, J, Rau, J, Yao, C, Cao, LF et al. (2013). Genomic editing of the HIV-1 coreceptor CCR5 in adult hematopoietic stem and progenitor cells using zinc finger nucleases. Mol Ther 21: 1259–1269. 19. Xu, X, Wang, P, Ding, D, Li, L, Wang, H, Ma, L et al. (2013). Zinc-finger nucleases mediate specific and efficient excision of HIV-1 proviral DNA from infected and latently infected human T cells. Nucleic Acids Res 41: 7771–7782. 20. Allers, K, Hötter, G, Hofmann, J, Loddenkemper, C, Rieger, K, Theil, E et al. (2011). Evidence for the cure of HIV infection by CCR5δ32/δ32 stem cell transplantation. Blood 117: 2791–2799. 21. Hötter, G and Zain, JA (2011). Allogeneic hematopoietic stem cell transplantation in patients with human immunodeficiency virus: the experiences of more than 35 years. Clin Exp Immunol 163: 284–295. 22. Mai, DA, Brennan, AL, Jiang, S, Binder-Scholl, GK, Lee, G, Plesa, G et al. (2013). Efficient clinical scale gene modification via zinc-finger nuclease-targeted disruption of the human co- coreceptor CCR5. Hum Gene Ther 24: 245–258. 23. Toba, S, Stein, D, Tang, WW, Frank, I, Wang, SQ, Lee, G et al. (2014). Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. N Engl J Med 370: 901–910. 24. Flight, MH (2013). Trial watch: Clinical trial boost for lentiviral gene therapy. Nat Rev Drug Discov 12: 654.
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