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Preclinical Evaluation of a Novel TALEN Targeting CCR5 Confirms Efficacy and Safety in Conferring Resistance to HIV-1 Infection

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Preclinical Evaluation of a Novel TALEN Targeting *CCR5* Confirms Efficacy and Safety in Conferring Resistance to HIV-1 Infection

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Therapies to treat patients infected with human immunodeficiency virus (HIV) aim at preventing viral replication but fail to eliminate the virus. Although transplantation of allogeneic *CCR5*Δ32 homozygous stem cell grafts provided a cure for a few patients, this approach is not considered a general therapeutic strategy because of potential side effects. Conversely, gene editing to disrupt the *C-C chemokine receptor type 5 (CCR5)* locus, which encodes the major HIV coreceptor, has shown to confer resistance to CCR5-tropic HIV strains. Here, an engineered transcription activator-like effector nuclease (TALEN) that enables efficient *CCR5* editing in hematopoietic cells is presented. After transferring TALEN-encoding mRNA into primary CD4+ T cells, up to 89% of *CCR5* alleles are disrupted. Genotyping confirms the genetic stability of the *CCR5*-edited cells, and genome-wide off-target analyses established the absence of relevant mutagenic events. When challenging the edited T cells with CCR5-tropic HIV, protection in a dose-dependent manner is observed. Functional assessments reveal no significant differences between edited and control cells in terms of proliferation and their ability to secrete cytokines upon exogenous stimuli. In conclusion, a highly active and specific TALEN to disrupt *CCR5* is successfully engineered, paving the way for its clinical application in hematopoietic stem cell grafts.

1. Introduction

As of today, there is no cure for infection with human immunodeficiency virus type 1 (HIV-1). The available antiretroviral therapies (ART) target different steps in the HIV replication cycle. Although ART manages to reduce the HIV burden below the detection limit as long as the medication is taken, it does not eradicate the virus. Inhibition of HIV entry has been considered a promising strategy that was explored in numerous ways. In particular, targeting C-C chemokine receptor type 5 (CCR5), the co-receptor of prevalent HIV-1 strains has been pursued using CCR5 inhibitors, or blocking antibodies.[1] But again, even when combined with other ARTs, they do not eradicate HIV.

On the other hand, the knowledge that a naturally occurring 32 nucleotide deletion (Δ32) mutation in the *CCR5* locus leads to resistance to CCR5-tropic (R5-tropic) HIV-1 strains,[2,3] has spurred some encouraging

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approaches.[4] People with a homozygous Δ32 mutation are rare, clinically unremarkable, and conduct a normal life.[5] In 2007, an HIV-positive patient, later referred to as the "Berlin Patient," was transplanted with an allogeneic stem cell graft of an human leukocyte antigen (HLA)-matched donor homozygous for the *CCR5*Δ32 mutation after diagnosis of acute myeloid leukemia. After stopping ART, the patient was closely monitored for virus titers. As of today, that is, 13 years later, no viral RNA or proviral DNA has been detected, so that he was declared the first patient to be cured of HIV.[6–8] Following the principle of the Berlin patient, two more patients, known as the London[9,10] and the Düsseldorf[11] patients, might be declared as cured. These observations underline the possibility to transfer HIV resistance from a donor to a patient. However, due to the potentially severe side effects of allogenic transplantations and the difficulty of finding matching homozygous Δ32 donors, this strategy is not considered a universal approach to treat patients affected by HIV.

Alternatively, *CCR5* gene editing in autologous cells represents a potent approach to provide patients with cells resistant to HIV.[12] To this end, genome editing with programmable nucleases, in particular, zinc-finger nucleases (ZFN), transcription activation-like effector nucleases (TALENs), MegaTALs, and clustered regularly interspaced short palindromic repeat/CRISPR-associated protein (CRISPR-Cas) nucleases, have been used in several preclinical and clinical studies to disrupt the *CCR5* gene in primary human CD4+ T cells or hematopoietic stem and progenitor cells (HSCs).[13–22] In a clinical trial conducted by Tebas et al., 12 individuals living with HIV received a single dose of *CCR5*-edited autologous CD4+ T cells. Although the effect lasted only transiently, the blood level of HIV provirus decreased in most patients and the infusion of *CCR5*-modified autologous CD4+ T cells proved to be safe.[15] To achieve long-lasting effects, however, *CCR5* must be modified in HSCs. This ensures that HIV resistance will be conferred to all CD4+ immune cells, that is, also macrophages and dendritic cells. In reported clinical scale validation runs, 25–71% of *CCR5* alleles were edited in HSCs but the used ZFN revealed high off-target (OT) activities,[18] suggesting a relevant genotoxic potential in the stem cell compartment. HSCs were also edited with CRISPR-Cas nucleases for the treatment of an HIV-positive patient with acute lymphocytic leukemia. The *CCR5* editing frequency in the graft was ≈18% and was further diluted by the concomitant transplantation of unedited HSCs. Not surprisingly, HIV rebound was observed during treatment interruption.[20] Hence, while the concept of conferring HIV resistance by disrupting *CCR5* has been known for over a decade, the employed designer nucleases revealed low to intermediated disruption efficiencies (usually 20–50% allelic knockout frequencies) and/or undesirable OT effects. We conjecture that for a *CCR5* editing approach to be successful in the clinic, *CCR5* editing in HSCs must be both highly efficient, >50% biallelic knockout, and highly specific, to provide the patient with a safe graft and a sufficient number of HIV-resistant immune cells.

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In our study, we validated a novel TALEN for its ability to disrupt the *CCR5* allele with both high activity and high specificity, using settings transferrable to clinical-grade manufacturing. To demonstrate resistance to infection with HIV-1, *CCR5* editing was performed in primary CD4+ T cells. While TALENs were employed to disrupt *CCR5* in human cells before,[16,23,24] here we show that an improved TALEN design in combination with novel RNA transfer technologies[25] enabled us to knockout *CCR5* in CD4+ T cells with high activity and high specificity. The manufactured T cells were HIV resistant, genetically stable, and maintained their potency.

2. Results

2.1. *CCR5* Knockout is Genetically Stable in CD4+ T Cells

The target locus in exon 3 of the *CCR5* gene was chosen to be in the region coding for the N-terminus of the CCR5 protein (**Figure 1**A). The rationale was twofold: 1) out-of-frame insertion/deletion (indel) mutations would cause frameshift mutations that lead to early termination of translation, and 2) this sequence stretch codes for a conserved region of CCR5 that is bound by HIV-1 gp120. Even in-frame indel mutations will remove critical residues necessary for HIV-1 to bind to CCR5.[26,27] We first established the optimal conditions to achieve high *CCR5* disruption in CD4+ T cells from different donors with TALEN encoding mRNA via electroporation. Cells were cultivated for several days and subjected to genotyping at days 7 and 21 of culture to monitor gene disruption frequencies. To this end, we employed the T7 endonuclease I (T7E1) assay, a simple test for detecting targeting efficiency in gene-edited cells based on the T7E1's ability to recognize indel mutations on PCR fragments generated from the target locus. A transient temperature shift to 32 °C increased allelic *CCR5* disruption frequency from 50% to 85% (Figure 1B). The indel frequencies ranged between 50–90% at day 7 of culture and stabilized around 85% after 21 days, demonstrating stable editing in long term cultures. We also evaluated the effect of the gene disruption on the surface expression of CCR5. CCR5 expression varies between cell subsets and donors[28] as well as during activation, therefore staining was performed at the same culture stage. The percentage of CCR5 positive cells was significantly decreased ($p = 0.0009$) after editing with TALEN while no significant decrease in CCR5 expression was detected in control samples treated with only the left TALEN arm (Figure 1C). Reduction of CCR5 protein expression remained significant ($p = 0.0314$) and stable for 21 days, which paired well with the observations made on the genomic level (Figure 1B).

In parallel, we edited cells from a Δ32 heterozygous donor. Allelic gene disruption rates reached 69% on day 7 and 86% on day 21 (Figure 1B), so well within the ranges of the homozygous CCR5 wt donors. When performing a phenotypic analysis, a drop of 50% in CCR5 expression was also observed for this donor (Figure 1C, red dots), in line with the results seen with the homozygous donors.

To better understand the type of indels generated by the nucleases, we performed next-generation sequencing (NGS) analysis on the edited *CCR5* target locus in three different donors, one of which being Δ32 heterozygous, and analyzed the indels at days

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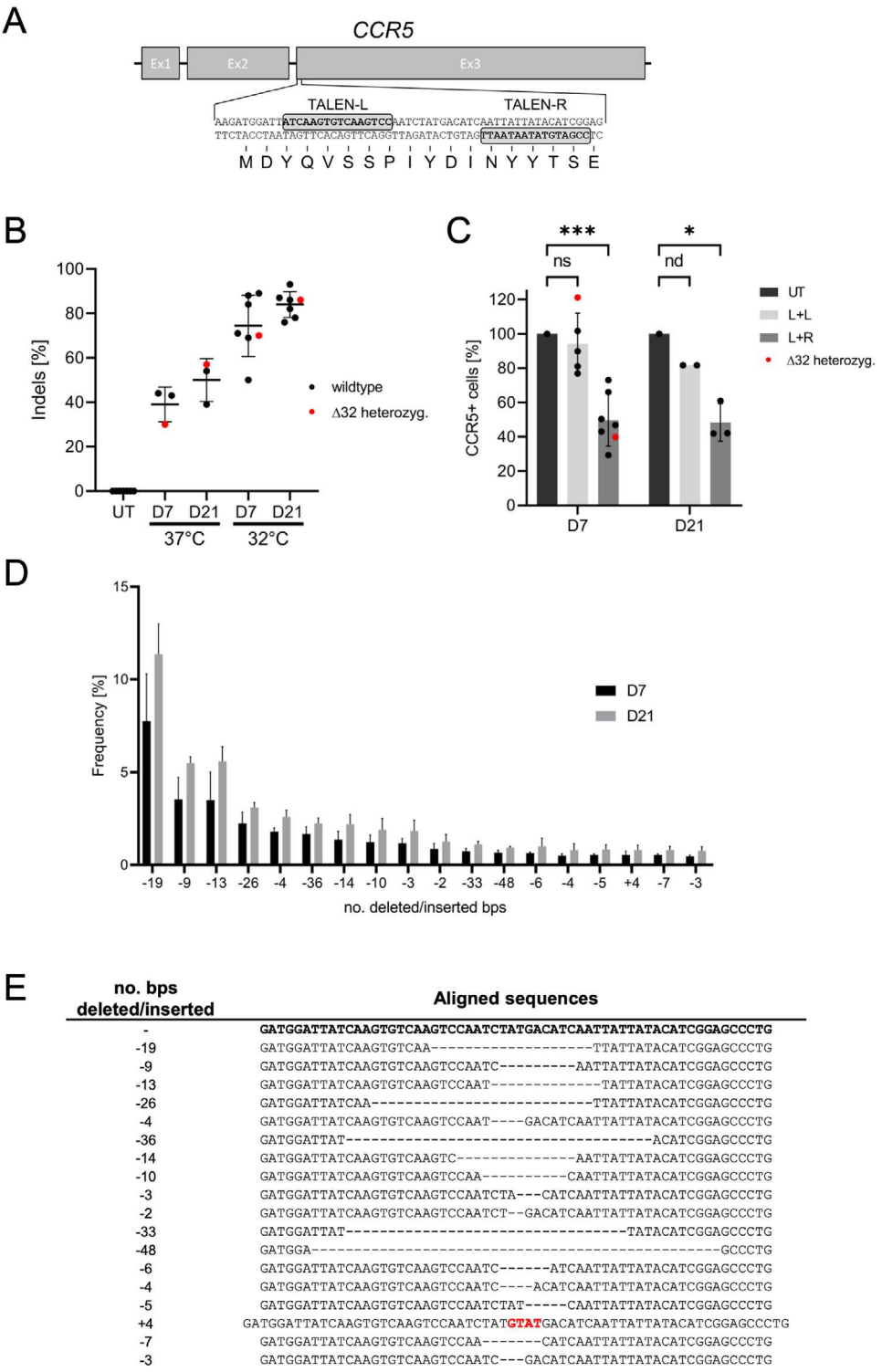

Figure 1. Stable *CCR5* knockout in CD4+ T cells. A) Schematic of the*CCR5*locus with TALEN binding sites (boxes). B) Quantification of TALEN activity. T7E1 assays were performed in CD4+ T cells at days 7 or 21 post-electroporation. Where indicated, cells were transiently incubatedat 32 °C for 24 h. Samples derived from a Δ32 heterozygous donor are highlighted in red (n= 3–7 donors; ±SD). C) CCR5 expression in CD4+ T cells. Cells were electroporated with only left TALEN arm (L+L) or both TALEN arms (L+R). Data is shown relative to untreated (UT) cells. Each dot represents a different donor (n= 2–7; ±SD; ns = not significant, nd = not determined, *$p \leq 0.05$; ***$p \leq 0.001$, paired$t$-test). D) Frequency of mutations. Targeted amplicon sequencing of the target site was performed after 7 and 21 days of culture (n= 3 donors; ±SD). Displayed are the frequencies of all indels with a frequency 1 in at least one of the three experiments. E) The sequence of induced mutations. Displayed are all sequences quantified in (D).

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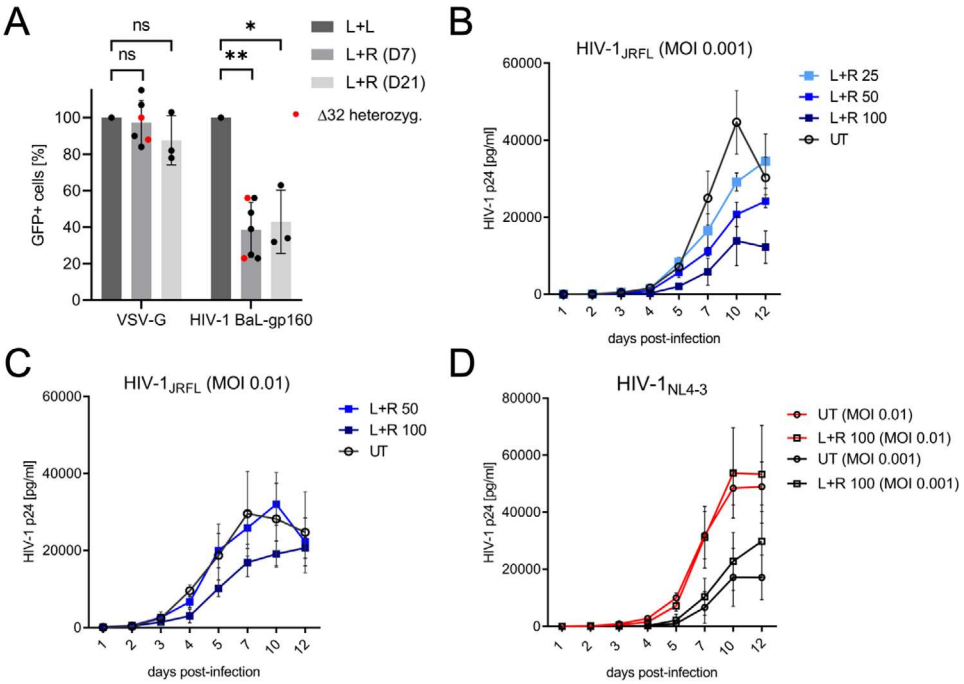

Figure 2. *CCR5*disruption confers HIV-1 resistance. A)Challenge withlentiviralvector. Untreated (UT) cells as well as TALEN-edited cells (L+L; L+R) were transduced with GFP-expressing lentiviral particles (6 transducing units per cell) that were either pseudotyped with VSV-G or HIV-1BaL-gp160. Challenge was performed at days 7 and 21 of culture. Indicated is the percentage of GFP-positive cells relative to the corresponding untreated cells. Samples derived from two Δ32 heterozygous donors are marked in red. Each dot represents a different donor (n= 3-7; ±SD; ns = not significant, *$p \leq$ 0.05; **$p \leq 0.01$, paired$t$-test). B–D) Challenge with HIV-1. Shown is the p24 kinetics over 12 days of infection in untreated (UT), TALEN edited cells (L+R 100), and edited cells spiked with UT at ratios of 1:1 (L+R 50) or 1:3 (L+R 25). Cells were challenged with HIV-1_{JRFL} at MOIs of 0.001 (B) or 0.01 (C), or with HIV-1_{NL3-4} at MOIs of 0.01 or 0.001 (D) in triplicates ±SD.

7 and 21, respectively (Figure 1D,E). The indel patterns and frequencies remained comparable between samples and over time. There was no significant enrichment or depletion of a specific indel type detected by a paired t-test during the culture period. This indicates that the clonal distribution of the edited cells between the two timepoints remained stable. In all three donors, the most frequent indel was a 19-nt deletion, followed by a 9-nt deletion, that combined represented about 20% of the total indel mutations. Of note, the four most frequent deletions −19, −9, −13, and −26 are likely due to microhomologies, as detected by the Microhomology-Predictor tool.[29]

2.2. *CCR5*-Edited CD4+ T Cells are Resistant to HIV-1 Infection

As a proof of concept, we challenged the edited cells at an early and late culture timepoint with GFP expressing lentiviral vectors pseudotyped with either the gp160 glycoprotein of the HIV-1 R5-tropic strain BaL or VSV-G as control. The *CCR5* edited CD4+ T cells showed ≈50% reduced susceptibility to transduction with the HIV-gp160 pseudotyped vector when compared to control cells (**Figure 2**A). When challenging T cells from two Δ32 heterozygous donors, a decreased susceptibility—similar to cells from the other donors—was observed. As a CD4/CCR5-independent transduction control, we transduced the cells in parallel with VSV-G pseudotyped lentivectors. Under these conditions, we did not see any alteration in transduction efficiency.

Next, we tested the resistance of *CCR5*-edited CD4+ T cells to replication-competent HIV-1 by infecting them with either R5-tropic HIV-1_{JRFL} or CXCR4-tropic (X4-tropic) HIV-1_{NL4-3}. HIV-1 p24 was quantified in the supernatant of infected cells as a measure of viral replication (Figure S1A,B, Supporting Information). In samples infected with HIV-1_{JRFL} (high or low multiplicity of infection [MOI]), a significant protective effect ($p = 8.8 \times 10^{-4}$ and $p = 5.7 \times 10^{-5}$) was observed when compared to infection of unedited cells. As expected, infections with X4-tropic HIV-1_{NL4-3} did not reveal significant differences (Figure S1B, Supporting Information). Furthermore, to investigate the impact of the editing rate on the viral replication, we spiked edited CD4+ T cells with unedited cells at ratios of 1:1 (50% edited cells) and 1:3 (25% edited cells). Low MOI infection with HIV-1_{JRFL}, as determined by p24 in the supernatant, was significantly ($p = 2.72 \times 10^{-14}$) reduced in non-diluted edited cells (100%) when compared to unedited samples (Figure 2B). The protective effect was also observed in both the 50% and 25% mix ($p = 8.38 \times 10^{-11}$, $p = 1.13 \times 10^{-4}$), suggesting that a cell population with ≈20% of edited *CCR5* alleles was able to slow down virus replication. A significant protective effect ($p = 3.06 \times 10^{-3}$) was also observed when edited cells (100%) were infected with a high MOI (Figure 2C), albeit to a lower degree as compared to the low MOI infection. After dilution (50% mix), the protective effect was lost when cells were infected at high MOI ($p = 0.98$). In the control settings with HIV-1_{NL4-3} infections, we did not see any impairment of virus replication in the edited cells (Figure 2D), as expected. We conclude

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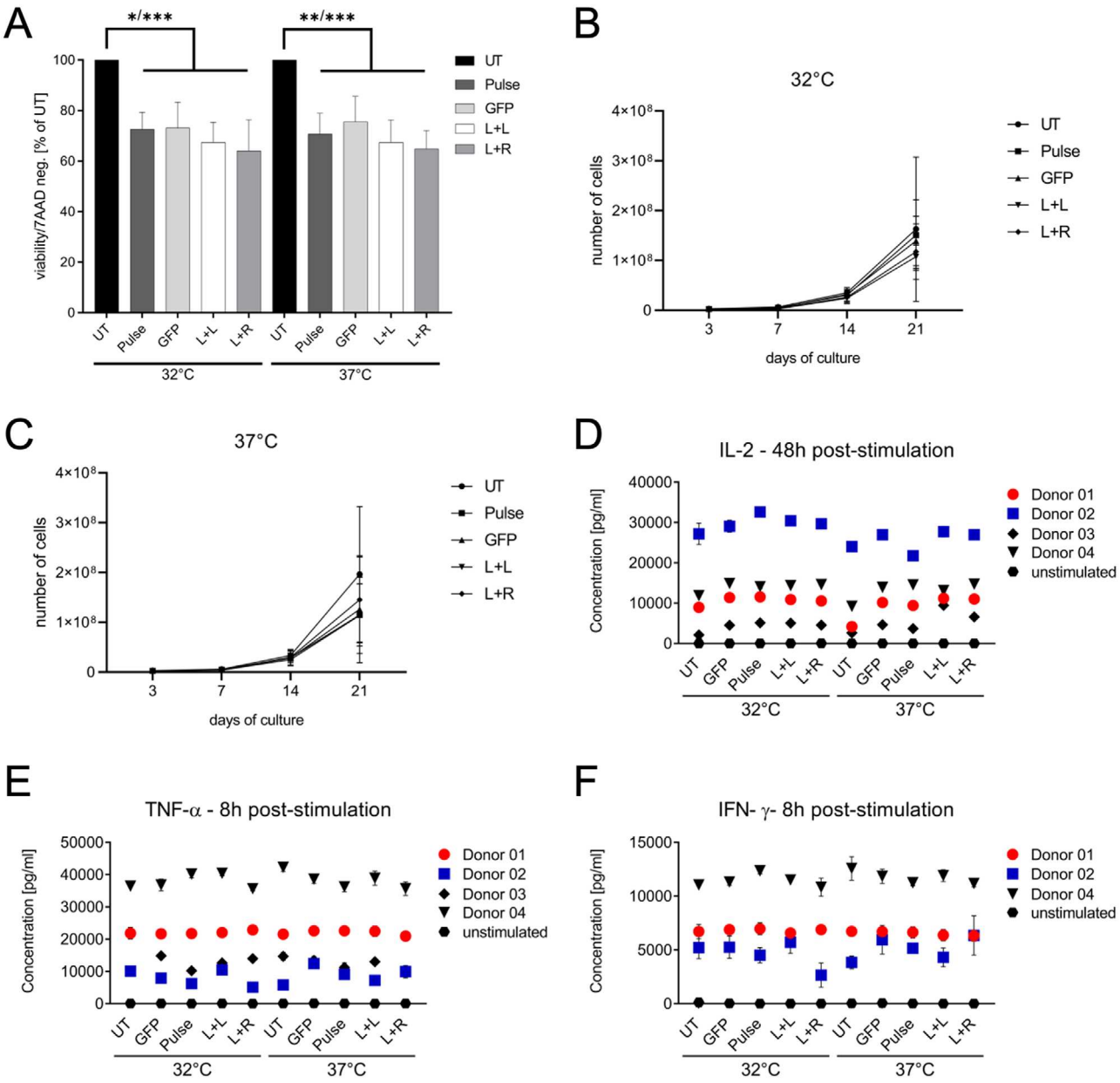

Figure 3. *CCR5*disruption does not impair CD4+ T cell functions. A) Viability. Shown is the viability of CD4+ T cells 24 h after mock-electroporation (Pulse), or electroporation with mRNA (GFP/TALEN L+L/TALEN L+R) relative to untreated (UT) cells (n= 4–12; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ±SD). B,C) Proliferation. Shown are the growth curves of CD4+ T cells subjected to transient temperature shift to 32 °C (B) or kept at 37 °C (C), after mock-electroporation (Pulse) or electroporation with mRNA (GFP, TALEN arms L+L/L+R), or left untreated (UT). Cells were counted at the indicated timepoints. Error bars represent ±SD. D–F) Cytokine secretion. Cytometric bead array was used to assess the release of TNF-α (D), IFN-γ (E), or IL-2 (F) at indicated timepoints after stimulation (n= 3, ±SD).

that editing of CD4+ T cells with *CCR5* targeting TALEN leads to protection against R5-tropic HIV-1 in an editing dose-dependent manner.

2.3. *CCR5*-Edited CD4+ T Cells Maintain Potency

Transfer of mRNA to T cells via electroporation can potentially affect cell viability and/or functionality. We addressed this point by evaluating viabilities 24 h post-treatment. CD4+ T cells electroporated with TALEN mRNA (both arms, or left arm only), GFP mRNA, or mock pulsed, showed a decrease in viabilities of ≈30% when compared to untreated samples (**Figure 3**A), implying that the electric pulse but not the RNA transfer had an impact on T cell viabilities. On the other hand, the transient temperature shift to 32 °C did not affect viability.

Next, we wanted to investigate if *CCR5*-edited cells retain their proliferative potential. To this end, the edited CD4+ T cells and

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control samples were cultivated under proliferation activating conditions. The total number of cells at days 7, 14, and 21 were comparable for all tested conditions (Figure 3B,C). A time-series analysis[30] confirmed that the samples, independent of cultivation temperature or treatment, did not show significant differences in proliferation. Hence, we conclude that editing *CCR5* did not affect the expansion capabilities of CD4+ T cells.

To explore if editing *CCR5* has an impact on CD4+ T cell function in terms of cytokine release upon stimulation, we stimulated the cells with either phorbol 12-myristate 13-acetate (PMA), ionomycin or CD3/CD28/CD2 to trigger the release of TNF-α, IFN-γ or IL-2, respectively. Supernatants of electroporated T cells were harvested 8–48 h after stimulation and analyzed by cytometric bead array. While we observed variations in the amounts of secreted cytokines among the four donors, the values did not significantly (two-sided *t*-test) differ when comparing the various treatment regimens (Figure 3D–F). Unstimulated cells did not release measurable amounts of cytokines. In sum, these results demonstrate that editing *CCR5* did not impair the ability of the engineered CD4+ T cells to respond to the different stimuli.

2.4. Editing *CCR5* in CD4+ T Cells with High Specificity

To assess the genotoxic potential of TALEN mediated editing of *CCR5*, we investigated the occurrence of OT activity by two different means: targeted amplicon sequencing of *in silico* predicted OTs or an unbiased *in cellula* assay. The top 20 potential OTs were predicted using the PROGNOS tool.[31] Amplicons from edited samples were compared to amplicons of untreated samples (**Figure 4**A). Two of the top 20 predicted OTs revealed some low but significant OT activity over background (Table S2, Supporting Information). However, both OT01 (0.12% versus 0.10% background, $p = 0.000\,211$) and OT10 (0.08% versus 0.07% background, $p = 0.004422$) are situated in intronic regions. These data were complemented with an unbiased OT detection approach termed oligonucleotide capturing assay (OCA), which is based on GUIDE-seq.[32] The highest score (OCA1) was obtained for the *CCR5* target site (Figure 4B). While OT01 was also found by OCA (OCA03), OT10 did not match with any of the top 24 OCA hits (Table S3, Supporting Information). On the other hand, *CCR2* did not come up as a potential OT in PROGNOS but a weak activity at *CCR2* was picked up by OCA (OCA4). Importantly, however, all identified OCA sites had a considerably lower score than the *CCR5* on-target site (OCA1). Together, these OT analyses demonstrate that the employed TALENs are highly specific designer nucleases with minimal OT activity.

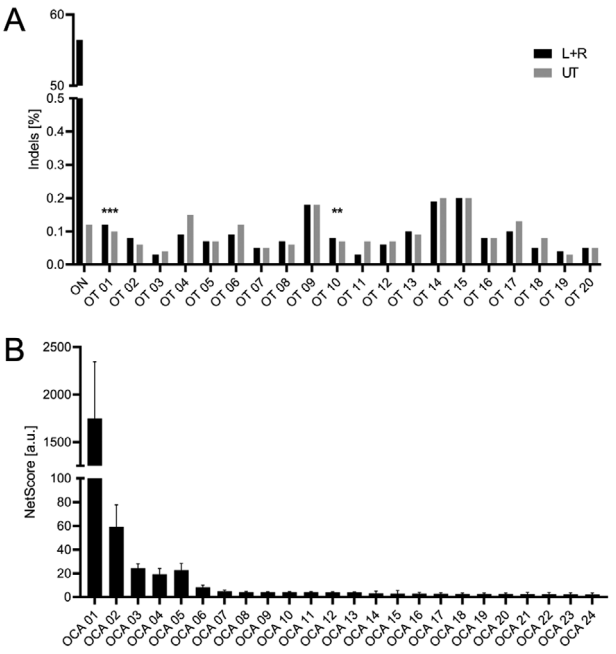

Figure 4. Off-target analysis in TALEN-edited CD4+ T cells. A) Targeted amplicon sequencing of the top 20 OTs predicted by PROGNOS and analyzed by CRISPResso2 (**$p \leq 0.01$, ***$p \leq 0.001$). B) Oligonucleotide-capture assay (OCA) performed in CD4+ T of three donors and measured in triplicate. Bars represent mean ± SD of all nine measurements after subtraction of scores obtained for mock-treated cells.

3. Discussion

Approaches to disrupt *CCR5* in human primary CD4+ T cells and HSCs have been previously described.[13–22] These reports demonstrated the feasibility of abrogating HIV entry, albeit with some drawbacks. This includes low gene disruption efficiencies, high off-target effects or absence of specificity analyses, missing potency analyses, and/or lack of compatibility with good manufacturing practice (GMP).[16,33–35] In this study, we present efficient editing of the *CCR5* locus in primary CD4+ T cells in a GMP-compatible manner. GMP compatibility is defined as a condition that uses GMP-compliant devices and research-grade reagents that are available as GMP-grade material as well. Under these specifications, we disrupted up to 90% of *CCR5* alleles with no notable off-target activity, suggesting that the employed TALEN are highly specific. Disrupting the region that encodes the N-terminus of the CCR5 protein in CD4+ T cells gave rise to cells resistant to infection with R5-tropic HIV-1 but, as expected, not X4-tropic virus. Importantly, *CCR5*-edited CD4+ T cells did not show any differences to non-edited control cells in the applied proliferation and potency assays. This indicates that TALEN-mediated editing of *CCR5* is efficacious and safe, opening a window for therapeutic applications in CD34+ HSCs.

Our data further demonstrate that low *CCR5* editing frequency is not sufficient to abrogate HIV infection, putting forward that efficient disruption that mediates bi-allelic gene knockouts are paramount to see clinical effects.[15] This is in line with a recently published case report confirming that a *CCR5* knockout frequency of 18% in transplanted HSCs is not sufficient to achieve clinical benefit.[20] Another crucial point for a clinical trial is to recruit patients exclusively positive for R5-tropic HIV variants since the absence of CCR5 would favor the propagation of X4-tropic viruses if present in the patient. The importance of proper examination was seen in the "Essen patient"[36] who suffered from an X4 rebound after allogenic transplantation of a Δ32 homozygous HSC graft. Performing a knockout of both coreceptor encoding loci, *CCR5* and *C-X-C chemokine receptor type 4* (*CXCR4*), can be considered for CD4+ T cells[19,37,38] but not for HSCs, as

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CXCR4 is essential, for example, for HSC homing and B cell development.[39,40]

A side effect of gene editing approaches is genotoxicity as a consequence of OT effects. OT activity can induce mutagenesis but also provoke chromosomal rearrangements, both potentially inducing cellular transformation. Employing highly specific designer nucleases is therefore paramount to mitigate the risk of such effects. Several tools are available to identify OT events, including *in silico* predictions which were used in[16,34] and known to miss important OTs. Another study[33] performed OT analysis using whole-genome sequencing, which is unable to detect rare OT events. In our study, we carefully evaluated OTs using both *in silico* prediction as well as by employing the unbiased, cell-based oligonucleotide capturing assay (OCA). We detected two rare OT events occurring in introns of genes, thus minimizing the risks of adverse effects. For instance, OT1/OCA3 in *CNOT10* was cleaved in 0.12% of cells. OCA identified three additional OTs, one of them in the *CCR2* gene. The OCA scores of these OTs were similarly low as for OCA3, suggesting OT activities in the range of 0.1%. While editing in terminally differentiated cells, like CD4+ T cells, mitigates the risk of developing malignancies, this is not the case for genome editing in long-lived multipotent stem cells. Importantly, all functional assays we performed in this study demonstrated that the *CCR5*-edited cells behaved as unedited cells, suggesting that TALEN expression did not have any negative impact on these cells. All in all, we demonstrate that our novel designed TALEN mediates *CCR5* disruption with high frequency and with low genotoxicity, so paving the way for clinical translation in CD34+ hematopoietic stem cells.

4. Experimental Section

CD4+ T Cells Cultivation and Editing: PBMCs were isolated from the leukocyte reduction system chambers obtained from the Blood Donation Center after informed donor consent. CD4+ T cells were isolated using CD4 MicroBeads (Miltenyi, Germany) and cryopreserved. After thawing, cells were cultivated in X-Vivo15 (Lonza, Switzerland) supplemented with 20 U mL^{-1} IL-2 (Miltenyi) and activated with T Cell Activation/Expansion Kit (Miltenyi). After 3 days, beads were removed and electroporation was performed by combining 5×10^6 CD4+ T cells with 10 µg of each TALEN mRNA or 5 µg of GFP mRNA.[41] Cells were electroporated in 100 µL electroporation buffer using P3 Primary Cell 4D-Nucleofector kit (Lonza) and program EO-115 or in 200 µL of BTXpress Cytoporation medium T (BTX, Harvard Bioscience, USA) (1 pulse 1200 V, 0.1 ms, 0.2 ms interval, 1 pulse 1200 V, 0.1 ms, 100 ms interval and 4 pulses 130 V, 0.2 ms, 2 ms intervals). For long-term culture, CD4+ T cells were reactivated with T Cell Activation/Expansion Kit every 7 days. The number of cells was determined on an automated cell analyzer (NucleoCounter NC-250, ChemoMetec, Denmark). Genomic DNA was extracted with DNeasy Blood & Tissue Kits (Qiagen, Netherlands). NGS or T7E1 assay was performed and analyzed as previously described.[42]

mRNA Production: For in vitro RNA transcription, 10 µg of the TALEN encoding plasmid containing a T7 promoter and an encoded poly(A) was linearized, purified with QIAquick Gel Extraction Kit (Qiagen), and RNA transcribed with mMESSAGE mMACHINE T7 Ultra kit (ThermoFisher, USA). The RNA pellets were resuspended in EB buffer (Qiagen) and stored at −80 °C.

Lentiviral Transduction: For production,[43] HEK293T cells (ATCC, CRL-3216) were grown in DMEM GlutaMAX I (Life Technologies, USA) supplemented with 10% FCS (GE Healthcare, UK), 10.000 U mL^{-1} penicillin, and 10 mg mL^{-1} streptomycin (GE Healthcare), and 100 mM sodium pyruvate (PAA, Austria). 15×10^6 cells were seeded per 15 cm plate 24 h before transfection. Before transfection, the medium was supplemented with chloroquine 1:1000 (Sigma-Aldrich, Germany), and polyethyleneimine transfection performed with 15 µg pMDLg/pRRE (Addgene), 3 µg pRSV-Rev (Addgene), 15 µg of the lentivirus encoding plasmid, and 5.1 µg of the envelope encoding plasmid (Bal_gp120, a kind gift of Dr. Boris Fehse, or pMD2.G, Addgene). After 12 h, the transfection mix was exchanged with a medium containing 10 mM sodium butyrate (Sigma-Aldrich). Supernatants were harvested 32 h and 56 h post-transfection, pelleted at 20 000 × *g* at 4 °C for 2 h on a 20% sucrose cushion using a swingout rotor. Pellets were resuspended in 50 µL of PBS and stored at −80 °C. For titration, lentiviral EGFP vectors were titrated on 5×10^4 PM1 cells (NIH AIDS Reagent Program[44]), cultivated in RPMI 1640 medium containing L-Glutamine (Life Technologies), 10% FCS (GE Healthcare), 10.000 U mL^{-1} penicillin, and 10 mg mL^{-1} streptomycin (GE Healthcare), and supplemented with 4 µg mL^{-1} polybrene, by spinoculation for 1 h at 200 × *g* at 32 °C. For transduction, 5×10^4 CD4+ T cells were cultured in X-Vivo15 medium (Lonza) containing 4 µg mL^{-1} polybrene with 6 transducing units per cell by spinoculation as above.

Flow Cytometry: GFP expression after lentiviral transduction and/or cell viability (7AAD, AppliChem, Germany) were assessed using BD Accuri C6 Flow Cytometer (BD Biosciences, USA). CCR5 expression was detected by labeling 1×10^5 cells in 50 µL of PBS with 2 µL of APC-labeled mouse anti-human CD195 antibody (BD Biosciences) for 20 min at room temperature. Cells were analyzed on BD FACS Canto-II (BD Biosciences).

HIV-1 Infection: HIV-1 provirus encoding plasmids were obtained from the NIH AIDS Research and Reference Reagent Program.[45,46] Virus stocks HIV-$1_{JR\text{-}FL}$ (R5-tropic) and HIV-$1_{NL4\text{-}3}$ (X4-tropic) were generated and titrated as previously described.[47] For infection, 2×10^5 CD4+ T cells were activated as above and infected with either HIV-$1_{JR\text{-}FL}$ or HIV-$1_{NL4\text{-}3}$ at MOIs of 0.01 and 0.001. At indicated timepoints, 50 µL of cell culture supernatant was harvested and used to determine p24 concentration by ELISA.[48]

Cytokine Release: 200 µL of supernatants were harvested from 1×10^6 cells per sample at indicated timepoints. Cytokine concentrations were determined using the Cytometric Bead Array for IFN-γ, IL-2, or TNF-α (BD Biosciences). Cells were stimulated with CD3/CD28/CD2 beads (Miltenyi), 1 µg mL^{-1} ionomycin (Merck-Millipore, Germany), or 10 ng mL^{-1} PMA (Sigma-Aldrich).

Off-Target Analyses: Potential OTs were predicted with PROGNOS (http://bao.rice.edu/cgi-bin/prognos/prognos.cgi) using the TALEN v2.0 algorithm.[31] Five mismatches per half-sites were allowed. Loci were PCR amplified using primers listed in Table S1, Supporting Information. Libraries were prepared with NEBNext Ultra II DNA Library Prep Kit (NEB), quantified with ddPCR Library Quantification Kit for Illumina TruSeq (Biorad, Germany), sequenced on Illumina MiSeq platform using MiSeq Reagent Kit v2, 500-cycles (Illumina, USA), and data analyzed with CRISPResso2.[49] *p* values were adjusted with the Benjamini & Hochberg[50] method. For OCA, 1×10^6 PBMCs (ALLCELLS, USA)/mL in X-Vivo15 medium (Lonza), supplemented with 5% human AB serum (Gemini, USA) and 20 ng mL^{-1} of IL-2 (Miltenyi), were activated using human T activator CD3/CD28 (ThermoFisher). 4 days later, 5×10^6 T cells in cytoporation medium T were electroporated with 20 µg of TALEN mRNA (10 µg per subunit) and 10 µL of pre-annealed oligodeoxynucleotides (dsODN, 100 µM) using AgilePulse MAX system (Harvard Apparatus, USA) and a 0.4 cm cuvette. Genomic DNA was extracted 3 days later, randomly sheared to 300 bp fragments by sonication (Covaris LE220-plus), fragments end-repaired/A-tailed (NEBNext Ultra End Repair/dA-Tailing Module), and NGS Y-adapters (TruSeq Annealed Adapter) added. Two rounds of anchored PCR using dsODN-specific and adapter-specific primers were performed. Adapter-specific (P5_1) and dsODN-specific primers were used in the first PCR. Adapter-specific (P5_2) primers, dsODN-specific primers P7, and primers adding the barcode and P7 sequence to the ends of the PCR product were used in the second PCR. PCR products were pooled and sequenced using Illumina NextSeq (2 × 150 bp). The resulting sequences reads were mapped to the human genome to identify potential OT sites.

Statistical Analyses: A time series analysis was performed to compare the samples in both the HIV-1 challenge and growth curves

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experiments.[30] The goodness of the fit between the full and the reduced models was tested by Anova where *p*-value indicates the probability that the two models were the same. *p*-values are indicated with * ≤ 0.05; ** ≤ 0.001; *** ≤ 0.0001. For all other analyses, the Student's *t*-test was applied.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

A.J., A.D., and P.D. are employees of Cellectis. J.G. and C.R. are employees of Ethris. T.I.C. and T.C. have sponsored research collaborations with Cellectis and Miltenyi Biotec. K.J.M. received travel grants and honoraria from Gilead Sciences, Roche Diagnostics, GlaxoSmithKline, Merck Sharp & Dohme, Bristol-Myers Squibb, ViiV, and Abbott. The University of Zurich received advisory board honoraria from Gilead Sciences and research grants from Gilead Science, Roche, and Merck Sharp & Dohme for studies in which K.J.M. serves as principal investigator.

Author Contribution

M.R. contributed to the formal analysis, investigation, methodology, validation, visualization, and writing the original draft. A.J. contributed to the formal analysis and methodology. Y.L.K. formal analysis, investigation, and methodology. M.H. contributed to the formal analysis, methodology, software, and validation. M.R. contributed to the formal analysis, visualization, and writing the original draft. G.A. contributed to the formal analysis and software. J.G., C.R., and C.M. provided the resources. A.D. contributed to the formal analysis and investigation. K.M. contributed to the conceptualization, formal analysis, funding acquisition, methodology, supervision, and writing the original draft. P.D. contributed to the conceptualization, funding acquisition, and resources. T.C. contributed to the conceptualization, funding acquisition, supervision, and writing the original draft. T.I.C. led the conceptualization, methodology, supervision, and visualization and contributed to the formal analysis, funding acquisition, and writing the original draft.

Data Availability Statement

The data that support the findings of this study are mainly available in the supplementary material of this article. Additional data are available upon request.

Keywords

C-C chemokine receptor type 5 editing, CCR5 knockout, human immunodeficiency virus type 1, HIV cure, transcription activator-like effector nuclease

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