Editing *CCR5*: A Novel Approach to HIV Gene Therapy

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Abstract Acquired immunodeficiency syndrome (AIDS) is a life-threatening disorder caused by infection of individuals with the human immunodeficiency virus (HIV). Entry of HIV-1 into target cells depends on the presence of two surface proteins on the cell membrane: CD4, which serves as the main receptor, and either CCR5 or CXCR4 as a co-receptor. A limited number of people harbor a genomic 32-bp deletion in the *CCR5* gene (*CCR5Δ32*), leading to expression of a truncated gene product that provides resistance to HIV-1 infection in individuals homozygous for this mutation. Moreover, allogeneic hematopoietic stem cell (HSC) transplantation with *CCR5Δ32* donor cells seems to confer HIV-1 resistance to the recipient as well. However, since *Δ32* donors are scarce and allogeneic HSC transplantation is not exempt from risks, the development of gene editing tools to knockout *CCR5* in the genome of autologous cells is highly warranted. Targeted gene editing can be accomplished with designer nucleases, which essentially are engineered restriction enzymes that can be designed to cleave DNA at specific sites. During repair of these breaks, the cellular repair pathway often introduces small mutations at the break site, which makes it possible to disrupt the ability of the targeted locus to express a functional protein, in this case CCR5. Here, we review the current promise and limitations of *CCR5* gene editing with engineered nucleases, including factors affecting the efficiency of gene disruption and potential off-target effects.

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Introduction

As HIV research continues to identify novel druggable viral and host factors that promote virulence and latency, the long-term clinical management and survival of HIV-positive individuals has improved considerably. Combination therapies, like highly active antiretroviral therapy (HAART), continuously suppress HIV replication while attenuating the development of escape mutants. However, because HAART is unable to clear latent viral reservoirs [1, 2], patients require lifelong treatment, which not only is expensive but has been associated with multiple adverse side effects and the development of drug-induced diseases [3–5]. The sustained antiviral efficacy of these therapeutic regimens is also strongly influenced by the compliance of each patient, which remains a key factor in managing not only the HIV infection but also the development of any accompanying disease [6]. Ideally, a therapy aimed at eliminating both the replicating and latent viral populations would provide a long awaited cure.

HIV-1 fusion with the cell membrane and ensuing virus entry is an intricate process that requires the expression of both the CD4 transmembrane glycoprotein as well as an associated seven-pass G-protein coupled chemokine co-receptor, CCR5 or CXCR4 (Fig. 1), a receptor combination typically found on CD4+ T cells, macrophages and dendritic cells [7]. Virus attachment is mediated by gp120, a viral surface glycoprotein located in the lipid membrane of the HIV-1 virion. Initially gp120 binds to CD4, which then facilitates the sequential attachment of gp120 to either the CCR5 or CXCR4 co-receptors. R5-tropic viruses, most prominently detected during the early stages of HIV-1 infection, bind to the CCR5 co-receptor, whilst X4-tropic viruses bind to CXCR4. The subsequent conformational change of the viral envelope protein exposes the viral gp41 glycoprotein, which mediates fusion with the target cell membrane. The resulting formation of a transmembrane pore enables the delivery of the viral capsid, which initiates viral integration and replication. Whereas the majority of the population is susceptible to infection, a small percentage of individuals are protected from infection with particular HIV strains. This resistance to HIV infection has been linked to naturally occurring genetic variations, including polymorphisms within the locus encoding the CCR5 co-receptor [8–14]. As a consequence, rational design of novel therapeutic strategies has also focused on blocking viral entry with small molecule drugs or genetic engineering to generate HIV-resistant T cells.

CCR5 as a Target for HIV Antiretroviral Therapy

CCR5 was first identified as the prominent co-receptor for R5-tropic viruses following the discovery that three chemokines, RANTES (CCL5), MIP-1α (CCL3), and MIP-1β (CCL4), impede HIV-1 binding [15]. Ever since, pharmaceutical companies have focused heavily on the development of HIV antiretroviral therapies based on entry and fusion inhibitors. One such drug, Maraviroc, binds to the

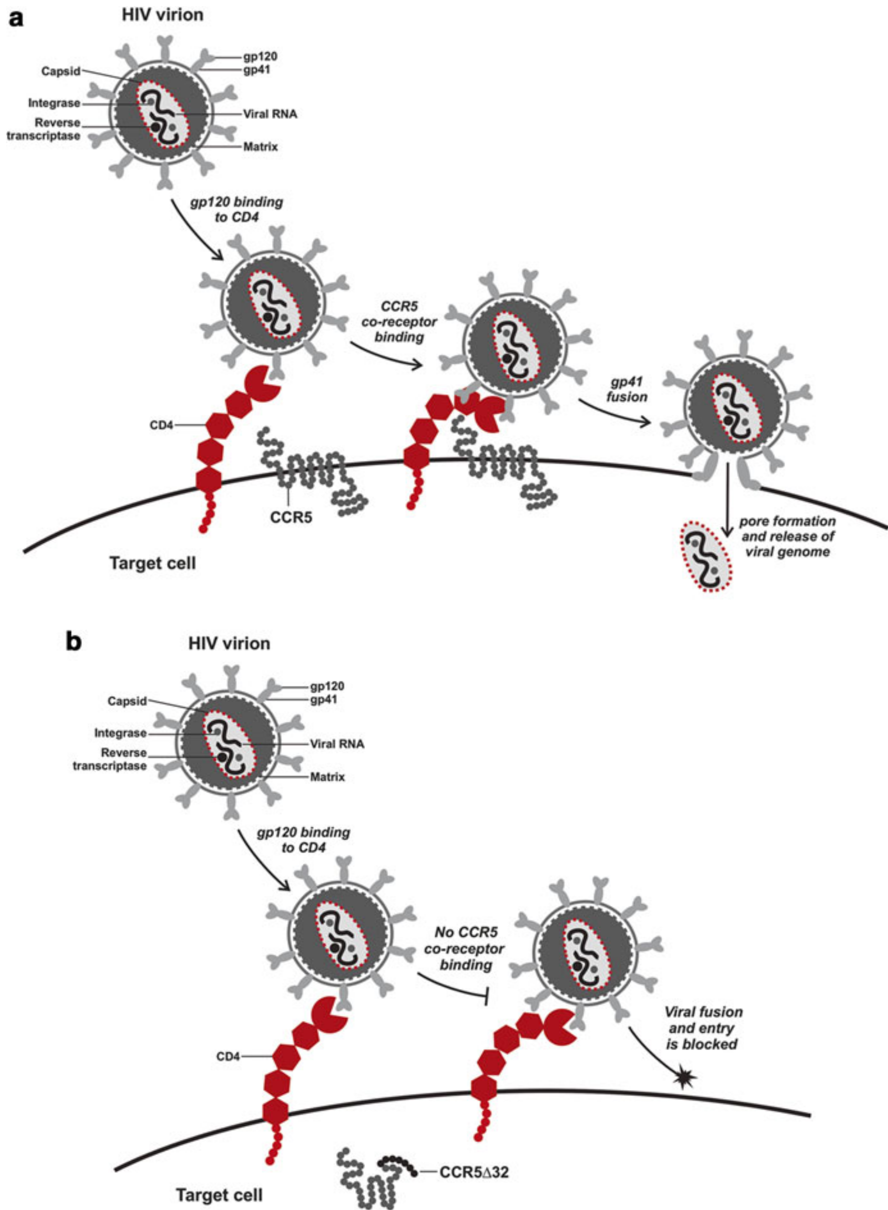

Fig. 1 Attachment and entry of R5-tropic HIV-1. (**a**) The HIV virion initially binds to target cells through interactions between the viral gp120 surface glycoprotein and the CD4 receptor. The CD4 receptor then draws the virion closer to the target cell, facilitating the interaction between the CCR5 co-receptor and gp120. This triggers a conformational change, allowing the gp41 glycoprotein to fuse to the cell membrane in order to create a transmembrane pore. The viral capsid, which contains the HIV RNA, integrase and reverse transcriptase, is then released into the target cell. (**b**) Initial binding of the HIV-1 virion occurs as described above; however, the CCR5Δ32 mutant form of this co-receptor is severely truncated and remains cytosolic, ultimately eliminating the gp120 binding site. As there is no co-receptor binding, the conformational change required to expose the gp41 protein is blocked, preventing viral fusion and entry

transmembrane domains of CCR5, ultimately preventing viral attachment and fusion [16]. Based on successful clinical trials, Maraviroc has been approved for HIV-1 treatment in both Europe and the USA. However, as with most currently available HIV therapies, viral escape mutants have been isolated [17–19]. Furthermore, as Maraviroc binds to the CCR5 co-receptor, it is not effective against X4-tropic viral infections.

Moving away from traditional HAART therapy, the adoptive transfer of synergistic T cells and allogeneic stem cells has been investigated as potential curative treatments. Initially, the efficacy of synergistic or autologous transplantation of hematopoietic stem/progenitor cells (HSPCs) was described in HIV-positive patients that had developed lymphomas [20–23]. Whilst patients remained on antiviral therapy, the myeloablative conditioning required prior to transplantation facilitated the reconstitution of the T cell compartment. In 2007, Hoffmann and colleagues reported that the adoptive transfer of T cells between HIV-1 discordant twins resulted in improved $CD4^+$ T cell counts [24]. The patients remain on antiretroviral therapy and required a total of 12 transfers to achieve a sustained expansion of $CD4^+$ cells. Since myeloablative conditioning was not performed before adoptive transfers, these results suggest that HLA-matched T cells could help reprise aspects of the immune system, provided that HIV viral loads are continuously repressed. Nonetheless, neither approach is curative, as patients still require continuous antiretroviral therapy post-transplantation.

In contrast, an allogeneic HSPC transplantation from a donor homozygous for the *CCR5Δ32* mutation has given rise to the first described permanent “cure” for HIV [25–27]. The *CCR5Δ32* mutation was originally identified in a small group of people who, despite being repeatedly exposed to HIV, did not contract the disease [12, 13]. This 32 base pair deletion in the *CCR5* gene induces a frameshift mutation and the resulting truncated protein does not support gp120 binding, ultimately preventing HIV-1 infection (Fig. 1). Although individuals who are homozygous for this mutation are resistant to R5-tropic HIV-1 infection, they remain susceptible to X4-tropic strains [28]. A number of studies showed that HIV-positive patients, who are heterozygous for the *CCR5Δ32* mutation, have reduced disease progression and better overall prognosis than patients who are homozygous for the wild-type *CCR5* gene [10, 11, 29, 30]. In 2009, Hütter and colleagues described the first curative allogeneic HSPC transplantation using an HLA-matched donor who was homozygous for the *CCR5Δ32* mutation [26]. Timothy Brown (alternatively referred to as the “Berlin patient”), an HIV-positive patient on HAART therapy, received the initial HSPC transplant after developing acute myeloid leukemia (AML), which was refractory to induction and consolidation chemotherapy. As his AML relapsed, a second HSPC transplantation from the same homozygous *CCR5Δ32* donor was performed. To date, the patient remains cancer-free and HIV negative in the absence of HAART [25–27], suggesting that homozygous *CCR5Δ32* HSPC transplantation could be used to cure not only the blood-related malignancy but also HIV-1 infection. Although this presents an idealistic approach, the number of homozygous *CCR5Δ32* donors is low, since only approximately 1 % of the Caucasian population has this HIV-1-resistant genotype [30, 31]. Accordingly, much research has focused on engineering homozygous *CCR5Δ32*-like mutations in patient-derived HSPCs and T cells using designer nucleases.

Gene Editing with Designer Nucleases

Designer nucleases are engineered enzymes that are comprised of a DNA binding domain, tailored to bind to a specific target sequence, and a DNA cleavage domain (Fig. 2). Binding of the engineered nuclease to a defined genomic target site results in the formation of a DNA double stranded break (DSB) which, in turn, elicits cellular DNA repair mechanisms that can be exploited to achieve targeted and permanent genetic modifications. Mammalian cells rely on two major DSB repair pathways: non-homologous end joining (NHEJ), which is active throughout the cell cycle, and homologous recombination (HR) based repair, which is restricted to the S/G2 phase.

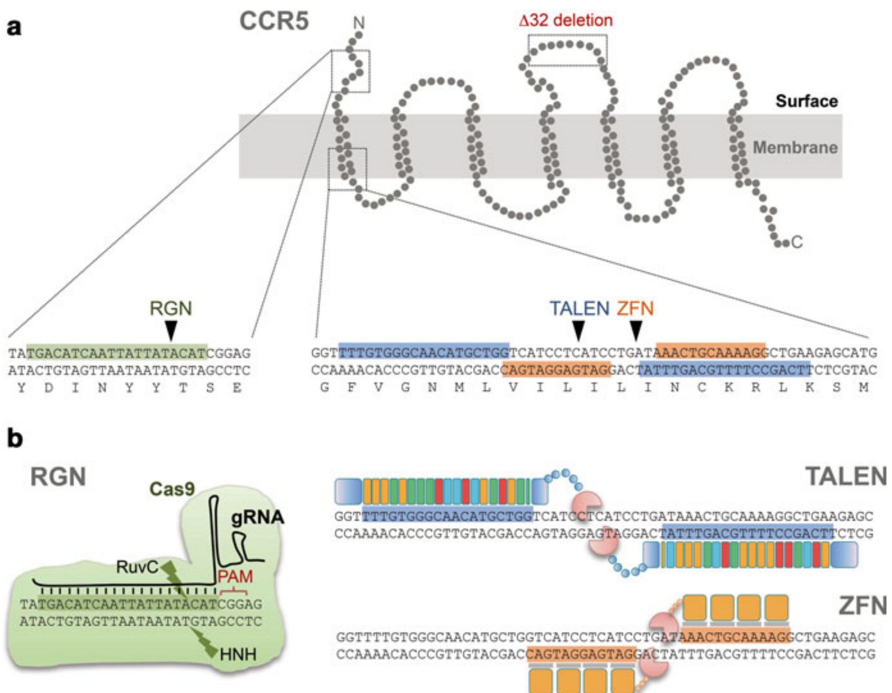

Fig. 2 Designer nucleases to disrupt *CCR5*. (**a**) Schematic of the CCR5 protein localized to the cellular membrane. The *dotted boxes* indicate the corresponding regions of the genomic locus targeted by designer nucleases as well as the location of the Δ*32* deletion. Three different designer nuclease platforms have been efficiently engineered to knock out *CCR5* and the corresponding DNA target sites are indicated in *green* (RGN), *light blue* (TALEN), and *orange* (ZFN). The putative cleavage sites are indicated (*black triangles*) (**b**) Designer nucleases. RGENs are composed of the Cas9 nuclease and a guide RNA (gRNA) that directs the enzyme to the target site. The protospacer adjacent motive (PAM) required by the Cas9 enzyme to recognize and cleave the target site is indicated in *red*. The two nuclease domains within the Cas9 protein (RuvC and HNH) are highlighted. TALEN or ZFN monomers include a modular DNA binding domain that is engineered to recognize a specific DNA target sequence. Each TALE module specifically recognizes one nucleotide in the target subsite, while a ZF module binds to a nucleotide triplet. A short linker connects the respective DNA binding domain to the cleavage domain of the *Fok*I restriction enzyme (*light red*), which cuts the DNA upon dimerization of the two monomers at the target site

As compared to HR, NHEJ is an error-prone pathway, which can be harnessed to insert small insertion/deletion (indel) mutations at the DNA break in order to inactivate a target gene, such as *CCR5*. Conversely, HR relies on the genetic information contained in the sister chromatid for the accurate repair of a DSB. For gene editing, this pathway can be exploited by including a donor DNA template with specific sequence homology during the generation of nuclease-mediated DSBs [32, 33]. In this setting, the genetic information is transferred from the donor DNA to the target locus, thus allowing precise genomic modifications.

Dimeric zinc-finger nucleases (ZFNs) have been traditionally used for genetic modifications [34]. The DNA binding domain is comprised of multiple zinc-finger modules, each recognizing three to four nucleotides in a sequence-specific manner. However, generating highly active ZFNs with novel specificities is challenging and cumbersome, as context-dependent interactions between individual modules within the zinc-finger array affect the overall binding efficiency [35]. In the last 15 years, ZFNs have been successfully used in basic research to study gene function [36–44] and to correct genetic defects underlying human disorders for therapeutic purposes [45–47] in preclinical settings. Their relatively small size has allowed ZFNs to be delivered using the most common viral and non-viral platforms as well as a direct protein delivery [48].

For therapeutic applications, a high specificity of the designer nuclease is of utmost importance, as off-target cleavage activity poses obvious concerns with regard to genotoxicity. Two studies assessing the genome-wide specificity of the *CCR5*-specific ZFN pair revealed a considerable level of non-specific off-target activity [49, 50]. In view of the complexity of generating highly specific ZFNs, the discovery of a novel modular DNA binding domain identified in transcription activator-like effectors (TALEs) of plant pathogens has provided new momentum to the genome engineering field. TALE-based nucleases (TALENs) can be easily customized to target any given sequence (Fig. 2) due to their simple recognition code in which a TALE module specifically recognizes one nucleotide [51–53]. When compared to an existing ZFN, some *CCR5*-specific TALENs showed similar activity but lower cytotoxicity [54, 55]. While more work needs to be invested to dissect the specificity signature of designer nucleases, initial results suggest that TALENs seem to harbor a rather high specificity [55–57]. TALENs have hence evolved as a valid alternative for the generation of transplantable HIV-resistant T cells. Unlike ZFNs, TALENs are relatively large proteins with a highly repetitive structure. While adenoviral vectors can be used to deliver single TALEN monomers, lentiviral vectors have failed to transfer intact TALEN encoding expression cassettes [58]. As a consequence, many labs have relied on in vitro transcribed mRNA or plasmid DNA to deliver the TALENs.

The newest addition to the toolbox for genome engineers is of bacterial origin as well. The clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system is used by prokaryotes to defend themselves against invading DNA [59]. It consists of the Cas9 cleavage enzyme complexed to a guide RNA strand that directs the enzyme to a 20-nucleotide long target site [60, 61]. Exchanging a specific portion of the gRNA molecule allows researchers to redirect the Cas9 cleavage activity

to a user-defined target sequence (Fig. 2). This versatile platform, also known as RNA-guided nuclease (RGN) technology, holds many advantages over both ZFNs and TALENs. The most obvious one is the simplicity to customize the enzyme to target any sequence of choice by simple molecular cloning techniques [62, 63]. Moreover, delivering the Cas9 protein with more than one gRNA molecule allows multiplexing, i.e., to target several sites simultaneously [64]. Although RGNs have been shown to target *CCR5* efficiently [65, 66], concerns regarding their specificity have been raised [67–69]. On the other hand, further advances, such as Cas9 nickases [70], the use of truncated guide RNAs [71], and dimeric RNA-guided *Fok*I nucleases [72], have shown promise to generate more specific RGNs.

Target Cells

Two potential cellular targets have been envisioned for a *CCR5* disruption-based HIV therapy: CD4+ T cells, which are the mature lymphocytes infected by HIV, or CD34+ HSPCs, which would give rise to HIV-resistant T cells and macrophages.

In the first scenario, patient derived CD4+ T cells will be collected by apheresis and modified ex vivo using designer nucleases [47]. Modified cells will then be amplified in vitro and subsequently reintroduced in the patient (Fig. 3). For the therapy to be effective, a large number of cells are required to retain proficient proliferative and effector functions. Consequently, patients enrolled in such trials should have a CD4+ T cell count above a set threshold that allows collection of enough CD4+ T cells to be genetically altered and subsequently expanded ex vivo. Transfer of the *CCR5* modified T cells will at least temporarily restore T cell immunity of the patients. Discontinuation of antiretroviral medication would allow the virus to infect

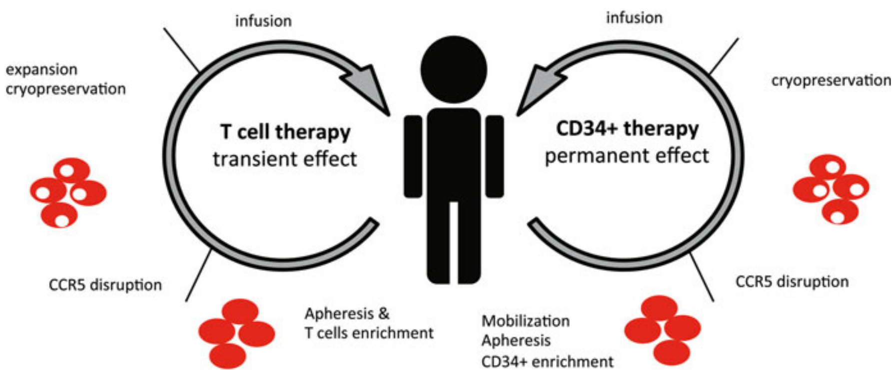

Fig. 3 Clinical application of modified T cells and CD34+ cells. After collection of cells by apheresis, CD4+ T cells or CD34+ hematopoietic stem cells are enriched and *CCR5* disruption is accomplished by expression of designer nuclease. T cells are expanded ex vivo before adaptive transfer. In case of CD34+ cells, chemotherapy of AIDS lymphoma patients will assist the engraftment of the modified cells

and replicate in susceptible cells. Over time, only cells devoid of CCR5 will be able to expand in presence of the virus. Once the modified pool of T cells is depleted as a result of cellular senescence, the transfer of modified T cells can be repeated. Of note, the *CCR5* disrupted cells remain susceptible to CXCR4-tropic strains and discontinuing HAART could result in a flare of these X4-tropic strains. Importantly, however, a viral rebound was not observed in the "Berlin patient" although he was positive for CXCR4-tropic strains [26, 73]. Nonetheless, to overcome this potential limitation, a simultaneous disruption of *CCR5* and *CXCR4* has been reported in primary CD4+ T cells, and protection from both R5 and X4-tropic virus was verified in a mouse model [74].

The second approach is directed towards the targeting and manipulation of CD34+ HSPCs [45]. The main advantage of this strategy when compared to CD4+ T cell targeting is the ability of modified CD34+ cells to engraft and produce a long-lasting effect. HSPCs continuously differentiate in all the hematopoietic lineages, including T cells and macrophages that can be infected by HIV. The downside is that stem cells are difficult to manipulate and tend to lose their differentiation potential when cultured ex vivo. In addition, transplantation of HSPCs requires a mild preconditioning regimen to provide adequate space in the bone marrow for engraftment of the modified HSPCs. In this setting, leukopoiesis will occur from both modified and non-modified CD34+ cells, and the survival advantage in the presence of replicating HIV will occur on the level of CD4+ T cells and macrophages.

The advantages of the two approaches are apparent: since the genetic modification is performed in autologous cells, there is no need for HLA matching, which significantly decreases the risk of developing graft-versus-host-disease or graft rejection. Additionally, there is no need for post-transplantation immunosuppressive therapy. The patients will be provided with an autologous pool of HIV-resistant cells, which restores the immune system either transiently or permanently. An open question is whether active clearance of HIV reservoirs will occur in an autologous setting where the graft-versus-host effect is not present.

Applying Designer Nucleases for HIV Gene Therapy

Many HIV gene therapy trials based on the ex vivo modification of CD4+ T cells or HSPCs have used ribozymes, aptamers, and siRNAs [75]. Although none of these studies have reported clinical benefit in terms of decreased viral load or protection from HIV replication so far, they showed promising outcomes in terms of safety, long-term engraftment and survival of modified peripheral cells [76, 77], including maintenance of the genetic modification in mature myeloid and T cells [77, 78]. These positive aspects were the basis for the clinical trials aimed at disrupting the *CCR5* co-receptor gene with designer nucleases. This strategy has a major advantage over conventional knockdown approaches using RNA interference, since it permits the generation of HIV-resistant cells after a single treatment. Indeed, when *CCR5*-specific ZFNs were delivered to primary human T cells by adenoviral

transduction, a population of HIV-resistant T cells was observed in vivo 50 days after transplantation in a murine HIV infection model [47]. A similar approach was applied to human CD34+ HSPCs by nucleofection of DNA expression plasmids encoding *CCR5*-specific ZFNs. Following transplantation in a humanized HIV mouse model, *CCR5* disrupted cells showed selective survival after challenge with HIV [45]. However, nucleofection of plasmid DNA into primary cells, and in particular into stem cells, can be associated with considerable cytotoxicity. This drawback has been recently overcome by delivering ZFNs in the form of in vitro transcribed mRNA [79].

Based on these preclinical accomplishments, the use of ZFNs as an HIV gene therapy for the generation of transplantable autologous HIV-resistant T cells has entered phase I/II clinical trials. The protocol was similar in all studies (Fig. 3): CD4+ T cells were isolated from HIV patients and transduced with an adenoviral vector expressing a ZFN pair targeted to *CCR5*. After ex vivo expansion, the cells were reinfused into the patients. In the first published study [80], 12 patients were recruited and received one infusion of 10 billion CD4 T cells. Six patients underwent a 12-week treatment interruption 4 weeks after infusion. The primary objective was the assessment of safety, while secondary objectives included the evaluation of increased CD4+ T cell counts, the trafficking of *CCR5*-modified cells to the gut mucosa, and a decrease in viral load. The modified CD4+ T cells engrafted and were detected in the patients up to 42 months after transfer. Moreover, modified cells were detected in all biopsies of the rectal mucosa, revealing successful trafficking. Treatment was prematurely discontinued and HAART reinitiated in two patients because of a rise in HIV RNA levels above the threshold. In four patients who completed the 12-week HAART interruption, a relative survival advantage of the modified cells was observed. The decrease in virus load correlated with the number of circulating cells carrying biallelic modifications at the *CCR5* locus. Actually, the one patient with undetectable HIV load after treatment interruption was found to be heterozygous for the *CCR5Δ32* allele. In summary, this first-in-human application of ZFN designer nucleases showed infusion of *CCR5*-modified T cells to be safe and well tolerated, and led to reduced virus loads in some patients. However, complete eradication of HIV could not be achieved, probably due to suboptimal engraftment and the low number of cells carrying a biallelic disruption. It will be interesting to learn what further safety evaluations involving a larger sample size and a long-term follow-up will reveal.

Based on these promising results, more studies have been initiated, including one which specifically enrolled ten patients heterozygous for *CCR5Δ32* (NCT01044654). As expected, the biallelic modification frequency in the *CCR5Δ32* cohort was doubled as compared to normal, and three out of eight subjects with high levels of engraftment had virus loads below detection limit up to 20 weeks following interruption of HAART (Sangamo Biosciences Inc., Richmond, CA: press release on Dec. 6, 2013). To improve engraftment and increase of CD4+ T cell counts, another study involving 12 patients has evaluated the use of escalating doses of cyclophosphamide (NCT01543152), a drug used for non-myeloablative lymphodepletion to enhance adoptive T cell transfer [81]. Conditioning with cyclophosphamide was

reported to be safe and well tolerated, and a dose-dependent increase was observed for both normal and modified CD4+ T cells (Sangamo Biosciences Inc., Richmond, CA: press release on Dec. 6, 2013).

Since HIV can also use the *CXCR4* co-receptor for viral entry, an alternative strategy for HIV treatment using *CXCR4*-specific ZFNs delivered by adenoviral vectors has been investigated [82]. However, while *CCR5* disruption seems to be well tolerated by the immune system, the CXCR4 receptor plays an important role in immune regulation, especially in B cell development [83], and its disruption raises concerns of potential deleterious effects. ZFNs have not only been used to create HIV-resistant cells but novel strategies have also been developed to eradicate the provirus from infected cells [84]. While promising, this approach may be limited by the difficulties associated with targeting the integrated provirus, especially in rare cells like resting T cells or latently infected cells.

Concluding Remarks

The presented clinical results are encouraging and validate the *CCR5* knockout strategy as an important development in fighting HIV infection. Furthermore, the data underline the number of T cells with biallelic *CCR5* disruption to be a key factor for clinical success. On the other hand, off-target cleavage of designer nucleases is a major concern. This is especially true if applied in multipotent stem cells predestined to be transplanted in patients, as the potentially mutagenic events could prompt a malignant phenotype. Hence, specificity of engineered nucleases will be the second key factor required to pave the road for this new line of gene therapy into the clinic.

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