Engineering T Cells to Functionally Cure HIV-1 Infection

Rachel S Leibman and James L Riley

Department of Microbiology and the Abramson Family Cancer Research Institute, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania, USA

Despite the ability of antiretroviral therapy to minimize human immunodeficiency virus type 1 (HIV-1) replication and increase the duration and quality of patients’ lives, the health consequences and financial burden associated with the lifelong treatment regimen render a permanent cure highly attractive. Although T cells play an important role in controlling virus replication, they are themselves targets of HIV-mediated destruction. Direct genetic manipulation of T cells for adoptive cellular therapies could facilitate a functional cure by generating HIV-1–resistant cells, redirecting HIV-1–specific immune responses, or a combination of the two strategies. In contrast to a vaccine approach, which relies on the production and priming of HIV-1–specific lymphocytes within a patient’s own body, adoptive T-cell therapy provides an opportunity to customize the therapeutic T cells prior to administration. However, at present, it is unclear how to best engineer T cells so that sustained control over HIV-1 replication can be achieved in the absence of antiretrovirals. This review focuses on T-cell gene-engineering and gene-editing strategies that have been performed in efforts to inhibit HIV-1 replication and highlights the requirements for a successful gene therapy–mediated functional cure.

Received 9 January 2015; accepted 13 April 2015; advance online publication 19 May 2015. doi:10.1038/mt.2015.70

INFLUENCING MORE HIV-1–SPECIFIC T CELLS FAILS TO CONTROL HIV-1 INFECTION

Attempts to manufacture T cells as therapeutic agents to treat the human immunodeficiency virus type 1 (HIV-1) disease have been ongoing for over two decades. After discovering the critical role that cytotoxic T cells (CTLs) play in controlling HIV replication in vivo, researchers sought to augment the activity of natural CTLs. When reinfusion of a single expanded Nef-specific CTL clone led to virus escape, it became clear that approaches that limited HIV escape would have to be employed, including more broadly targeted CTL populations. Along these lines, Lieberman et al. expanded polyclonal CD8 T cells from patients by using autologous B-LCL lines pulsed with a mixture of Env, Gag, and Nef peptides prior to reinfusion. However, the decreases in plasma and cell associated virus were minimal and not statistically significant at 24 weeks postinfusion. Similarly, Tan et al. infused two CTL clones that were rapidly eliminated upon reinfusion, possibly due to an overstimulated and overly-mature Fas+/CD28- phenotype, exacerbated by the lack of CD4 T cell help. Toxicity concerns then led to the incorporation of the hygromycin phosphotransferase-thymidine kinase (HyTK) suicide gene, which would lead to cell death in the presence of ganciclovir. Riddell et al. selected Gag-specific CTLs and incorporated a retrovirally delivered HyTK suicide vector; however, this evoked a CTL response against the modified gene itself and elimination of infused CTLs. To track homing and persistence, Brodie et al. retrovirally modified Gag-specific CTLs to express the neomycin phosphotransferase (neo) gene. Although a transient decrease in productively infected cells in the LN was observed, and neo-marked CTLs were colocalized with HIV RNA+ cells in the lymph nodes, CTL persistence declined rapidly. Altogether, these early studies highlight the difficulty of engineering effective, sustained, and safe HIV-specific T cell therapies.

A CASE FOR GENE THERAPY TO TREAT HIV-1 INFECTION

With 20 plus years of additional wisdom, it is interesting to consider why these initial clinical trials failed to show durable control of HIV replication. For one, the technology to expand T cells for adoptive T-cell therapy was still in its nascent stage and has subsequently improved significantly, accelerated by success in cancer adoptive immunotherapy. Initial efforts to expand single cells in the presence of high IL-2 levels over a period of months have evolved into procedures that manufacture equivalent or higher numbers of cells over a period of 10 days, with better engraftment potential and more robust effector activity. Technological advances in vector design have optimized transgene expression by incorporating strong promoters, enhancer elements, nuclear translocation signals, and posttranscriptional regulatory elements. Conceptually, the field has a better understanding of the immunological challenges surrounding adoptive therapy for HIV. It is now known that the immune system makes a robust response to HIV infection, and HIV-specific CD8 and CD4 T cells can be readily identified in untreated, HIV-positive individuals. However, HIV has largely figured out how to evade the natural cell-mediated immune response. Thus, the rationale to infuse

Correspondence: James L Riley, Department of Microbiology and the Abramson Family Cancer Research Institute, University of Pennsylvania, Perelman School of Medicine, Philadelphia, Pennsylvania, USA. E-mail: riley@exchange.upenn.edu
more HIV-specific T cells is not as sound as the rationale to infuse better HIV-specific T cells. Specifically, T cells that persist in vivo, maintain cytolytic activity, and target epitopes required for virus replication so that escape would be accompanied by a decrease in viral fitness. Moreover, infusing billions of HIV-specific CD8 T cells without HIV-specific CD4 T cell help is akin to deploying soldiers without the necessary supplies to sustain the fight. There might be some short-term benefit, but it is difficult to imagine how a war might be won with this strategy. Unfortunately, rapid infection and depletion of these HIV-specific CD4 T cells negates and complicates the benefits of infusing CD4 T cells to support CD8 cytolytic effector functions. This review discusses how the use of genetic engineering could address how to build a better CD8 effector T cell and how to restore HIV-specific CD4 T cell help.

REDIRECTING POLYCLONAL T CELLS TO TARGET HIV-1

Once the propensity for CTL-induced virus escape was established in the early adoptive therapy trials, researchers next sought to improve upon the natural cell-mediated cytotoxic responses by engineering artificial antigen receptors. Conceptually, these receptors have advantages over traditional T-cell receptors (TCRs) in that they can be affinity enhanced to exhibit broader epitope reactivity or can be designed to bind intact, nonprocessed viral proteins, similarly broadening the scope of HIV-specific CTL antigens. Importantly, these receptors need to be designed to target sequences from which escape leads to a significant loss of viral fitness.

AFFINITY ENHANCED TCRs FOR ADOPTIVE T-CELL THERAPY

Joseph et al. demonstrated that polyclonal CD8 T cells could be redirected to target HIV-infected cells upon transduction with a lentivirus expressing the HIV Gag-specific SL9 TCR, which recognizes an HLA-A*02 restricted P17 epitope SLYNTVTATL (A2-SL9) and is associated with lower plasma virus levels during chronic HIV infection.19 These cells lysed A2-SL9-expressing target cells and were able to greatly reduce HIV infection in a SCID mouse model of HIV infection. The low inherent affinity of TCRs for their peptide-major histocompatibility complex (MHC) binding partners, in the general range of 1–100 μmol/l, led researchers to hypothesize that enhanced TCR binding affinity could lead to more powerful and sustained CTL responses.20 Affinity enhancement was invoked successfully to modify the HLA-A*01 restricted TCR specific for the NY-ESO-1 testes-specific tumor antigen. In early clinical trials, 5 out of 11 melanoma patients and 4 out of 6 synovial cell sarcoma patients experienced complete or partial responses to NY-ESO-1 TCR-transduced T-cell infusions.21 Varela-Rohena et al.22 reported a similar method to enhance the A2-SL9 TCR, producing a TCR with picomolar affinity for its cognate antigen that could control both CCR5- and CXCR4-utilizing strains of HIV better than the wild-type (WT) A2-SL9 TCR. Furthermore, the enhanced affinity TCR produced higher levels of the cytokines IL-2, MIP-1β, and IFNγ in response to A2-SL9-expressing K562 artificial antigen presenting cells (aAPCs) and could control common SL9 escape variant presentations not recognized by WT SL9 TCR. A clinical trial (NCT00991224) was initiated to examine the effects of infusing WT or affinity enhanced SL9 TCR-transduced T cells in HIV-infected patients.

Unfortunately, off-target toxicity that resulted in the death of two participants in a different trial with an affinity-enhanced HLA-A*01 restricted MAGE-A3-specific TCR for myeloma and melanoma patients caused concern over the ability of investigators to predict and model the target specificity of affinity enhanced TCRs.23 In the MAGE-A3 trial, the high affinity TCRs gained the capacity to bind an epitope from the unrelated protein titin, expressed on contracting cardiac tissue, and resulted in cardiac toxicity.24 As this protein is only expressed on beating cardiomyocytes and not traditionally cultured cardiac myocytes, this off-target killing capacity was not identified in preclinical toxicity tests. The results of this trial underscored the need for thorough pre-clinical bioinformatics screening to identify all proteins containing the minimal amino acid sequences required for TCR recognition,24 combined with sophisticated cell culture techniques to identify off-target CTL reactivity to these proteins. After careful discussions with NIH, FDA, and IRB, NCT00991224 was closed before any patient received T cells transduced with a high affinity A2-SL9-specific TCR. The use of T cells expressing high-affinity TCRs to target HIV, and particularly the HIV reservoir, is still an attractive concept, predicated upon a better understanding of the safety and specificity of these approaches.

CHIMERIC ANTIGEN RECEPTORS FOR ADOPTIVE THERAPY

Chimeric antigen receptors (CARs) are hybrid antigen receptors in which an extracellular antigen binding domain is linked to intracellular T-cell activation domains, most commonly the CD3 zeta chain.25–27 More recently, second- and third-generation CARs have been developed by including additional costimulatory domains such as 4-1BB and/or CD28 to increase proliferation and survival of modified cells.12,28,29 Unlike a TCR, where antigen binding is physically uncoupled from signal transduction, CARs accomplish both these functions from a single protein. While this limits the repertoire of potential targets to surface expressed proteins, it allows T cells to bind targets in a high affinity, TCR-independent, MHC-unrestricted manner. Unlike TCRs, CARs avoid the potential danger of mispairing with endogenous alpha and beta chains to generate off-target specificities and do not compete with TCRs for limited levels of endogenous CD3 complexes.25 CARs have been employed successfully for cancer adoptive therapy, mediating remission in ~80% of acute lymphocytic leukemia patients,30–32 and development for use in solid tumors is well under way.33,34

CARs that bind the HIV Env glycoprotein, which is expressed on the surface of virions and HIV-infected cells, have been created using HIV-specific antibody single-chain variable fragments or the host protein CD4 as the antigen binding moiety.35–36 Preclinical experiments determined that CAR-transduced CD8 T cells specifically lysed Env-expressing cells in vitro, and a gammaretroviral vector incorporating the CD4-based CAR entered four clinical trials.37–40 Despite the possibility that surface expression of CD4 from the CD4 CAR could render transduced CD8 T cells susceptible to infection, the persistence of CAR T cells was striking, with the modified cells detectable in 98% of samples.
tested up to 11 years postinfusion. Linear mixed-effects modeling predicted a decay rate of over 16 years, with gene expression in 11 out of 13 patients. This persistence was speculated to be the result of culture methods that resulted in a high proportion of central memory cells, combined with expression of a nonimmunogenic transgene that may have been intermittently stimulated in vivo through low-affinity interactions with MHC class II molecules or HIV Env due to bursts in virus replication. The low affinity of CD4 for MHC class II likely prevented modified cells from attacking normal host cells. Although CAR-transduced cells could not be sorted in the postinfusion patient samples due to the inability to distinguish CAR CD4 from endogenous CD4, patient peripheral blood mononuclear cells (PBMCs) were stimulated with anti-CD4 loaded K562 aAPCs and zeta chain copy number was found to increase, suggesting the ability to proliferate in response to antigen. While none of the clinical trials led to durable reductions in viral loads, an important outcome of these trials was the lack of related serious adverse events, indicating the safety of utilizing gammaretroviral vectors for T cell directed gene therapy approaches. Moreover, the prolonged persistence of the transduced cells is promising, as earlier T-cell infusion trials led to much more rapid decay rates. Thus, with the proper technological advances, CAR T cell expansion and functionality could be improved to facilitate sustained control over HIV replication.

THE CHALLENGES OF RESTORING HIV-1–SPECIFIC CD4 T-CELL HELP
HIV preferentially infects HIV-specific CD4 T cells, which are required for generating effective HIV-specific CD8 T-cell responses. Untreated HIV infection depletes the majority of total body CD4 T cells through virus-induced apoptosis and immune-mediated deletion mechanisms. While HAART dramatically slows down the loss of CD4 T cells, full reconstitution of CD4 T cell activity typically does not occur. Moreover, the HIV-specific CD4 T cells that evade deletion often show functional impairment reminiscent of what has been described as exhaustion. Recent work has dissected the molecular signatures of CD4 versus CD8 T cell exhaustion and found that, while commonalities exist, exhausted CD4 T cells have many distinct features from both effector CD4 T cells and exhausted CD8 T cells. Efforts to reverse exhaustion in the context of HIV infection have largely centered on blocking PD-1 signaling. However, much more work is required to delineate how to effectively manipulate exhaustion phenotypes, which are dependent on environmental context. Restoration of CD4 T cell activity, whether by immune augmentation or by protection from deletion, will be a critical factor to enable long-term control of HIV replication in the absence of highly active antiretroviral therapy (HAART). While efforts to protect engineered T cells from exhaustion are less well developed, much progress has been made on protecting T cells from HIV infection (discussed below) within the last several years.

INHIBITING HIV-1 PROPAGATION WITH TRANSDOMINANT PROTEINS
The first gene engineered T cells constructed to fight HIV-1 infection that advanced to the clinic expressed transdominant (TD) proteins that competitively inhibited their cognate viral protein counterparts. While they retained functional binding and protein-interacting domains, they were mutated so that they did not maintain their native function in virus replication. Transdominant versions of HIV Env, Gag, Tat, and Rev have all been developed. One such protein, a TD Rev termed M10 was explored in clinical trials. Initially gold microparticles were used to deliver plasmids for M10 expression to autologous CD4 cells. Although preferential survival of Rev M10 transduced cells was seen relative to empty plasmid controls, persistence was poor and the cells persisted with a half-life of 3–15 days. Retroviral delivery prolonged survival duration (ranging from 4 to 9 months), but again no reductions in viral load were seen.

INHIBITING HIV-1 REPLICATION WITH ANTIVIRAL RNAs
Antisense RNAs are single-stranded (ss) RNAs that impair virus replication by hybridizing to complementary viral RNA sequences and physically hindering translation and by targeting RNA for adenosine deamination by double-stranded RNA-specific adenosine deaminases. As the HIV genome is positive ssRNA, antisense RNAs also have the potential to directly interfere with genome replication. A number of antisense RNAs have been used to downregulate HIV gene expression when transfected into target cells or expressed from viral vectors including retroviral, lentiviral, and adeno-associated vectors. A clinical trial was performed to monitor the effects of a combinatorial vector expressing an antisense TAR element along with TD Rev M10, using transduced CD4 T lymphocytes from HIV-uninfected identical twin donors. Survival of therapeutic vector-containing cells was detected up to 154 weeks postinfusion, whereas control vector-transduced cells were not detected at later time points. This survival difference was pronounced in a patient who discontinued HAART during the trial and had high viral loads, which decreased during HAART interruption.

Another phase 1 clinical trial utilized a conditionally replicating lentiviral vector (VRX496) that expressed an antisense Env RNA under the HIV LTR promoter, so vector propagation relied on HIV to supply Tat and Rev. Thus, expression of the antisense message was targeted to HIV-infected cells. Patients in this trial were failing antiretroviral therapy (ART) and had detectable plasma virus before receiving a single dose of ten billion autologous transduced CD4 T cells with an average of one to two vector copies per cell. After the trial, 2 out of 5 patients had more than half a log decreases in their plasma viral loads at 1 year postinfusion and 4 out of 5 patients had increased CD4 T cells compared to baseline. Importantly, there were no serious adverse events or integration related toxicities such as clonal expansion, and modified cells were detected in two patients at 1 year postinfusion.

A subsequent trial enrolled 17 patients with controlled viremia and included 3–6 infusions of 5–10 billion modified cells in an attempt to increase persistence and antiviral efficacy. Thirteen patients underwent an analytical treatment interruption (ATI) and the viral set point and time to viral rebound was determined. Most patients experienced viral rebound less than 4 weeks post-ATI, but 2 out of 13 had delays of 4 and 14 weeks before viral RNA was detectable. Six out of eight patients with previously recorded viral setpoints had decreased setpoints following ATI, although...
this did not reach statistical significance. Evidence of conditional replication, measured by vector RNA in plasma, was seen in 5 out of 6 patients. ATI led to enriched A to G substitutions in the Env targeted region, consistent with targeting by double-stranded RNA-specific adenosine deaminase. Multiple infusions did not increase persistence of the modified cells (half-life remained ~5 weeks) and vector frequency did not increase during ATI, indicating a lack of protection or a selective advantage. Trafficking to rectal tissue was seen and gene marking in the blood correlated with gene marking in GALT, yet persistence of the vector did not correlate with magnitude of antiviral effects.

Ribozymes are antisense RNAs with intrinsic enzymatic activity. Many of the viral RNAs that were targeted using antisense RNAs were also targeted with ribozymes. A ribozyme against the U5 LTR region expressed from a retroviral vector in human primary blood lymphocytes inhibited HIV replication by 100-fold. A small phase 1 clinical trial was conducted using autologous CD4 T cells transduced with this construct. Safety was demonstrated, but transduced cells did not persist in vivo. Another clinical trial utilized a ribozyme directed toward Tat, transferring retrovirally-transduced syngeneic CD4 T cells from an HIV-negative twin donor. Modified cells were detected at all follow-up times in the four recipient patients at 28 to 44 months postinfusion at a consistent frequency of 0.005–0.1% of PBMCs. No escape mutations were detected 24 weeks postinfusion in proviral DNA isolated from PBMCs.

RNA interference (RNAi) inhibits HIV through homology-directed enzymatic digestion by the RNA-induced silencing complex. Transfection of double-stranded siRNAs complementary to fully spliced early viral mRNAs such as Tat and Rev impairs HIV replication early in the lifecycle postintegration, and siRNAs that target late transcripts including the incompletely spliced Gag, Vif, and Nef prevent production of components required to produce infectious virions. Lentiviral vectors were subsequently utilized to stably deliver short hairpin RNAs (shRNAs), the precursor of siRNAs, to primary blood lymphocytes. Unfortunately, the short length of siRNAs (21–22 nucleotides) and the requirement for complete homology to bind the target transcript facilitates viral escape. Recently, Choi et al. used a lentiviral vector to deliver seven multiplexed shRNAs to hit multiple HIV targets simultaneously to minimize the potential for escape and showed that primary CD4 T cells could be protected in vitro and in vivo in infected NOD/SCID/IL2Rγc-/- mice.

RNA decoys and high affinity RNA aptamers have also been developed and tested in a number of cell lines and primary cells. An Env-targeting aptamer plus Tat/Rev siRNA combination inhibited HIV infection of transduced primary human PBMCs and protected CD4 T cells from HIV-mediated depletion in a RAG-hu mouse model of HIV infection.

ADDITIONAL ANTIVIRAL PROTEINS

Besides TD proteins, additional antiviral proteins have been generated to prevent HIV virion production. Stably expressed, intracellular versions of single chain antibody fragments, termed intrabodies, have been developed to block integration and interfere with HIV replication. Harrison et al. used retroviral vectors to stably express the diphtheria toxin A (DT-A) under control of the LTR promoter so that DT-A expression would be activated in HIV-infected T cells expressing Tat and Rev. Richardson et al. demonstrated that rhesus macaque TRIM5α and a chimeric human TRIM5α in which five amino acids in the PRY/SPRY (B30.2) domain were replaced with the corresponding rhesus residues restricted cell-free but not cell-associated HIV spread to untransduced cells within primary human CD4 T cell cultures. More recently, stabilized human TRIM5α proved to be a potent antiretroviral, suggesting that efforts to augment endogenous TRIM5α in individuals may be an effective way to block HIV replication.

An obstacle for all these antiviral RNA and protein methods is that they all rely on systemic delivery and sustained expression of the transgene to exhibit durable antiviral effects. Thus, for these gene engineering methods to effectively control HIV in the absence of HAART a combination of requirements must be met including modified cell survival, expansion, trafficking to key sites of HIV replication, and stable and abundant transgene expression. Moreover, the rapid mutation rate of HIV leaves the potential for escape, particularly from strategies such as RNAs, which only recognize a short target sequence. Similar to HAART, combinatorial approaches targeting multiple steps in the viral lifecycle may prove the most effective when considering strategies to interfere with HIV replication.

PROTECTING LYMPHOCYTES FROM HIV-1 ENTRY

Preventing HIV entry is an attractive strategy because it blocks virus propagation earlier in the lifecycle and prevents formation of CD4 cells carrying integrated proviruses, which constitute the HAART-refractory reservoir. The selective advantage of HIV-resistant CD4 T cells allows for expansion in the presence of the virus, due to cytotoxicity to the unprotected CD4 T cells. Preserved key CD4 T-helper cells could then potentially boost CD8 T cell responses to restore functional CTL control in vivo. Reducing expression of the CD4 receptor for HIV and the equally required CCR5 or CXCR4 coreceptors has been explored as a method to prevent infection of host cells. While deleting CD4 expression is not a viable therapeutic option because of its necessary functions, strategies that disrupt coreceptor expression or block the virus-coreceptor interaction are feasible and have been explored clinically.

FUSION INHIBITORS

Peptides derived from the C-terminus of the GP41 domain of Env (C-peptides) can be utilized to prevent the fusion of the virion with the host cell membrane. C-peptides interact with the viral GP41 N-terminus to disrupt six-helix bundle formation, which contains the energy required for fusion. Membrane-anchored C-peptides block HIV infection in vivo when expressed from retroviral or lentiviral vectors. Perez et al. showed a 15-fold reduction in HIV replication when C-peptide transduced primary CD4 T cells were challenged with the highly virulent BK132 strain of HIV. Resistance mutations to both soluble and membrane bound forms of the C-peptide–based inhibitor T-20/Enfurvertide did not result in insensitivity to an optimized C-peptide vector, termed M87o, which included 10 additional amino acids than T-20 (ref. 99). Partial resistance to M87o could be forced after repeated passeg
of HIV on cells expressing sub-optimal expression vectors and was accompanied by a complex pattern of mutations in both GP120 and GP41 regions of Env.100 M870-transduced cells were given to 10 patients with drug-resistant HIV infection and advanced disease.101 Gene-modified cells were detected at 1 year postinfusion in both lymph nodes and peripheral cells, and CD4 T-cell counts increased significantly from baseline. Four out of seven patients who altered their antiretroviral therapy regimens 4 months into the trial experienced over a log decline in plasma virus, and the patients who remained viremic throughout the study had the best persistence of gene modified cells at 1 year postinfusion. As these therapeutic peptide sequences are virally derived and overlap with GP41 sequences known to be bound by human antibodies, they have the potential to trigger immune-mediated deletion. Therefore, a modified peptide was more recently designed to remove potential MHC-I and antibody recognition epitopes while retaining antiviral efficacy.101

CORECEPTOR EDITING

The discovery that people who lacked functional CCR5 were both healthy and resistant to CCR5-tropic strains of HIV prompted researchers to recreate this phenotype with methods designed to disrupt the CCR5 gene loci. A naturally occurring 32 base pair mutation (delta32) in the second extracellular loop of CCR5 leads to a translational frameshift and subsequent protein truncation, resulting in a nonfunctional receptor not amenable to HIV fusion.102,103 With an allele frequency of ~10% in Caucasians (although more commonly found in people of Western European descent), the majority of the population lacks this nonfunctional CCR5 allele.104-106 This HIV-resistant phenotype has been successfully transferred in the only known case of an HIV cure, in which an HIV-infected patient with acute myeloid leukemia received an allogeneic hematopoietic stem cell transplant from a delta32 homozygous donor and was determined to be virus-free more than 3 years after discontinuing antiretroviral therapy.93 Gene-modified cells were detected at 1 year postinfusion. The permanent and heritable nature of gene disruption allows selective advantage. With enough HIV-resistant cells present, that could expand in the presence of replicating virus due to their ability to uninfected controls, 1 month postinfection.124 Fifty days postinfection, the majority of mice had more than 50% of their CCR5 disrupted and a 0.72 log reduction in plasma viremia.125

CORECEPTOR DISRUPTION WITH ZFNs

ZFNs are customizable, hybrid restriction enzymes that fuse the FokI endonuclease to DNA-binding zinc finger domains. Amino acid modifications in the zinc fingers alter DNA binding specificity, particularly when multiple zinc fingers are combined and can be selected with phage display.117,118 ZFNs are designed in pairs that bind a precise genomic locus in a bidirectional manner to dimerize the FokI domains with the correct orientation and spacing required for cleavage.119 This directs the indiscriminate FokI cleavage domain to induce dsDNA breaks into the desired target sequence, which can lead to gene knockout by error-prone non-homologous end joining (NHEJ) or gene insertion by homologous recombination (HR), provided that a homologous substrate is introduced along with the ZFNs.120,121 Utilizing two unique ZFNs that are active as heterodimers promotes sequence specificity, but also introduces the possibility that each of the individual ZFNs will homodimerize and cleave off-target genomic loci. Thus, design modifications should be incorporated to ensure ZFNs function as obligate heterodimers.122 An attractive aspect of ZFNs is that they can be transiently added to cells as transcripts, proteins, or nonintegrating vectors to avoid the safety concerns associated with integrating viral vectors, namely insertional mutagenesis.123 The permanent and heritable nature of gene disruption allows for long-term therapeutic benefit to be achieved from short-term ZFN expression.

Perez et al.124 employed an adeno viral (Ad5/35) nonintegrating vector to deliver ZFNs targeting CCR5 loci to primary human CD4 T lymphocytes. The ZFN pair recognized a 24 base pair site within the first transmembrane domain of CCR5. Sequence analysis of the CCR5 target site revealed a 5 bp duplication of the spacer between the ZFN binding sites that occurred in over 30% of the mutated sequences and introduced an early stop codon within the first transmembrane domain of CCR5. In vitro analysis of primary CD4 T cells revealed that 33% of CCR5 disrupted cells were homozygous prior to expanding in an HIV-infected environment. Ultra-deep pyrosequencing was used to detect off-target cleavage in the 15 potential sites determined by systematic evolution of ligands by exponential enrichment (SELEX), and a small frequency of off-target disruption was identified in the CCR2 locus (4% of CCR2 alleles modified compared to 36% of CCR5 alleles modified), which is located near CCR5 on the same chromosome and has a single nucleotide difference in the sequence targeted by each of the ZFNs. While CCR2 is important for monocyte trafficking, CCR2-/- mice develop without overt signs of physical or immune impairment, so it is likely that deletion of CCR2 alleles in a small proportion of lymphocytes would not lead to noticeable defects. In vivo studies using an immunodeficient NOD/Shi-scid/IL-2Rgamma(null) (NSG) mouse model of HIV infection resulted in threefold enrichment of ZFN-modified cells in infected mice relative to uninfected controls, 1 month postinfection.124 Fifty days postinfection, the majority of mice had more than 50% of their CCR5 alleles disrupted and a 0.72 log reduction in plasma viremia. Over time, CD4 counts increased in ZFN treated mice relative to controls. Yi et al.125 used an alternative method to deliver
CCR5 ZFNs via a nonintegrating lentiviral vector pseudotyped with envelope from the CXC4-tropic HIV strain LAI, in order to selectively transduce CD4 cells within a culture of unstimulated or PHA stimulated primary PBMCs. Reduced viral loads and preserved CD4 T cells were observed in NSG mice engrafted with transduced PBMCs from HIV-infected patients with either high or suppressed viral loads.

Although CCR5 can be considered the primary HIV coreceptor and is required by most viruses during early infection stages, viruses can gain the ability to utilize CXC4, and this is associated with increased disease progression. Thus, to fully protect cells from HIV-infection, strategies should aim to disrupt both CXC4 and CCR5 expression. ZFNs targeting the second extracellular loop of CXC4 were employed in a NOD.Cg-Prkdcs×ld Il2rg<sup>−/−</sup> SzJ (NSG) mouse model of HIV infection and did not appear to impact trafficking, as CXC4 disrupted cells were found in equal proportions in the blood and spleen. A caveat is that trafficking in a humanized mouse model does not entirely reflect the conditions in a human or nonhuman primate. Protective effects were not seen when mice were challenged with the X4-tropic HIV BK132 strain, as the virus mutated in vivo to gain the capacity for CCR5-mediated entry. In another NSG mouse study using CXC4-targeted ZFNs, better control over HIV replication was seen when the X4-tropic HIV strain NL4-3 was used, and this was accompanied by concomitant increases in CD4 T-cell count. A subsequent comparison of dual ZFN-treated primary CD4 T cells established that CCR5 and CXC4 could be knocked out in the same cell. Deep sequencing analysis of predicted off-target sites failed to detect modification of these nontarget sites, suggesting that if cross-heterodimerization between CCR5 and CXC4 targeted ZFNs occurs it does not result in mutation of additional loci. ZFN treated NSG mice were then challenged with a combination of HIV Bal and BK132 strains, and dual-ZFN treated mice had higher CD4 T cell counts than mice given untransduced cells or cells treated with ZFNs that solely targeted CCR5. Over time, the proportion of coreceptor-negative cells increased.

Clinical feasibility of CCR5 ZFNs was demonstrated in a study that reliably generated populations of more than 10<sup>10</sup> ZFN treated CD4 T cells from HIV-infected donors, upon stimulation with anti-CD3 and anti-CD28 coated beads. Analysis of expanded ZFN transduced cells revealed that these cells retained similar phenotypes, cytokine production levels, and TCR diversity. Karyotype analysis detected no chromosomal abnormalities, which might result from the high proportion of ZFN induced dsDNA breaks. Three clinical trials using transduced T cells have been performed with these CCR5 ZFNs led by the University of Pennsylvania (NCT00842634) and Sangamo Biosciences (NCT01252641 and NCT01044654). The results of NCT00842634, in which 12 patients received ten billion autologous, modified CD4 T lymphocytes (SB-728) were recently published by Tebas et al. The gene modified cells could be detected in all patients throughout the duration of the study up for 42 months, with a median half-life of 64 weeks, and trafficking to the rectal mucosa was observed. Four out of six patients who underwent a structured treatment interruption (STI) completed the 12-week interruption, during which the viral load decreased by an average of 1.2 logs. One patient’s viral load decreased to undetectable levels during STI, and it was later determined that this patient was heterozygous for the CCR5 delta32 mutation. During the STI, the decline of CCR5 modified cells was significantly less than the unmodified cells. The rates of HIV DNA decay were found to be 10 times as rapid for SB-728-treated patients who maintained suppressed viremia (did not undergo STI) compared to control aviremic patients who did not receive ZFN-modified cells.

While safety has been demonstrated in these trials, extensive in vitro characterization identified over a dozen sites (besides CCR2) in human genome to which the CCR5 ZFNs had enzymatic activity towards. Notably, Pattanayak et al. found ZFN-mediated cleavage in the promoter of the BTBD10 gene, and downregulation of this gene has been associated with malignancy and pancreatic beta-cell apoptosis. However, these studies also demonstrated that decreasing the concentration and DNA binding affinity of the ZFNs can reduce off-target cleavage activity. Nevertheless, the possibility exists for the dsDNA breaks to induce transformation, whether through gene knock out or chromosomal translocations.

A similar CCR5 knockout approach was taken with the designer restriction enzymes known as TALENs. TALE DNA binding proteins from the plant pathogens <i>Xanthomonas</i> sp. can be fused to the FokI endonuclease, as done for ZFNs, to promote site-specific cleavage. While ZFNs recognize three nucleotides per zinc finger domain (comprised of 30 amino acids), TALENs recognize a single nucleotide per 34 amino acids. Side by side comparisons of ZFNs and TALENs were performed in 293T cells and comparable CCR5 disruption frequencies were obtained. TALENs had restricted CCR2 cleavage and a twofold increase in cell survival compared to ZFNs. As off-target effects vary in different cell types due to structural differences in chromatin, an important safety assessment of ZFNs and TALENs should include an analogous comparison in primary human CD4 T cells, the target cell employed in human clinical trials.

**CORECEPTOR DISRUPTION WITH CRISPRs**

The CRISPR system was adapted for eukaryotic genome engineering from bacteria and archaea, in which it functions as a prokaryotic immune system. Foreign DNA sequences are incorporated as spacers between the CRISPR repetitive DNA elements and subsequently transcribed and processed into a CRISPR-RNA (crRNA). The crRNA forms a complex with CRISPR-associated (Cas) nuclease proteins that digest invading sequences complementary to the crRNA. For eukaryotic gene editing applications, a type II CRISPR system was adapted from <i>Streptococcus pyogenes</i> that only requires two components to function: a guide RNA (gRNA) complementary to the target genomic site and its associated Cas9 nuclease. Similar to ZFN-mediated cleavage, Cas9 leaves a dsDNA break that can be repaired by NHEJ or HR. An attractive feature of this system is that only the gRNA must be altered to adapt it to a particular genomic site of interest, making it an inexpensive and simple procedure. Moreover, multiple gRNAs can be introduced to simultaneously target multiple loci within the same cell. The only target sequence requirement is that it be followed by a protospacer adjacent motif “NGG” sequence. Cradick and colleagues designed CCR5-specific gRNAs to assess gene editing frequencies and off-target effects in 293T cells.
CCR2 cleavage was seen at a low frequency in this system, even when two nucleotide mismatches existed. Additional work has demonstrated that Cas9 tolerates mismatches between the gRNA and target DNA, depending on the dose of gRNA administered and the number and location of mismatches.\(^{143,144}\) However, a different study utilizing CRISPR gRNAs was able to eliminate CCR5 expression in 68% of transduced TZM.bl cells, without any detectable off-target cleavage at the highly homologous sites identified by bioinformatics screening.\(^{143}\)

Success of coreceptor editing strategies is contingent on generating homozygous knockout cells, as demonstrated by the viral load reduction in the delta32 heterozygote patient whose cells received CCR5-directed ZFNs.\(^{95}\) Which method becomes the more useful clinical tool will depend foremost on safety, followed by efficacy as well as economic and engineering considerations. Bioinformatic software is readily accessible for researchers to identify off-target sites where designer endonucleases may cleave, but in vitro and in vivo preclinical testing must follow to verify the results and demonstrate safety.\(^{144-147}\) Additionally, nickase versions of ZFNs and Cas9 have been designed to promote HR while avoiding the potentially detrimental effects of NHEJ.\(^{148,149}\) Using a pair of Cas9 nickases with two gRNAs that bind close on the chromosome may facilitate genome editing in a manner more analogous to ZFNs and TALENs and reduce off-target effects to comparable levels.\(^{150}\) Unlike the FokI endonuclease, Cas9 does not require dimerization for functional enzymatic activity, so fusion of catalytically inactive Cas9 and active FokI have been designed to impart dimerization requirements on Cas9 while maintaining gRNA specificity.\(^{151,152}\) Analysis of off-target modifications in human genomic DNA sites introduced by the FokI-Cas9 chimeras showed 140-fold and 8-fold reduction relative to WT Cas9 or Cas9 nickases, respectively.\(^{152}\)

### DISRUPTING HIV-1 PROVIRAL GENOMES

An alternative HIV cure strategy targets designer endonucleases to viral DNA rather than host genes, disrupting HIV genes required for replication or excising the entire integrated provirus. Early proof-of-principle experiments infected Cre recombinase-transduced CEM and 293 cells with mutant HIV that had loxP sites incorporated into the LTR, and a reduction in viral replication compared to WT virus was shown.\(^{153}\) Subsequently, a mutant Cre recombinase was developed to recognize a sequence within the HIV strain TZB0003 LTR, and provirus was successfully excised from infected HeLa and CEM-SS cells.\(^{154,155}\) However, this atypical LTR recognition sequence prevents widespread applicability of this system.

### GENOME DISRUPTION WITH ZINC FINGER PROTEINS/ZFNs

The earliest attempts to use zinc finger proteins to disrupt HIV replication prior to the creation of ZFNs fused the DNA binding domains to KRAB transcriptional repression domains. Segal \emph{et al.} created such proteins to bind conserved regions of the HIV LTR and was able to repress HIV replication 10- to 100-fold in transduced primary human PBMCs and PM1 cells.\(^{156,157}\) Zinc finger proteins were also generated to bind to the integrase recognition site, in an effort to prevent the end-processing reaction required for integration.\(^{158}\) More recently, ZFNs targeting the R region located within 5’ and 3’ LTRs have been designed to excise the proviral genome.\(^{159}\) ZFNs were able to excise proviral genomes in transfected Jurkat cells infected with NL4-3-EGFP reporter viruses, with ~40% reduction in Gag copy number and a loss of GFP expression in ~50% of cells. Primary CD4 T cells were also transfected with ZFNs following NL4-3 infection in culture conditions mimicking acute and latent infections, and the amount of provirus and supernatant p24 was reduced by roughly 30%.

### GENOME DISRUPTION WITH CRISPRs

The CRISPR system was employed by Ebina \emph{et al.}\(^{160}\) to inhibit HIV expression and excise proviral genomes with gRNAs complementary to the LTR. Two gRNAs were transfected which targeted the TAR sequence within the R region and the NFkB binding site within the U3 Region. The gRNA targeting the TAR sequence was more effective at inhibiting HIV expression, and was able to disrupt HIV expression in ~30% of TNFα-stimulated LTR-GFP reporter Jurkat T cells, with greater reductions following multiple transfections. LTR-directed gRNAs have the capacity to promote excision of the proviral genome if simultaneous Cas9 cleavage occurs at both LTRs. Following triple rounds of transfection, ~32% of Jurkat reporter clones had proviral DNA excised, as determined by a decrease in the GFP copy number present.

Hu \emph{et al.}\(^{161}\) designed gRNAs to target a U3 region of the LTR that does not overlap with conserved transcription factor binding sites, in an effort to minimize off-target toxicity by altering host gene expression. Activity was assessed using the microglial, monocytic, and T cell lines CHME5, U1, and J-LAT, respectively, all of which harbor at least one integrated provirus, with the rationale that these cell types comprise the blood and tissue reservoirs of HIV. CHME5 cells harboring a latent GFP reporter virus were treated with the histone deacetylase inhibitor trichostatin A to induce GFP expression and a 70% reduction in the percent of GFP positive cells was seen in the CRISPR treated cells relative to mock treated controls. Simultaneous expression of two different gRNAs targeting LTR U3 sites led to deletion of the proviral genome in the majority of treated cells, as well as a number of indels in CHME5, U1, and J-LAT cells. No cleavage of predicted off-target sites was seen using a surveyor nuclease assay and whole-genome sequencing confirmed gRNA specificity.

While these data demonstrate the exciting potential of CRISPRs, the results must be recapitulated in resting, primary human CD4 T cells from HAART patients to more accurately represent excision of proviral genomes. The rare occurrence of latently infected cells \emph{in vivo} makes excision of proviral genomes a substantial challenge to surmount. It has been postulated that 2,000- to 10,000-fold reductions in the reservoir are required to prevent viral rebound for multiple years after discontinuing HAART,\(^{162}\) and latently infected cells comprising tissue reservoirs will not be subjected to gene-editing therapies that rely on \emph{ex-vivo} transductions. However, it is possible that combinatorial approaches that incorporate gene editing techniques to render cells uninfected, to excise provirus, and to augment the T cell cytotoxicity would lead to a functional cure in the absence of HAART. Along these lines, Voit \emph{et al.}\(^{163}\) used a combinatorial approach to generate a Jurkat-based HIV-resistant cell line by using ZFNs to disrupt the...
Conclusions

While major strides have already been made in the field of T cell engineering for adoptive therapy, including demonstrations of safety and feasibility, no clinical trial has resulted in durable and consistent control over HIV-replication in the absence of HAART. The advent of designer nucleases holds great promise for generating a functional cure, whether by deleting host genes with NHEJ or in the nuclear or cytoplasmic stages of virus replication and virion assembly. Designer nucleases have been employed to disrupt expression of integrated proviruses and to generate HIV-resistant cells by knocking out the coreceptors required for virus entry. CD8 and CD4 T cells can be redirected to kill infected cells with HIV-specific TCRs and CARs. Immune-mediated control over HIV in the absence of HAART will likely require the protection of key CD4 T-cell helper subsets from HIV infection so that HIV-specific activation will result in effector functions including IL-2 and IL-21 production and expression of CD40 ligand.

CCR5 locus while at the same time delivering a combination of HIV-restriction factors including TD Rev M10, human-rhesus chimeric TRIM5α, and a Vif-resistant APOBEC3G mutant and detected an additive benefit for inhibiting replication of both R5 and X4-utilizing strains of HIV.

Figures

Figure 1 Summary of gene-engineering and gene-editing strategies to functionally cure HIV-1 infection. A variety of antiviral proteins and antiviral RNAs have been intracellularly expressed in CD4 T cells to interfere with virus propagation early or late in the virus lifecycle, upon entry or in the nuclear or cytoplasmic stages of virus replication and virion assembly. Designer nucleases have been employed to disrupt expression of integrated proviruses and to generate HIV-resistant cells by knocking out the coreceptors required for virus entry. CD8 and CD4 T cells can be redirected to kill infected cells with HIV-specific TCRs and CARs. Immune-mediated control over HIV in the absence of HAART will likely require the protection of key CD4 T-cell helper subsets from HIV infection so that HIV-specific activation will result in effector functions including IL-2 and IL-21 production and expression of CD40 ligand.

Figure 1: Summary of gene-engineering and gene-editing strategies to functionally cure HIV-1 infection. A variety of antiviral proteins and antiviral RNAs have been intracellularly expressed in CD4 T cells to interfere with virus propagation early or late in the virus lifecycle, upon entry or in the nuclear or cytoplasmic stages of virus replication and virion assembly. Designer nucleases have been employed to disrupt expression of integrated proviruses and to generate HIV-resistant cells by knocking out the coreceptors required for virus entry. CD8 and CD4 T cells can be redirected to kill infected cells with HIV-specific TCRs and CARs. Immune-mediated control over HIV in the absence of HAART will likely require the protection of key CD4 T-cell helper subsets from HIV infection so that HIV-specific activation will result in effector functions including IL-2 and IL-21 production and expression of CD40 ligand.

REFERENCES


22. Varela-Rohenca, A, Molloy, PE, Dunn, SM, Li, Y, Suhoski, MM, Carroll, RG et al. (2008). Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor.


