Site-specific host gene modification by zinc finger nucleases: pointing the way to drug free control of HIV-1?

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Anti-retroviral therapy (ART) for human immunodeficiency virus-1 (HIV-1) infection has transformed its clinical course with spectacular reductions in morbidity and mortality, turning this once fatal diagnosis into a manageable chronic infection. However, ART has its limitations. Current ART does not eliminate the virus. Interruption of therapy results in rapid rebound of the virus, and such rebounds are associated with excess morbidity and mortality. This means that therapy once started is for life. This raises the issues of drug resistance due to suboptimal compliance, cumulative toxicities and mounting costs. Efforts to control the virus through novel interventions, particularly through cell or gene therapy have had a resurgence of interest as a single patient was apparently cured by an allogeneic stem cell transplantation from a donor who carried homozygous mutations that disable expression of the HIV-1 co-receptor CCR5. This paper reviews the state of play of gene therapy for HIV infection in the context of a recent paper showing the safety and feasibility of an approach that involves the \textit{ex vivo} disruption of the \textit{ccr5} gene in autologous CD4 T cells using a virally delivered zinc finger nuclease, before their expansion and reinfusion. Although there are still considerable challenges, this approach may point towards a future drug free therapy for HIV-1 infection.

The success of combination anti-retroviral therapy (ART) for human immunodeficiency virus (HIV) infection has transformed a near universally fatal illness into a chronic disease with good long-term survival for patients with access to gold-standard diagnostic tests, ART and health infrastructure for long-term care.¹ However, the disadvantages of such treatment include: life-long daily therapy, associated compliance issues, drug resistance, cost, cumulative drug side-effects and residual excess morbidity and mortality.²⁻⁵ With a vaccine for HIV remaining elusive, research has turned towards possible mechanisms for a 'functional cure', where patients still infected with viral DNA are able control HIV replication without ART in the absence of disease progression or the potential to infect others.

POSSIBLE PATHWAYS TO A FUNCTIONAL CURE

Insights as to how viral control and functional cure might be obtained come from long-term non-progressors and particularly a subgroup of these patients dubbed 'elite controllers' who, despite HIV infection, are able to maintain adequate CD4⁺ T-cell counts and very low or rarely, absent, plasma viremia without ART. Research has partially elucidated both host and viral factors contributing to the desirable clinical course of a subset of these patients. There are two genes in which naturally occurring deletions can contribute substantially to elite controller status: disruption of the viral gene \textit{nef},⁶ which reduces the pathogenicity of the virus, and the Δ32 mutation of the host gene \textit{ccr5},⁷ which results in the expression of defective version of CCR5, a chemokine receptor that acts as an HIV co-receptor required for viral entry.⁸⁻¹⁰

People who are homozygous for the CCR5Δ32 mutation are highly resistant to HIV-1 infection,¹¹,¹² while \textit{ex vivo} CD4⁺ T cells lacking CCR5 are difficult to infect \textit{in vitro}.¹³ Patients infected with HIV-1 who are heterozygous for the CCR5Δ32 mutation have slower disease progression to acquired immunodeficiency syndrome.¹¹ In addition, Maraviroc, a small molecule inhibitor that blocks the function of CCR5 has been licensed for use in combination ART regimens.¹⁴,¹⁵

TARGETS FOR GENE THERAPY

Initial attempts at gene therapy for HIV-1 targeted viral gene products. Some of these, such as a hammer-head ribozyme targeting conserved sequences in \textit{tat}, showed promise in Phase I/II clinical trials.¹⁶ More recently clustered regularly interspaced short palindromic repeats that cause deletions within the integrated virus, or short hairpin ribonucleic acids (shRNAs) that target highly conserved sequences within the 5′LTR inducing transcriptional gene silencing and enforcing viral latency through epigenetic change¹⁷⁻¹⁹ have been explored as novel gene therapies that directly target and

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enforce long-term viral silencing. However, viral targets have proved difficult because of the inherent variability in viral sequences and propensity of the virus to develop escape or resistant mutants.

Therefore, CCR5 has remained at the forefront of HIV gene therapy targets, in the hope that genetically interrupting its expression leads to CD4⁺ T-cell resistance to HIV-1 infection in the absence of ART. This strategy has enjoyed a surge of renewed interest since reports of the apparent cure of the ‘Berlin Patient’, have been disseminated. This patient has had no sign of HIV-1 infection as he received a second allogenic haemopoietic stem cell transplant for relapsing acute myeloid leukemia that complicated pre-existing HIV-1 infection.26–28 Although the patient was initially heterozygous for the CCR5Δ32 mutation, in his second transplant, the patient received stem cells from an human leukocyte antigen-matched donor who was also homozygous for the CCR5Δ32 mutation. The latter is postulated to have been the critical factor in the lack of reappearance of virus in this patient’s blood or tissues, despite him having discontinued ART for over 5 years. Indeed, others who have received similar therapy but have not received CCR5Δ32 mutated stem cells, have not had sustained remissions.21–23

These clinical examples and the inherent difficulties associated with allogenic bone marrow transplantation, have driven the resurgence of interest in gene therapy candidates aimed at interrupting CCR5 gene expression. The challenge is to silence or mutate a single gene in the human genome with high specificity and little collateral damage or ‘off target’ effects. The majority of this work has involved production of small interfering (si) or short hairpin interfering RNAs (shRNAs) targeting CCR5 message with some of these constructs entering Phase I/II clinical trials.27 These strategies target CCR5 transcripts and result in reduced CCR5 expression and host cell resistance to HIV-1 infection by degrading the mRNA before it can be translated into protein.

An alternative approach has been to disrupt the ccr5 gene itself, but this requires cutting double-stranded DNA in a targeted and limited fashion. Initially, this approach has employed zinc finger nucleases (ZFN; reviewed in Kelleher and Purcell26). These engineered paired constructs combine sequence binding specificity of zinc finger domains with FokI restriction endonucleases. Although the zinc fingers bind specific sequences within the double-stranded DNA, the dimerized restriction endonuclease creates a double-strand break. As a result, the damaged DNA undergoes either homologous end joining, in which the original sequence is maintained but remains a persisting target, or imperfect non-homologous recombination that results in the insertion or deletion of base pairs leading to a frameshift mutation, disabling gene expression. Importantly, the non-homologous recombination does not occur uniformly. In vitro, only 50–80% of cell lines and ≈ 30% of primary human CD4⁺ T cells had both alleles disrupted using this approach.29 Therefore, cells treated with ZFN will contain populations heterogeneous for target gene expression. ZFN targeting CCR5 have been shown to be effective in in vitro and in vivo in mouse models of HIV infection where severe combined immunodeficient-mice engrafted with human CCR5-modified CD4⁺ T cells displayed lower HIV viral loads and higher CD4 counts compared with untreated counterparts, as well as selection of CD4⁺ T cells carrying the ccr5 deletion mutants.29,30

**A PHASE I TRIAL OF ZFN-MEDIATED KNOCKDOWN OF CD4⁺ T-CELL CCR5 IN HIV INFECTION**

Recently, Tebas et al.31 reported a small-scale Phase I/II study in the New England Journal of Medicine, which describes the feasibility and safety of cell based gene therapy for HIV-1 using CCR5-targeting ZFN. In this study, 12 HIV-1-infected patients virally suppressed on ART, received reinfusion of ex vivo expanded, autologous CD4⁺ T cells that had been transduced with a construct encoding a ZFN aimed at mutating the ccr5 gene.29,30

All patients were infected with a CCR5 tropic virus with no evidence of infection with CXCR4 or dual (CXCR4 and CCR5) tropic virus. The patients were recruited into two groups. All patients were on stable ART with suppressed plasma virus. In general, group 1 patients, labeled as ‘immunological responders’, had been treated with ART earlier in the disease, commencing therapy at higher nadir CD4⁺ T-cell counts, and had greater CD4⁺ T-cell reconstitution compared with patients in group 2. Both groups received single infusions consisting of a large number of autologous CD4⁺ T cells (approximately 10¹⁰ cells). These cells were the result of an ex vivo expansion protocol based on co-stimulation with CD3 and CD28 antibodies32,33 that followed transduction with the CCR5-targeting ZFN delivered via a recombinant adenoviral vector. The transduced adenovirus produced transient expression of the ZFN from non-integrating DNA encoded within the recombinant vector. Group 1, but not group 2 patients, underwent an ‘analytical treatment interruption’ from weeks 4 to 12 post infusion.31

The numbers of cells infused were so large that despite redistribution to the tissues, there was a substantial, immediate increase in the peripheral blood CD4⁺ T-cell counts from a median of approximately 450 CD4⁺ T cells μl⁻¹ to just over 1300 cells μl⁻¹ at 1 week post infusion. There was substantial heterogeneity in the degree of increase in CD4⁺ T-cell counts between patients. In both groups, cell numbers underwent a biphasic decay to a plateau level, with the inflection point being approximately 12 weeks post infusion. After this time, peripheral blood CD4⁺ T-cell counts decayed more slowly, remaining 250 cells μl⁻¹ above baseline at 36 weeks post infusion.31

Interestingly, despite similar total numbers of cells being reinfused in both groups, there was a non-significant trend for post-infusion CD4⁺ T-cell counts to be higher in group 1. This may suggest that harvested cells from patients with more replete immune systems have a greater chance of survival or engraftment compared with cells from more lymphopenic hosts. Certainly there is evidence for progressive destruction of lymphoid architecture with progressive disease.34–36 Alternatively, it may illustrate saturation of the extravascular compartment. Consistent with the fact that CD8⁺ T cells were depleted before in vitro expansion, there was no significant change in the number of CD8⁺ T cells.

CCR5-modified CD4⁺ T cells were detected in all patients with estimates of between 10–27% (median 22%) of reinfused cells carrying a modification of the ccr5 gene.31 The half-life of the CCR5-modified CD4⁺ T cells in the peripheral blood compartment was 48 weeks at the median follow-up time of 64 weeks. Thereafter, CCR5-modified CD4⁺ T cells could be detected in all patients with the longest follow-up being 42 months. At that point, these represented <2% of CD4⁺ T cells in the peripheral blood. Gene-modified cells could be detected in rectal gut-associated lymphoid tissue, representing <1% of mononuclear cells in this compartment.

Group 1, the immunological responders, underwent a planned analytical treatment interruption between weeks 4 and 12 post infusion during which ART was ceased. There was a rapid recrudescence of plasma viremia in all six patients. Two patients were recommenced on ART at week 8 due to high viral loads. In contrast, another group 1 patient had a relatively low, late peak level of viremia. This patient then controlled plasma viremia to an undetectable
level in the absence of ART until week 12. Subsequent analysis found that this patient was in fact heterozygous for the CCR5Δ32 mutation.31

Group 2 patients remained on ART and aviremic throughout the study. Given the current dogma that there is no active viral replication in patients on effective ART, it would be expected that in these patients the CCR5-modified CD4+ T cells would have no selective advantage over the patients’ unmanipulated CD4+ T cells, because no new CD4+ cells should be infected in patients on effective therapy. Despite this, there was a relatively rapid initial decay in CD4+ T-cell numbers between weeks 1 and 12. This may be explained by trafficking of cells into tissues, or a homeostatic contraction in response to the supra physiological ex vivo expansion these cells have undergone before reinfection.

**DO THE ZFN CARRYING CD4+ T CELLS HAVE A SELECTIVE ADVANTAGE?**

There was a statistically significant difference in the rate of decay of the gene-modified cells compared with either the total or unmodified CD4+ T cells in group 1 when determined by quantile regression. However, this difference could not be demonstrated using a mixed linear effects model. This inconsistency is likely due to the small sample size and large interpatient variability.31 Interestingly, no formal comparison of the relative decay rates of CCR5-modified cells between group 1 and 2 is presented, but the shape and slope of the biphasic decay curves for the two groups are similar.

Certainly one interpretation of the data presented is that the cell decay dynamics are largely independent of viremia. CD4+ T-cell survival appears to be determined more by the biology of the cells reinfused and/or the receptiveness of the immune system into which they are reinfused. This is suggested by the observation, detailed above, that those patients with better immune reconstitution before reinfusion also had larger CD4+ T-cell count increases to peak and maintained higher levels of CD4+ T cells after the infection point in the decay curves after 12 weeks. Given these constraints, if the half-life of these cells is relatively short in the absence of viremia, it is unlikely to be longer in the presence of higher viral loads.

The cause of this relatively limited selective advantage of the transduced cells in the presence of viremia is unclear. The mechanism may relate to the limited extent of CCR5 gene disruption, which was estimated to occur in 21% of the reinfused cells. As there was no direct measurement of proportion of cells successfully modified, this estimate was based on the fact that approximately 25% of mutated cells displayed a specific target sequence. The estimate of total mutated cells was determined by multiplying the detectable mutated cells by four.31 Therefore, the overwhelming majority of reinfused cells were unaltered in either allele of the ccr5 gene. Furthermore, unlike in previous studies,39 the effectiveness of the ZFN in knocking down CCR5 is not directly assessed by quantification of CCR5 mRNA or CCR5 protein expression on the cell surface.

The relatively low level of CCR5-modified CD4+ T cells may not have impacted on CCR5 surface expression to a degree likely to alter cellular survival through resistance to HIV-1 infection. Indeed, for maximal efficacy the ZFN must disrupt both copies of the ccr5 gene in transduced cells. Certainly, HIV-infected patients who are CCR5Δ32 heterozygotes have slower progression of disease11 and rates of infection within this group are reduced by up to 65%. Thus, while heterozygotes are partially protected, their rate of infection is substantially higher than in homozygotes who are highly protected from infection.11 The authors estimate the frequency of biallelic disruption and relate this to efficacy. In group 1, the reduction in HIV viral load from peak correlated with the estimated rate of effective biallelic disruption of ccr5. However, for a more pronounced difference in CCR5-altered CD4+ T-cell survival compared with unaltered cells a higher level of CCR5 knockdown may be required. This is indicated when closer analysis of the patient who had the most effective reduction in viral load, from a peak below the previous viral set-point to an undetectable level during treatment interruption, found them to be heterozygous for the naturally occurring CCR5Δ32 mutation. Therefore, unlike wild-type counterparts, this patient only required a ‘single-hit’ of one ccr5 gene to be modified in order for CCR5 expression to be completely disrupted in any transduced cell. This provides a feasible explanation for the superior viral control in this patient. It is unclear if the remaining 11 patients were genotyped for CCR5 expression and this information would be valuable.

Certainly there is evidence that even low level, transient expression of CCR5 is sufficient to make cells susceptible to HIV infection. This is the likely mechanism by which apparently CCR5 negative cells (for example, follicular T helper cells) are infected at the same or higher rates than other CD4+ T-memory T cells of patients with HIV-1 infection.37,38 Furthermore, various inflammatory and infective stimuli such as a vaccination or viral infections transiently upregulate CCR5 expression on the surface of CD4+ T cells in vivo, providing an opportunity for infection.39–41 This is likely to occur in patients with single allele disruption of CCR5 after ZFN treatment.

A related hypothesis is that the viremic and/or study period may not have been sufficiently long enough to allow for a signal of selective advantages to be detected. Indeed as argued above, it is likely that reinfused cells were decaying from the peripheral blood compartment at a rate determined by factors other than viremia, particularly the overcrowded lymphatic space. This is suggested by the similar rates of cellular decay between groups 1 and 2. Phenotyping of reinfused cells, particularly in terms markers for proliferation, activation, apoptosis and longer-term survival would provide useful information regarding their homeostatic drive and likely fate.

**CAN GENE THERAPY CONTRIBUTE TO A FUNCTIONAL CURE?**

An intervention that induces a functional cure of HIV must either manipulate the host immune system into inducing potent viral control, or disable viral replication, assembly and export, infectivity or general fitness. Although gene therapies targeting both have been explored, the targeting HIV nucleic acids either in RNA or in the integrated human genomes remain an ongoing challenge.

In terms of host targets for gene therapy, the HIV co-receptor CCR5 remains an attractive target especially given the well-established observation that naturally occurring CCR5Δ32 homozygotes are resistant to infection and heterozygotes display slower disease progression to acquired immunodeficiency syndrome and the tanta-lizing functional cure of the ‘Berlin patient’. A number of si/shRNA constructs targeting CCR5 gene to be modified in order for CCR5 expression to be completely disrupted in any transduced cell. This provides a feasible explanation for the superior viral control in this patient. It is unclear if the remaining 11 patients were genotyped for CCR5 expression and this information would be valuable.

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The recent report by Tebas et al.\textsuperscript{31} has in the first instance demonstrated the safety of using ZFN targeting CCR5 expression in autologous CD4\textsuperscript{+} T-cell ex vivo before expansion and reinfusion. The process was tolerated well in 11 out of 12 studied patients with one patient suffering a severe transfusion reaction. Although this appears promising, the efficacy of CD4\textsuperscript{+} T-cell transduction and consequent biallelic knockdown of CCR5 expression will need to be greatly improved for a convincing impact on CD4\textsuperscript{+} T-cell selective survival in the setting of HIV viremia to be demonstrated.

Currently, constructs are delivered by a replication incompetent, recombinant adenovirus vector that transiently expresses the ZFN. Although this confers a measure of safety, because the gene is not integrated into the host genome as it is with lentiviral delivery, increasing rates of transduction and resulting gene expression is a major hurdle facing the field of gene therapy.

Further, the rates of engraftment and the long-term survival of these cells is unclear and seems affected by homeostatic factors unrelated to viremia. Other gene therapies that don’t expand the CD4\textsuperscript{+} T cells so greatly before reinfusion are being explored. If the transduced cellular decay reported by Tebas et al. is a common feature of this method, repeat treatments may be necessary. Current strategies to improve engraftment include use of relatively large doses of alkylating agents such as cyclophosphamide or busulphan.\textsuperscript{32} The alternative is to transduce and reinfuse autologous CD34\textsuperscript{+} hematopoietic stem cells, but obtaining sufficient cells and attaining high-level transduction rates remain significant challenges to the field. However, even if these can be maximized, efficient engraftment of the transduced cells requires conditioning with agents such as busulphan, which can be complicated by short-term toxicities, particularly cytopenias, and a longer-term risk of malignancy. The early promise of CXCR4 inhibitors in improving CD34\textsuperscript{+} hematopoietic stem cell engraftment has not yet resulted in a viable alternative. In addition, if this particular methodology is pursued, the long-term safety and off-target effects, particularly those that alter generation of hematopoietic stem cells, needs to be assessed. Given naturally occurring CCR5\Delta32 homozygotes have otherwise normal hematopoietic and immunological profiles, it is anticipated that this may not be a major issue.

**WILL ZFN-MEDIATED CCR5-MODIFIED CD4\textsuperscript{+} T CELLS CONTROL THE HIV RESERVOIR?**

Finally, in order for an HIV gene therapy to succeed in facilitating a functional cure the viral reservoir must be addressed. In the proposed model of acquired CCR5\Delta32 gene therapy as presented by Tebas et al., the success of ZFN-mediated CCR5-altered CD4\textsuperscript{+} T cells would seem to rely upon absolute eradication of CCR5 expression. This is because latent HIV infection of long-lived quiescent cells would continue to produce virions after transduction of genetically altered cells. Therefore, any expression of CCR5 would facilitate ongoing infection. It could also be argued that ongoing viral replication in the setting of scarce or absent CCR5 expression may lead to outgrowth of CXCR4 tropic virus, a potentially more pathogenic strain, which would be highly undesirable. In the ’Berlin patient’, the process of myeloablation before haemopoietic stem cell transplant may have effectively ablated any long-lived quiescent HIV-infected cells as part of the treatment.\textsuperscript{20} Although on balance this potentially lethal conditioning regime cannot be considered as part of a wide-spread intervention for HIV infection, the process of eradicating viral reservoirs is likely to be necessary for long-term functional cure.

**CONCLUSIONS**

The field of HIV therapeutics continues to march forward, beyond strategies aimed at disabling viral replication and towards gene therapy options that offer longer-term control in the absence of daily medication. Presently, targeting host genes appears more feasible than viral genes, and can be induced by either interrupting RNA expression or disruption DNA. The use of ZFNs in this domain is in its genesis and improved rates of transduction and knockdown are likely required for true efficacy. In addition, the effects of ccr5 disruption especially in the context of autologous cell expansion, on the phenotype and functional behavior of these cells needs to be further explored. In any case, the safe use of ZFNs to selectively target and disrupt specific gene sequences \textit{in vivo} is an exciting prospect for immunovirology. In terms of HIV therapeutics, ZFN may indeed be pointing us in the right direction.

**CONFLICT OF INTEREST**

ADK is the Principle investigator on Phase I trial of an shRNA that targets CCR5 that will be conducted in Sydney, Australia, sponsored by Calimmune, that will commence later in 2014. SCS declares no conflict of interest.
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