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Novel AIDS therapies based on gene editing

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Abstract

HIV/AIDS remains a major public health issue. In 2014, it was estimated that 36.9 million people are living with HIV worldwide, including 2.6 million children. Since the advent of combination anti-retroviral therapy (cART) in the 1990s, treatment has been so successful that in many parts of the world, HIV has become a chronic condition in which progression to AIDS has become increasingly rare. However, while people with HIV can expect to live a normal life span with cART, lifelong medication is required and cardiovascular, renal, liver and neurologic diseases are still possible, which continues to prompt research for a cure for HIV. Infected reservoir cells such as CD4+ T-cells and myeloid cells allow persistence of HIV as an integrated DNA provirus and serve as a potential source for the re-emergence of virus. Attempts to eradicate HIV from these cells have focused mainly on the so-called “shock and kill” approach where cellular reactivation is induced so as to trigger the purging of virus producing cells by cytolysis or immune attack. This approach has several limitations and its usefulness in clinical applications remains to be assessed. Recent advances in gene editing technology have allowed the use of this approach for inactivating integrated proviral DNA in the genome of latently infected cells or knocking out HIV receptors. Here we review this strategy and its potential to eliminate the latent HIV reservoir resulting in a sterile cure of AIDS.

Keywords

Gene editing; CRISPR/Cas9; HIV-1; AIDS

INTRODUCTION

HIV/AIDS remains a major public health issue with an estimated 36.9 million people living with HIV worldwide in 2014, including 2.6 million children [1]. The development of

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combination anti-retroviral therapy (cART) in the 1990s has meant that in many parts of the world, HIV morbidity and mortality has been reduced and infection has become a chronic condition where progression to AIDS is rare [2]. However in spite of cART, virus persists in the form of integrated proviral DNA in latently infected cells [3] and inflammation continues to be sustained in chronic HIV infection. This is associated with pathological conditions such as HIV-associated neurocognitive disorder (HAND) [4] and other inflammatory co-morbidities including cardiovascular disease, non-AIDS malignancies and osteoporosis [5]. Moreover, discontinuation of cART almost always leads to the re-emergence of detectable viral replication, rebound in viral load and the progression of HIV infection [6]. For these reasons, much research has focused on strategies to eradicate HIV reservoirs and effect a functional cure for HIV [7].

The properties of the latently infected cells that constitute the HIV reservoir make viral eradication a formidable problem. Latent cellular reservoirs exist in circulating blood as well as the CNS, bone marrow and gut associated lymphoid tissue with CD4+ T-cells being the most important [8]. It has been estimated that latently infected CD4+ T-cells can live for many decades, are resistant to cART and are not susceptible to attack by the immune system [8, 9].

The field of HIV-1 cure research was energized by the apparent cure of an HIV-infected individual, the “Berlin patient”, who received an allogeneic hematopoietic stem cell transplant from a donor homozygous for the CCR5 Δ32 mutation as part of his treatment for acute myeloid leukemia [10]. CCR5 is a protein on the cell surface that serves as one of two main co-receptors, along with CXCR4, after CD4 binding for HIV-1 cell entry. CCR5 is the co-receptor usually used by virus strains that initially infect an individual and most virus present in the early stages of HIV-1 infection are CCR5-tropic. The Δ32 mutation renders the CCR5 protein inactive and incapable of binding HIV-1. Persons homozygous for this polymorphism are almost completely protected from acquiring HIV-1 infection; individuals heterozygous for the mutation have slower progression of disease when infected [11,12]. The Berlin patient received radiation, chemotherapy, and had evidence of graft-vs-host disease, but the replacement of the patient’s cells with CCR5 Δ32 cells was thought to have been the main factor in permitting the patient to remain undetectable for HIV-1 in blood and tissues off antiretroviral therapy more than nine years later.

Subsequently, two patients with HIV-1 infection, themselves heterozygous for the CCR5Δ32 mutation, received allogeneic hematopoeitic stem cell transplants from donors with homozygous functional, HIV-1-susceptible wild-type CCR5 after reduced intensity conditioning [13]. Despite achieving undetectable levels of total HIV-1 DNA in blood (and rectal tissue in the one patient assessed) and undetectable infectious virus by viral outgrowth assay, rebound of viremia occurred 12 and 32 weeks after the interruption of antiretroviral therapy in the two patients. These cases demonstrated the limitations of the current HIV-1 reservoir assays, and the value of the analytical treatment interruption (ATI) of antiretroviral therapy to ultimately determine whether HIV-1 infection has been eliminated or immunologically controlled. Although measurable levels of viral reservoir were reduced to undetectable levels by allogeneic stem cell transplant from CCR5 normal donors, HIV-1 infection was not eliminated.

Hopes were raised again when a baby, started on antiretroviral therapy (ART) 30 hours after being born to a woman with HIV-1 infection, maintained undetectable levels of plasma HIV-1 RNA, cell-associated HIV-1 DNA, and HIV-1 antibodies after ART was stopped at 18 months of age [14]. One hypothesis is that the early initiation of ART could prevent the establishment of the latent cell reservoir. Unfortunately, viremia returned 27 months after stopping ART. Each of these cases of late viral rebound were consistent with temporal models of the reemergence of virus upon discontinuation of ART that were developed by the Siliciano group [15–18], and demonstrate the challenges faced by HIV-1 cure researchers in developing measures of the success of potential cure interventions.

One approach to eradicate HIV from the latently infected cells is the so-called “shock and kill” approach in which cellular reactivation is induced with a chemical agent, such as a histone deacetylase inhibitor [8, 19]. *Ex vivo* evidence suggests that, for these cells to die, the activation of viral expression must occur in the context of an enhanced CD8 cytotoxic T lymphocyte response or other modality of immune attack targeting these cells [20]. This approach has limitations for usefulness in clinical applications because the efficacy of currently used chemical agents that reverse latency remains unsatisfactory due to their low efficiency of induction, non-specific effects and toxicity [21]. Cell-associated HIV-1 RNA has been only modestly increased in clinical studies with these agents and these increases have not translated into changes in the viral reservoir as measured by viral outgrowth assays [22–25].

The recent advances in gene editing technology have made available the possibility of using these approaches for inactivating the integrated HIV proviral DNA in the genome of latently infected cells. Several new techniques are available and we will discuss in subsequent sections how these have been deployed against HIV. One of the first gene editing technologies to be used was the Cre recombinase, which is a tyrosine-type site-specific recombinase from bacteriophage P1 that allows predictable modification of genomes and enables precise genome editing in heterologous hosts by carrying out site-specific recombination events between two DNA recognition sites known as LoxP sites [26]. The zinc-finger nuclease (ZFN) class of gene editing proteins are fusion proteins of the nonspecific endonuclease cleavage domain of the FokI restriction enzyme with a custom-designed Cys2-His2 zinc-finger protein, which gives and enzyme capable of making sequence-specific DNA double-strand breaks (DSBs)[27,28]. Another class of reagents are also FokI fusion proteins known as the transcription activator-like effector nuclease (TALEN) system and have a targeting domain that is taken from the Xanthomonas bacteria TAL effector proteins [29,30]. Another class of nucleases are the homing endonucleases, also known as the meganucleases and their megaTAL derivatives (meganuclease/TAL effector fusion proteins), which have also been used against HIV provirus [31].

The most powerful category of gene editing tool is the clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9), which provides unparalleled control over gene editing [32–35]. CRISPR/Cas9 is straightforward, easy to use and is flexible in that it can be adapted to different targets [36]. CRISPR loci and Cas proteins are present in ~90% of archaeae and ~50% of bacteria and evolved as a defense against viruses [37]. This prokaryotic adaptive immune system has been developed into a flexible and precise gene

editing tool, where a short guide RNA (gRNA) is used to direct the sequence-specific cleavage of a specific target DNA. There are two parts that make up the CRISPR/Cas9 system: a guide RNA (gRNA), which determines the target specificity, and an endonuclease (Cas9) that cleaves both strands of the target DNA when gRNA and Cas9 are co-expressed in the same cells. The gRNA is designed based on the sequence of the DNA target so as to contain a 20 base-pair guide sequence that associates with the target by Watson-Crick base-pairing and thus recruit the gRNA/Cas9 complex. Successful binding of Cas9 to the target and subsequent endonucleolytic cleavage also requires a Protospacer Adjacent Motif (PAM) trinucleotide sequence immediately following the target sequence. Cleavage of target DNA causes a double-strand break (DSB), which lies 3–4 nucleotides upstream of the PAM sequence. Since Cas9 is a general endonuclease, its specificity is conferred by the small gRNA and this can be either synthesized chemically or produced by in vitro transcription or cell expressed to provide a highly specific gene-targeted tool.

The DSBs that are generated by cleavage by ZFN, TALEN or CRISPR/Cas9 may be repaired by NHEJ pathway of DNA repair. Since this process is error-prone, it often results in the generation of inserts/deletions (InDels) or base substitutions at the site of the repaired DSB. This may lead to frameshifts and/or premature stop codons, which can effectively disrupt the open reading frame (ORF) of the target gene. Alternatively, if multiplex editing is applied, a section of DNA between two DSBs may be excised also leading to loss of gene function. Thus these gene editing approaches are suitable for inactivating and eliminating HIV proviral DNA. In the following sections, we will examine each of the four gene editing technologies that are available and how they have been adopted against HIV.

CRE RECOMBINASE AND OTHER TYROSINE-TYPE RECOMBINASES

The Cre recombinase from bacteriophage P1 carries out site-specific recombination events between two DNA recognition sites known as LoxP sites allowing precise manipulation of genomes and has been used widely in mouse genetics [26]. Cre target specificity can be altered to a moderate extent to generate new site-specific recombinases via directed evolution [38]. For example, a procedure known as substrate-linked protein evolution (SLiPE) places the recombination target site of interest next to the recombinase coding region allowing those DNA molecules carrying a successful recombinase coding region to be physically marked by that recombinase on the linked substrate and retrieved from a background of unsuccessful recombinase candidates by PCR [38]. SLiPE has been employed to evolve a tailored recombinase that recognizes an asymmetric DNA sequence within an HIV-1 proviral long terminal repeat (LTR) and efficiently excises integrated HIV proviral DNA from the genome of latently infected cells [39]. LTR-specific recombinase (Tre-recombinase) is proven to be a promising tool for excision of HIV-1 provirus from infected cells [39, 40]. However, efficient and safe delivery into infected cells *in vivo* is a prerequisite to their development as new antiviral agents [41]. Mariyanna et al [42] describe Tre-recombinases expressed in bacteria that are tagged either with the protein transduction domain (PTD) from HIV-1 Tat or the translocation motif (TLM) from the Hepatitis B virus PreS2 protein. These were able to translocate efficiently into human HeLa cells and showed recombination activity on HIV-1 LTR sequences present in an episomal form or stably integrated and were also able to excise full-length proviral DNA from chromosomal

integration sites of HIV-1-infected HeLa and CEM-SS cells. This may provide a basis for a non-genetic transient application of engineered TRE-recombinases for potential HIV eradication strategies [42]. Hauber et al [43] reported conditional expression of Tre-recombinase from a self-inactivating lentiviral vector in HIV-infected cells. Expression of the transgene resulted in HIV-1 provirus excision with no cytopathic effects and was effective *in vivo* in humanized $Rag2^{-/-}\gamma^{-/-}$ mice engrafted with either Tre-transduced primary $CD4^+$ or $CD34^+$ cells [43].

This Tre-recombinase recognition is restricted to HIV-1 subtype A isolates, which limited its broad application. To develop a broader antiviral agent that is able to eradicating a wider range of HIV-1 proviruses from infected cells, Karpinski et al [44] employed SLiPE to evolve a novel recombinase (Brec1) that recognizes a 34-bp sequence present in the LTRs of most clinically relevant HIV-1 subtypes and strains. Brec1 efficiently and precisely excises integrated HIV-1 provirus and was found to be efficacious on a number of clinical HIV-1 isolates both *in vitro* and *in vivo*, including in mice that were humanized with patient-derived cells [44].

ZINC FINGER NUCLEASES (ZFN) FOR NOVEL GENE EDITING AIDS THERAPIES

ZFN are fusion proteins between cleavage domain of FokI and a sequence-specific DNA recognition domain of a customized Cys_2-His_2 zinc-finger protein and deliver DSBs that can be repaired by NHEJ to yield small alterations at targeted genomic loci [27,28]. ZFN have allowed highly efficient disruption of genes in different cell types and organisms facilitating targeted gene therapy [45] including engineering resistance to HIV-1 [46]. While a few studies have targeted the viral genome itself via LTR-specific ZFN [47,48] similar to approaches used with CRISPR/Cas9 described below, most have targeted one or both of the coreceptors needed for HIV-1 infection: CCR5 and CXCR4. HIV infects CD4+ cells such as helper T-cells and macrophages and viral entry is mediated through interaction of HIV-1 gp120 and host CD4 and coreceptor. Macrophage- or M-tropic HIV-1 strains (R5 viruses) use CCR5, which is also used by nearly all primary isolates of HIV-1 of various genetic subtypes [49,50]. T-tropic HIV-1 strains (X4 viruses) use CXCR4 [49,50]. The requirement of HIV-1 for a coreceptor can be exploited through gene editing approaches to the CCR5 or CXCR4 genes to combat HIV-1 infection.

As noted above, individuals who carry a mutation in the CCR5 gene known as CCR5-Δ32, which encodes a truncated form of the receptor are protected against R5 strains of HIV-1 [11,12] prompting development of anti-HIV drugs that block viral interaction with CCR5. Maraviroc is currently approved by the FDA and maintains durable responses in patients with R5 HIV-1 [51,52]. Another approach is to use gene therapy approaches to reduce or eliminate the expression of CCR5 [53]. Holt et al [54] designed ZFN that disrupted CCR5 in human CD34+ hematopoietic stem/progenitor cells at a frequency of 17%. ZFN-treated cells engrafted immunodeficient mice and gave rise to multilineage progeny with stably disrupted CCR5. Control mice receiving untreated cells and challenged with R5 virus showed severe CD4+ T-cell loss, whereas mice transplanted with ZFN-modified cells underwent rapid

selection for CCR5$^{-/-}$ cells and had reduced HIV-1 levels [54]. Maier et al constructed a chimeric Ad5/F35 adenoviral vector encoding CCR5-specific ZFN, which allowed efficient delivery and transient expression to anti-CD3/anti-CD28 stimulated T cells [55]. This results in a robust *ex vivo* manufacturing process that can generate >10^{10} CCR5-modified CD4+ T cells from healthy and HIV+ donors and *in vivo* toxicity studies showed no detectable ZFN-specific toxicity or T-cell transformation indicating suitability for a clinical trial [55]. Li et al [56] engineered autologous CD34+ hematopoietic stem/progenitor cells by disruption of CCR5 using recombinant adenoviral vector for CCR5-ZFN and achieved >25% CCR5 gene disruption. The resulting cells engrafted a humanized mouse model and supported multilineage differentiation *in vitro* and *in vivo* [56]. An important aspect of this type of functional cure strategy is that HIV-resistant cells are expected to be selected for by the actions of the virus itself [57]. Yi et al [58] used a non-integrating lentivirus to transiently expression ZFN and pseudotyped the virus with HIV-1 envelope to targeted delivery to CD4+ T cells. Transduction with CCR5-ZFN NILV conferred resistance to HIV-1 in vitro and transduced CD4+ T cells from HIV-1 negative individuals became resistant to HIV-1 challenge in mice. Similarly, endogenous virus replication was suppressed mice reconstituted with transduced CD4+ T cells from HIV-1 positive patients [58]. Yao et al [59] disrupted CCR5 of human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) with specific ZFN and showed they retained their pluripotent characteristics and could differentiate into CD34+ cells in vitro, which were able to give rise to all types of hematopoietic colonies. This suggests patient-specific stem cells modified with ZFN may be potentially useful in treating HIV infection [59].

Finally, ZFN have also been used to disrupt the CXRC4. Yuan et al [60] found this approach conferred resistance to HIV-1s that utilizes CXCR4 for entry in tissue culture and *in vivo* in HIV-1-infected NSG mice with engrafted ZFN-modified CXCR4 CD4+ T cells. Didigu et al [61] used ZFN for simultaneous modification of both CCR5 and CXCR4 in primary human CD4+ T cells. The modified cells proliferated normally and were resistant to both CCR5- and CXCR4-tropic HIV-1 in vitro. They engraft and traffic normally when introduced into a humanized mouse model of HIV-1 infection where they are protected from infection with CCR5- and CXCR4-tropic strains of HIV-1.

TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASES (TALEN) FOR NOVEL GENE EDITING AIDS THERAPIES

TALEN are fusion proteins known that have a targeting domain from *Xanthomonas* TAL effector proteins and an endonucleolytic catalytic domain from FokI [29,30]. Targets for TALEN that have been exploited against HIV-1 include the provirus and cellular genes required for HIV infection such as the CCR5 coreceptor and lens epithelium-derived growth factor, LEDGF/p75 [62]. Some studies suggest that TALEN have the advantages that they have low cellular toxicity and off-target effects and are able to target methylated DNA, which is relevant to targeting latent HIV-1 provirus [62]. In addition, they are monomers with degenerate recognition sites that are able to target predicted escape mutations [63], which that will be discussed subsequently. Disadvantages are that TALENs take longer to construct compared to CRISPR, where you only need to make new gRNA. In addition,

TALENs are larger and hence are more difficult to deliver [62]. Ru et al [64] used a cell-penetrating peptide-based system for TALEN delivery by constructing a functional Tat-TALEN proteins with cell-penetrating HIV-1 Tat peptide fused to TALEN. Purified Tat-TALEN penetrated cells and disrupted the CCR5 gene with a 5% modification rate observed in human induced pluripotent stem cells [64]. Mock et al [65] used lentiviral particles containing genetically inactivated reverse transcriptase (RT) to package vector mRNAs encoding CCR5-specific TALEN to mediate efficient transduction of cells and transient transgene expression. Efficient targeted genome editing and abrogated expression of CCR5 was observed in different cell lines [65]. Mock et al [66] also efficiently delivered CCR5-specific TALEN into T cells by mRNA electroporation and obtained >50% CCR5 knockout in primary T cells and low off-target activity. CCR5-edited cells were protected from infection by HIV-derived lentiviral vectors and wild-type CCR5-tropic HIV-1 [66].

The TALEN approach has also been used to target the human PSIP1 (PC4 and SFRS1 interacting protein 1) gene, which encodes LEDGF/p75 a cellular protein used by HIV-1 as a chromosome docking and integration cofactor [67]. PSIP1 is a potential therapeutic target since, like CCR5, knockout of LEDGF/p75 is well tolerated by the immune system. Fadel et al [67] performed two types of PSIP1 knockouts: whole-gene deletion and integrase binding domain deletion and inhibited HIV-1 integration and viral replication in Jurkat cells even though the capacity to assemble infectious viral particles was normal in the $PSIP1^{-/-}$ cells. Thus, PSIP1-specific TALEN may have therapeutic potential in gene targeting for HIV-1 disease.

Finally, TALEN can also be used to target the HIV-1 provirus itself. Ebina et al [68] established an efficient TALEN-based strategy to excise HIV-1 proviral DNA targeting the HIV LTR. Transfection of in vitro transcribed TALEN-encoding mRNA, gave >80% removal of viral DNA from T cell lines. A lentiviral vector system was also developed to take advantage of the efficient proviral excision and permit straightforward selection of gene-transduced and HIV-excised cells in T cell lines [68]. In another study, Strong et al [69] used TALEN to target a highly conserved sequence in the HIV-1 proviral transactivation response element (TAR). A TAR-GFP reporter construct was efficiently inactivated by of TALEN plasmid and when HIV-infected cells containing full-length integrated proviral DNA were transfected with TALEN plasmid, the HIV TAR region sustained indels and a mutated HIV-1 proviral DNA was found to be incapable of expressing Gag protein [69]. Thus TALEN may have future potential for HIV-1 proviral DNA eradication.

CRISPR/CAS9 FOR NOVEL GENE EDITING AIDS THERAPIES

The clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9) provides unprecedented control over gene editing, is straightforward, facile and flexible and is perhaps the most powerful gene editing tool yet available [32–36]. The CRISPR/Cas9 gene editing approach has the capacity to disrupt both cellular genes necessary for HIV-1 infection and integrated HIV-1 proviral DNA [70,71]. It has been shown that CRISPR/Cas9 facilitates the excision of DNA segments of integrated HIV-1 provirus DNA in a variety of different latently infected cell types including CD4+ T-cells. CRISPR/Cas9 can be targeted to sequences within the HIV-1 LTR U3 region that flank the proviral genome and thus allow

the complete excision of the proviral DNA [72–77]. This approach also allows cells to be prophylactically protected by expressing CRISPR/Cas9 in uninfected cells to prevent them against being infected later by HIV-1 [73,74]. However, it should be noted that this approach requires longer, or more constitutive, expression of CRISPR/Cas9 in the cells and the issue of the immunogenicity of Cas9 has not yet been resolved.

In a recent study, tail-vein or intraperitoneal injection of two transgenic mouse models with recombinant adeno-associated virus 9 vector expressing Cas9 and a multiplex of gRNAs effected the cleavage of integrated HIV-1 DNA in the animal. Excision of a large essential DNA fragment from the HIV-1 provirus occurred in spleen, liver, heart, lung, kidney and circulating lymphocytes of the mice indicating proof-of-concept experiment for the *in vivo* eradication of integrated HIV-1 provirus by CRISPR/Cas9 in a wide variety of different cells and tissues [78].

As mentioned, an approach to eradicate HIV-1 from reservoir cells is the “shock and kill” approach where the latent virus is forced to emerge by cellular reactivation that is induced by a chemical agent and then killed as a result of viral cytolysis or immune attack [8,19]. As noted above, the limitations of this approach are low efficiency of induction and the occurrence of non-specific and toxic effects [21]. A novel means to activate HIV-1 is to use catalytically-deficient Cas9-synergistic activation mediator (dCas9-SAM) technology as a method to selectively and potently reactivate latent viral reservoirs. It has been possible to screen and identify gRNAs within the HIV-1 LTR that induce robust reactivation of provirus [79,80], which induced cellular suicide via toxic buildup of viral proteins suggesting that this might serve as a novel HIV-latency-reversing therapeutic tool for eliminating HIV-1 latent reservoirs.

GENERATION OF HIV-1 ESCAPE MUTANTS DURING GENE EDITING

The use of gene editing as described provides an attractive approach to the elimination of latent HIV-1 provirus. By targeting an essential HIV-1 gene, cleavage occurs and subsequent repair by error-prone NHEJ can result in the introduction of InDels that can disrupt the ORF of the target gene resulting in loss of function and viral elimination. A caveat to this that has emerged is the possible generation of escape mutants in which NHEJ repair has generated changes that allow the virus to replicate but are no longer subject to further cleavage because the target sequence has been altered. Recently, there have been three reports of HIV-1 escape mutants that have arisen when using gene editing approaches. De Silva Feelixge et al [81] tested HIV-1 pol gene-specific ZFN and identified a resistant, infectious, mutant virus, which had appeared after ZFN-mediated disruption of the reverse transcriptase gene. Although gene disruption of HIV protease, reverse transcriptase and integrase inhibits viral replication, a random amino acid insertion within reverse transcriptase had produced a virus that was able to actively replicate. This mutant was resistant to the endonuclease but remained susceptible to treatment with reverse transcriptase inhibitors [81]. Escape mutants have also been observed with the CRISPR/Cas9 approach [82–85]. Wang et al [82] reported profound inhibition of HIV-1 replication in T-cells with Cas9 and antiviral gRNAs but found that virus rapidly escaped from inhibition. Sequencing the HIV-1 escape mutants revealed InDels around the Cas9/gRNA cleavage site indicative of NHEJ DNA repair [82]. Similarly,

Wang et al [83] reported that many of the InDels generated by NHEJ after Cas9/sgRNA cleavage are indeed lethal but that others lead to the emergence of mutant replication competent viruses that are now resistant to Cas9/sgRNA. Yoder and Bundschuh [84] found that Indels localized to the CRISPR/Cas9 cleavage site and consisted of a single base pair in non-coding region targets but were usually 3 base pair indels when a coding region of HIV-1 was targeted allowing the reading frame to be conserved. These unexpected observations illustrate that Cas9 cleavage followed by NHEJ inactivates virus by introducing InDels but that a fraction of these retain viability [82,83]. Any therapeutic strategy for HIV eradication by gene editing must consider implications of generating viral escape mutants.

The occurrence of viral escape mutants can be minimized by appropriately choosing a suitable gene editing strategy. One approach is to target multiple sites. If multiplex gRNAs are used with the CRISPR/Cas9 system rather than a single gRNA, the chance of generating escape mutants is reduced since multiple mutations are less likely than one. Similarly, if two gRNAs are designed to produce DSBs that allow excision of DNA segment, the removal of that genetic material will prevent escape mutations from occurring. Such a multiplex approach has been demonstrated to give strong suppression of HIV-1 [73–77]. It should also be noted that approaches that cause the disruption of cellular genes, such as CCR5 as described above, would not be expected to contribute to the generation of escape mutants nor would approaches that use the dCas9-SAM technology, since dCas9-SAM has no nuclease activity.

DELIVERY OF THERAPEUTIC GENE EDITING AGENTS

Perhaps the biggest challenge for gene editing technologies is their efficient delivery to HIV-infected cells. A number of different viruses can be used for delivery and these include: adenovirus, adeno-associated virus (AAV) and lentiviruses [86]. Adenovirus vectors are useful but they have the limitation of being significantly immunogenic [87]. The presence to antibodies to adenovirus is quite common in the general population and probably more common in HIV-infected people. On the other hand, lentiviral vectors can also be used but the transduced nuclease must be present transiently or there is a risk of generating off-target events. For this reason, self-inactivating replication-incompetent or integrase-defective lentiviruses are more suitable since they give transient expression and can infect and transduce both dividing and nondividing cells [86,88]. Many studies have employed lentiviral CRISPR/Cas9 strategies including their use in eradicating latent infection by HIV-1 [71]. In the case of AAV, the virus transduces both dividing and nondividing cells without integrating and lacks an integration machinery thus remaining largely episomal. AAV vectors have limited pathogenicity and immunogenicity but suffer from the disadvantage that they are limited by the small size of the transgene that they are capable of accommodating. Choi et al delivered the Cas9 protein itself together with gRNA pre-packaged in lentiviral particles for transient exposure and showed effective gene disruption in cells [89]. Thus there are a number of possible solutions to delivery including delivering Cas9 and gRNAs separately or splitting the Cas9 enzyme into separately delivered subdomains as we have reviewed recently [90].

GENE EDITING: *EX VIVO* STRATEGY

The application of gene therapy approaches to HIV in human patients could be effected by two possible approaches: *ex vivo* approaches modify viral or cellular genes in cultured cells collected from the patient, which are then readministered while *in vivo* approaches deliver the gene therapy agent directly to the patient (Figure 1).

In an *ex vivo* approach, Tebas et al [91] adopted a strategy to test the safety of infusion of autologous CD4+ T cells in which CCR5 gene was disrupted by a ZFN. Twelve patients on cART who had chronic aviremic HIV infection were enrolled in an open-label, nonrandomized, uncontrolled study of single dose infusion of ZFN-modified autologous CD4+ T cells. Six of the patients then underwent interruption of cART 4 weeks after the infusion of 10^9 autologous CD4+ T cells, which had CCR5 disrupted by ZFN at a frequency of 11 to 28%. Safety was the primary outcome that was assessed as seen by the occurrence of treatment-related adverse events. One serious adverse event was observed which was attributed to a transfusion reaction. The median CD4+ T-cell count was 1517 cells/mm^3 after one week, which was significantly higher compared to the preinfusion count of 448 cells/mm^3 ($P<0.001$) while CCR5-modified CD4+ T cells was 250 cells/mm^3, which is about 9% of circulating PBMCs and 14% of CD4+ T cells. The half-life of the modified cells was estimated to be about 48 weeks. Secondary outcomes that were measured were immune reconstitution and HIV resistance. Treatment interruption resulted in viremia but the decline in circulating CCR5-disrupted cells was about 1.81 cells/mm^3 per day, which was significantly slower than the decline in unmodified cells, which was about 7.25 cells/mm^3 per day ($P=0.02$). In one of four patients who could be evaluated, HIV RNA became undetectable, while in most patients, the blood level of HIV DNA decreased [91]. Techniques have been developed to produce large numbers of *ex vivo* modified cells for treatment. As noted above, Maier et al developed a robust *ex vivo* manufacturing process allowing generation $>10^{10}$ CCR5-modified CD4+ T cells for ZFN modification that is suitable for clinical trials [55]. Recently, Adair et al developed a novel program for semi-automated cell isolation and culture equipment, which allows complete generation of gene-modified CD34+ blood cell products suitable for transplantation [92].

Another approach to *ex vivo* therapy is the use of chimeric antigen receptors (CAR). CAR are T-cell receptors that are genetically edited so as to graft an heterologous specificity onto an immune effector cell, usually in the context of grafting the specificity of a monoclonal antibody onto a T-cell modified by transfer of the antibody coding sequence using a vector such as a retrovirus. CAR have been used in *ex vivo* therapeutic approaches to a number of diseases including cancer [93]. Since HIV-specific cytotoxic T lymphocyte (CTL) responses are critical in controlling HIV infection, CAR can be used to augment HIV-specific CTL responses. For example, Zhen et al [94] reported the use of a protective CAR in *ex vivo* treatment of hematopoietic stem/progenitor cells (HSPC) to engineer immunity to HIV. CAR-modified HSPCs differentiated into functional T-cells and natural killer (NK) cells in humanized mice and conferred resistance to HIV infection and suppression of HIV replication. Pegu et al [95] generated a dual specificity antibody that both activated CD4 T-cells infected with HIV-1 and also facilitated their lysis where the first specificity was directed to the conserved CD4-binding site of HIV-1 Env and the second to CD3 antigen.

This antibody stimulated T-cell activation and induced proviral gene expression in infected T-cells while also stimulating CD8 T-cell effector function, which redirected T cells to lyse these cells by recognizing the newly expressed Env protein [95]. In a similar approach, Sung et al [96] generated a bispecific, antibody that bound HIV-1 Env and CD3 and found that it redirected polyclonal T cells to specifically engage with and kill Env-expressing cells, including CD4+ T-cells infected with different HIV-1 subtypes and mediated clearance of CD4+ T cells infected with HIV-1 by CD8+ T-cells [96]. Gardner et al [97] produced a fusion of CD4-Ig with a small CCR5-mimetic sulfopeptide, which bound HIV-1 (Env), which efficiently neutralized 100% of a diverse panel of neutralization-resistant HIV-1, HIV-2 and simian immunodeficiency virus (SIV) isolates. Rhesus macaques inoculated with an AAV vector expressing this antibody were protected from several infectious challenges with SIV and thus functioned like an effective HIV-1 vaccine [97]. The data from these studies and others [98–100] suggest that gene therapy with CAR may be a potentially effective therapy for chronic HIV infection.

CONCLUSIONS

New and powerful gene editing tools have become available for use against HIV-1 and they continue to be refined. A schematic of the major gene editing tools is shown in Figure 2.

While promising, significant obstacles lie in the way, such as the generation of viral escape mutants, avoidance of off-target effects and the technical demands of delivering the reagents to HIV-infected cells in patients. However, progress is already under way and there is an ongoing Phase 2 clinical trial (SB-728) to evaluate the safety and tolerability of a ZFN-CCR5-gene modification approach in T-cells in HIV-infected subjects, for which several trials have already been completed including a Phase 1 single-dose trial [94]. Since this initial demonstration of clinical safety [91], subsequent trials have sought to optimize the treatment parameters such as varying the input dose of cells and using multiple infusions of cells [101]. A summary of the experiments that have been performed on various systems relevant to the development of novel AIDS therapies is given in Table 1. In addition, gene editing approached can also be used to launch an immunological attack on chronic HIV infection as exemplified by the CAR approach described in the previous section. It will be interesting to follow the rapid pace of advancement in this field as it unfolds in the future.

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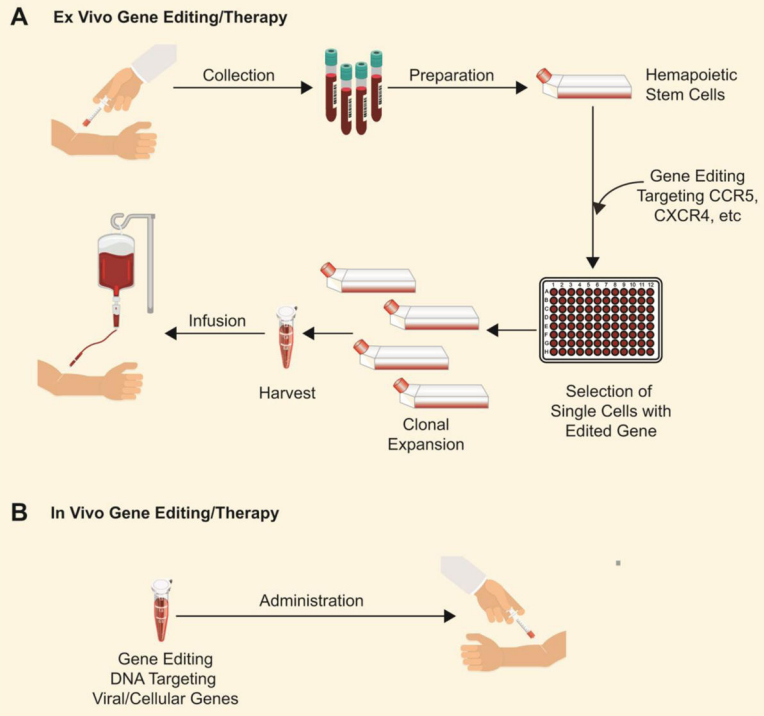

Figure 1. Proposed steps for clinical application of the gene editing strategy for elimination of HIV-1

A. Ex vivo approach involving propagation of hematopoietic cells for treatment with gene editing molecules followed by screening and selecting the identified cells with genetically inactivated, critically important cellular genes for viral infection, followed by cell expansion in the laboratory for infusion in the clinic. **B.** Direct administration of the gene editing molecule as created in the laboratory using an efficient delivery system to patients in the clinic.

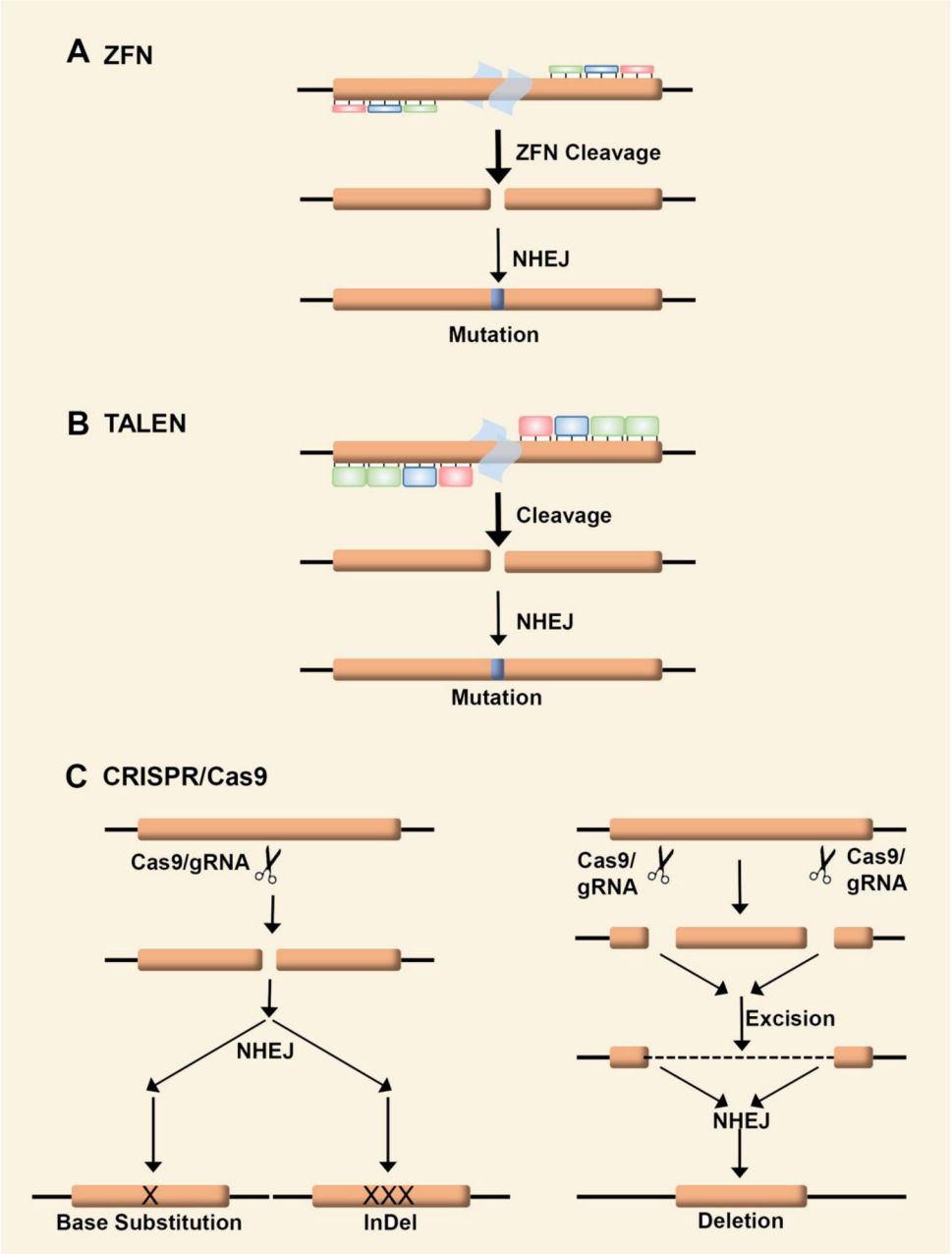

Figure 2. Schematic of the gene editing technologies

A. Zinc finger nucleases (ZFN) are a class of gene editing proteins, which are fusion proteins between the nonspecific endonuclease cleavage domain of the FokI restriction enzyme and a custom-designed Cys2-His2 zinc-finger protein, which confers specificity and gives an enzyme that can make sequence-specific DNA double-strand breaks. **B.** Transcription activator-like effector nucleases (TALEN) are another class of reagents also based on FokI fusion proteins and have a targeting domain that is taken from the Xanthomonas bacteria TAL effector proteins. MEG (MegaTAL) are derived from the homing endonucleases known as the meganucleases fused with the TAL effector proteins), **C.** Clustered regulatory interspaced short palindromic repeat (CRISPR)-associated 9 (Cas9)

is a two-component system consisting of a single-guide RNA (gRNA) that, when expressed with the Cas9 endonuclease enzyme, is able to find and cut a DNA target specified by the sequence of the guide RNA.

Table 1

Genetic Targeting toward HIV-1/AIDS Cure

A. Viral Genes					
	ZFN	**TALEN/MEG**	**CRISPR**	**Recombinase**	**CAR**
Cell Model	47, 48	68, 69, 92, 98	72–74, 76–80	39–42, 44	93–100
Ex Vivo	ND	ND	74	44	ND
Animal Models	ND	ND	75	43–44	93, 99, 100
Clinical Trials	NR	NR	NR	NR	NR
B. Cellular Genes					
Cell Model	46, 50, 59, 60, 61, 107	64–67	103–106, 108	ND	ND
Ex Vivo	55, 91	ND	ND	ND	ND
Animal Models	46, 54, 56–58, 61, 104	ND	100	ND	ND
Clinical Trials	91	NR	NR	NR	NR

ND - Not Done

NR - Not Reported