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REVIEW

OPEN

CRISPR/Cas9 for achieving postintervention HIV control

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Purpose of review
Recent advances in gene therapy have led to the first clinically approved CRISPR/Cas9 therapy for β-thalassaemia and sickle cell disease. Gene therapy could play an important role in targeting HIV persistence and achieving postintervention HIV control. Here, we review recent updates in CRISPR/Cas9-based HIV gene therapy approaches, including CCR5-editing (protect), proviral targeting (excise or modify), and immune cell engineering (attack).

Recent findings
Recent studies provide additional safety data for use of CRISPR/Cas9-based gene therapies, however low in vivo editing efficiency highlights the need for improved delivery methods. This is particularly relevant for strategies requiring transfection of all HIV-infected cells containing intact proviruses, such as proviral excision. For ex vivo editing approaches, poor engraftment and durability of edited cells present additional challenges. Newer methods such as lipid nanoparticle delivery could provide a mechanism to overcome current limitations with ex vivo and in vivo delivery. Several studies have demonstrated proof-of-concept of combination gene therapy approaches, including gene editing strategies to generate HIV-resistant cells with immune effector functions, providing novel approaches to control and durably suppress viral replication.

Summary
Several studies have demonstrated feasibility of gene therapy approaches in achieving postintervention HIV control. Improvements in both ex vivo and in vivo delivery methods are required to progress current gene therapy approaches to the clinic.

Keywords
B cell engineering, CAR T cells, CCR5, CRISPR/Cas, gene therapy, HIV latency

INTRODUCTION

Current management of HIV depends on lifelong antiretroviral therapy (ART) to suppress viral replication. There have now been ten reported cases of sustained HIV control in the absence of ART following stem cell transplantation [1–10], eight of which were related to use of donor cells lacking CCR5 (homozygous CCR5-Δ32 donor), which confers resistance to viruses that use CCR5 for viral entry (R5-tropic virus). Significant risks associated with stem cell transplantation limit scalability of this approach; however, these cases have provided proof-of-concept that long-term postintervention ART-free HIV control is achievable.

Recent advances in clustered regularly interspaced palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) and viral or mRNA delivery systems have seen a number of gene editing therapies proceed to the clinic, including the first U.S. Food and Drug Administration (FDA)-approved CRISPR/Cas9 cell therapy, Casgevy, for treatment of transfusion-dependent β-thalassaemia and sickle cell disease [11]. With the rapid development of gene and

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KEY POINTS

- CRISPR/Cas9 is being investigated as a tool to achieve postintervention HIV control, either through modification of host cells to protect from HIV infection, direct targeting of the HIV provirus, or immune cell engineering to enhance anti-HIV activity.
- A major barrier to progressing HIV CRISPR/Cas9-based therapeutics is an efficient delivery modality for both ex vivo and in vivo applications.
- Effective in vivo strategies will require targeted delivery of CRISPR/Cas9 to all cells with replication competent HIV (for direct proviral targeting approaches) or to a high number of progenitor immune cells for durable and effective activity.
- Combining gene editing strategies provides a novel approach to obtain postintervention HIV control.

cell-based therapies, target product profiles (TPPs) have been developed through a multistakeholder consensus process to define minimum and optimum targets for gene and cell-based therapies, including a recent TPP for HIV [12▪▪] and sickle cell disease [13]. For an HIV cure, the optimum TPP is a safe and effective therapy that can maintain viral control similar to optimally delivered ART, applicable to all populations [12▪▪].

There are three main gene therapy strategies being investigated for postintervention HIV control: protect: CCR5-editing to render host cells resistant to rebounding HIV infection; excise or modify: direct targeting of the HIV provirus to excise, reactivate, or silence the latent reservoir; attack: enhancing anti-HIV immunity through immune cell engineering (Fig. 1). Here, we review recent updates across these categories in achieving postintervention ART-free HIV control, with a focus on CRISPR-Cas9 based strategies.

PROTECT: GENE THERAPY TO RENDER HOST CELLS RESISTANT TO HIV INFECTION

Aiming to replicate the success of HIV cure using CCR5-Δ32 donors, several studies have used gene therapy to disrupt the CCR5 gene ex vivo followed by infusion of gene-edited cells lacking CCR5. Early

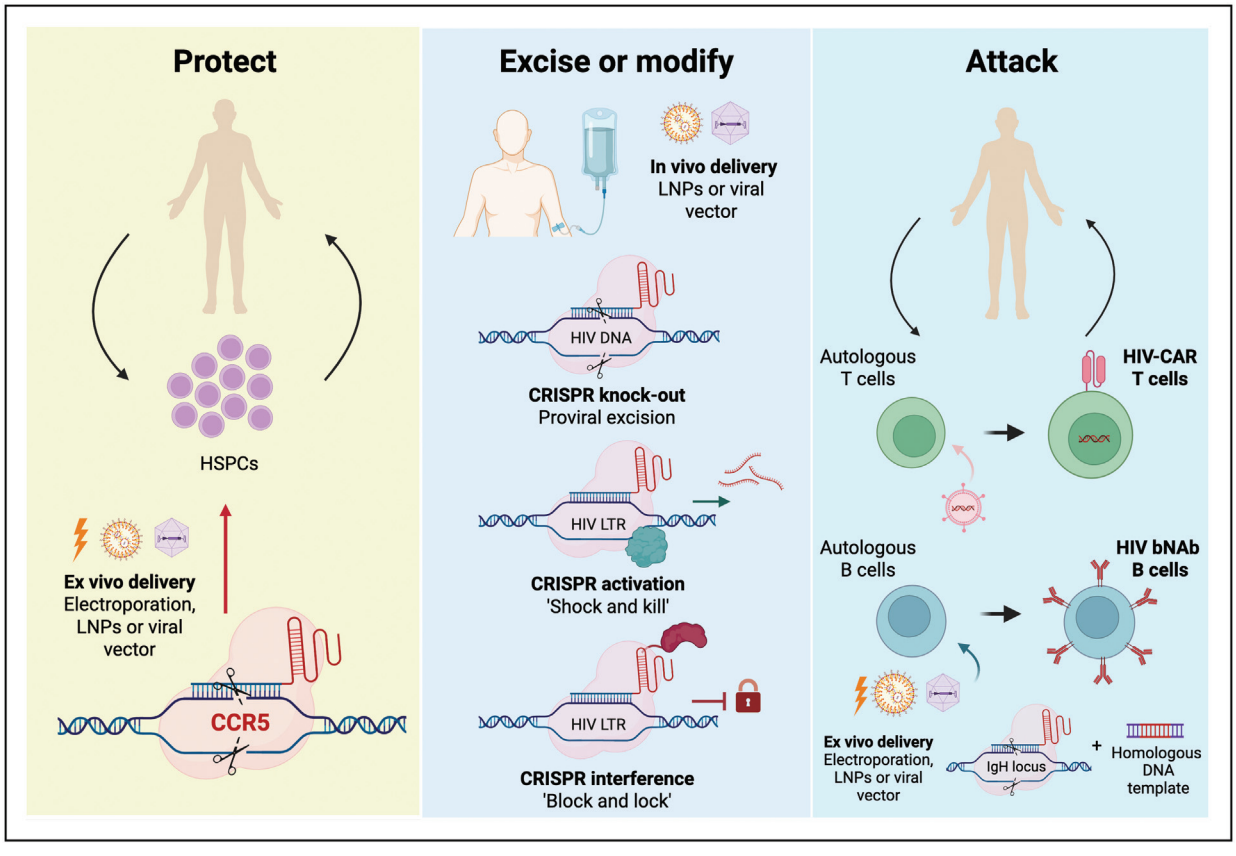

FIGURE 1. Current gene therapy approaches for achieving postintervention HIV control. bNAb, broadly neutralizing antibody; CAR, chimeric antigen receptor; HSPCs, haematopoietic stem and progenitor cells; LNP, lipid nanoparticle; LTR, long terminal repeat.

clinical trials used zinc finger nucleases for ex vivo editing of autologous $CD4^+$ T cells [14,15] and CRISPR/Cas9 has been used more recently for ex vivo CCR5 knock-out in haematopoietic stem and progenitor cells (HSPCs) as part of treatment of acute lymphoblastic leukaemia in a person with HIV [16]. In these studies, efficacy was limited by the low frequency of CCR5-edited cells, both in the preengraftment product (20–30%) and in peripheral blood $CD4^+$ T-cells obtained postengraftment (<5% for CRISPR/Cas9-edited cells at 19 months) [14,16]. These numbers fall far below the predicted frequency of approximately 90% CCR5-edited cells which would be required to protect from HIV rebounding following cessation of ART [17]. This limitation is likely a result of the low ex vivo editing efficiency and reduced capacity of edited cells compared to nonedited cells to re-populate in vivo [18].

A recent study evaluated CRISPR/Cas9 CCR5-edited human HSPCs that were subsequently transplanted at varying frequencies into immunodeficient mice after myeloablation [19▪]. High ex vivo editing efficiency of HSPCs (>90%) was reported in this study using electroporation of CRISPR/Cas9 with guide RNAs (gRNAs) targeting the CCR5 locus. The high level of editing efficiency achieved is a significant advance over the previously described studies using zinc finger nucleases [14,15] and initial CRISPR/Cas9 studies [16,20], and could be a result of the use of optimized gRNA target sites. Engraftment of CCR5-edited HSPCs was stable in vivo and cells were able to differentiate into myeloid and lymphoid cell lineages that lacked CCR5. Edited cells were resistant to HIV infection; however, decreased benefit was observed if the frequency of CCR5-edited cells was low, with negligible protection when frequencies ranged between 54% and 26%. These findings confirm modeling predictions that approximately 90% of CCR5-edited cells would be needed for this approach to provide long-term protection from viral rebound after stopping ART [17].

An alternative approach is to directly deliver the CCR5 gene editing tools to immune cells in vivo, for example through use of viral vectors or lipid nanoparticles (LNPs) (Fig. 2). However, this approach is critically dependent on the ability to transfect HSPCs and CD4 T cells with high efficiency in vivo. Targeted LNPs have been investigated using CD117 antibody-conjugation, for delivery of reporter mRNAs to HSPCs in mice [21,22]. These studies have provided proof-of-concept for in vivo gene editing of HSPCs without the need for a conditioning regimen, although LNP delivery of CRISPR/Cas9 mRNAs to HSPCs has not yet been evaluated.

One major limitation of CCR5 gene editing is the lack of protection against viruses that use CXCR4 for cellular entry (X4-tropic virus). Viral rebound from X4-tropic virus has been observed in people with HIV who received a CCR5-Δ32 stem transplantation and stopped ART [23,24], demonstrating that CCR5-editing alone is not a suitable intervention if X4-tropic virus is present, which is the case in roughly 25% of people with HIV [25,26]. To circumvent this, a strategy combining CRISPR/Cas9 knock-out of CCR5 with expression of a cell membrane-anchored HIV fusion inhibitor, C46, has recently been evaluated in cell lines [27] and primary human T cells [28] and shown to protect from both R5- and X4-tropic virus in vitro. However, editing efficiency of HSPCs was low, with only 15% of cells expressing C46 in edited HSPCs ex vivo, and fewer than 10% of bone marrow cells expressing C46 14 weeks postengraftment in immunodeficient mice, despite over 90% of cells carrying the CCR5 knock-out [28]. This study highlights the added complexity of multimodality gene editing and will require further optimization to improve efficiency of this gene editing approach.

EXCISE OR MODIFY: TARGETING THE HIV PROVIRUS

One approach to eradicate latent HIV is through direct proviral excision. Proof-of-concept of CRISPR/Cas9-mediated proviral excision has been demonstrated in both humanized mice [29,30] and nonhuman primate (NHP) models [31▪,32]. Safety and biodistribution of in vivo CRISPR/Cas9 targeting of the SIV provirus using an adeno-associated virus (AAV) vector (EBT-001) was recently assessed in ART-treated, SIV-infected rhesus macaques [31▪]. EBT-001 DNA was detected in all examined tissues, including mesenteric lymph nodes, the central nervous system (CNS) and the gastrointestinal tract and evidence of SIV proviral excision was detected in blood and tissues at both 3 and 6 months. However, there was high variability in excision efficiency between individual NHPs and across various tissues, with no detectable excision in the spleen or colon in some NHPs. Measures of intact proviral SIV DNA to quantify changes in the viral reservoir pre and postintervention were not assessed and an analytic treatment interruption was not performed. Off-target editing was assessed in pre and postintervention lymph node samples from two NHPs by sequence analysis of predetermined genomic regions with the highest sequence homology to the gRNAs. The authors found no off-target activity, although analysis was limited by low sequencing depth and reduced sensitivity for detection of single nucleotide polymorphisms. Importantly, there was no evidence of larger structural variants at nominated sites. Overall,

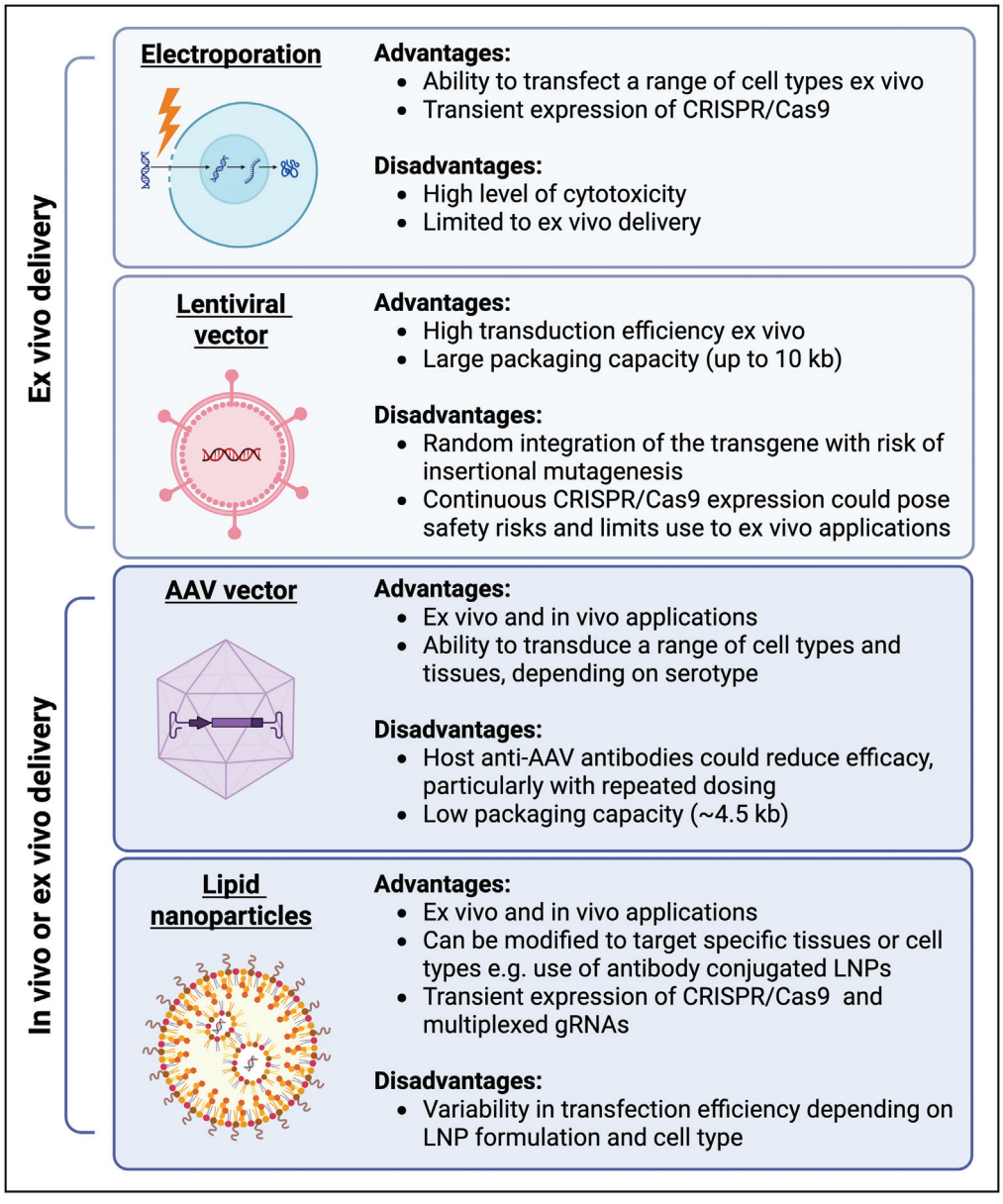

FIGURE 2. Ex vivo and in vivo delivery modalities for CRISPR/Cas9. AAV, adeno-associated virus; gRNA, guide RNA; kb, kilobase; LNP, lipid nanoparticle.

the study demonstrated safety and feasibility of CRISPR/Cas9 delivery to important anatomical reservoirs, albeit at insufficient levels to eliminate all latently infected cells.

A first-in-human phase 1 clinical trial of an AAV-vector containing HIV-targeting CRISPR/Cas9 therapy, EBT-101 (NCT05144386), has been evaluated in six participants with HIV and a suppressed viral load. EBT-101 was demonstrated to be safe and well tolerated; however, viral rebound was observed in all three participants who underwent analytical treatment interruption, suggesting insufficient activity following a single dose to achieve postintervention ART-free HIV control [33]. Further data are awaited, including outcomes on secondary endpoints including biodistribution and immunogenicity.

The 'shock and kill' approach to eliminate latently infected cells relies on latency reversing agents (LRAs) to induce virus production, leading to virus-mediated cytotoxicity or immune-mediated clearance of the latently infected cell [34]. LRAs evaluated to-date have lacked potency, owing to nonspecificity for the HIV provirus and potential adverse effects limiting adequate dosing [35–37]. One method to specifically target HIV is the use of CRISPR activation (CRISPRa), which comprises gRNAs targeting the HIV promoter and catalytically inactive 'dead' Cas9 (dCas9) fused to transcriptional

activators. CRISPRa has previously shown promise in cell line models of latency [38–40], including in a recent study that combined CRISPRa with a pro-apoptotic 'suicide gene', tBid, to enhance killing of reactivated cells [41▪]. Expression of the tBid gene was made HIV-specific through use of an HIV promoter and inclusion of a Rev response element, necessitating presence of HIV proteins, Tat and Rev, for tBid protein expression.

Our team recently demonstrated the successful delivery of CRISPRa mRNA and relevant guide RNAs to resting $CD4^+$ T-cells using a novel LNP that enhanced association of the LNP as well as endosomal release of the mRNA [42▪]. These CRISPRa-LNPs increased cell-associated HIV transcripts in $CD4^+$ T-cells from people with HIV on suppressive ART, albeit modestly and did not induce multiply-spliced transcripts. While these findings suggest CRISPRa delivery may require further optimization, this work highlighted that CRISPRa has potential beyond cell lines as an HIV-specific LRA. Further evaluation is required to assess safety and efficacy of this LNP formulation in vivo.

Another approach for direct proviral targeting is through proviral inhibition, aiming to permanently silence the HIV reservoir by blocking HIV transcription. Several 'block and lock' approaches have been described [43], including use of dCas9 variants to repress transcription through DNA or histone methylation, referred to as CRISPR interference (CRISPRi). CRISPRi reduced proviral reactivation following stimulation with mitogens or LRAs in cell line models of latency [44,45]. However, the durability of proviral silencing is unknown, as CRISPRi-mediated repression of transcription has been observed to wane once CRISPRi expression diminishes [46]. Further evaluation of CRISPRi is required in primary cells, including ex vivo testing, to assess effectiveness of this approach in achieving long-term HIV silencing.

A challenge for all direct proviral targeting approaches is the vast HIV sequence diversity within and across individuals. This introduces risk of failed gRNA binding to the HIV provirus due to sequence mismatches, leading to potential loss of activity [47,48]. In addition, incomplete HIV sequence coverage by gRNAs could lead to viral escape mutations, which has been described for CRISPR/Cas9 proviral excision [49–52]. For CRISPRa and CRISPRi, the choice of gRNA target site is restricted to regions immediately upstream of the transcription start site for effective activity [46,53]. This poses further challenges in identifying conserved regions across HIV subtypes for gRNA targeting. Use of multiplexed cross-clade gRNAs will likely be required for CRISPR/Cas9 systems, particularly if this therapeutic is to achieve the minimum targets set out in the TPP of at least 50–75% effectiveness across participants [12▪▪]. Alternatively, subtype-specific gRNAs could be used in combination with companion diagnostics to validate the proviral subtype and sequence compatibility. The ability to efficiently deliver multiple gRNAs could be aided through use of LNP delivery of CRISPR/Cas9 mRNAs encapsulated with presynthesized gRNAs [42▪], although the maximum capacity of gRNA multiplexing has not yet been established. Alternative Cas orthologues have also been explored to increase the breadth of targetable regions across the HIV genome, given the ability of different orthologues to recognize different protospacer adjacent motifs [54].

For direct proviral targeting approaches to be effective in achieving postintervention ART-free HIV control, delivery of CRISPR/Cas9 components to all cells harboring intact, replication-competent proviruses is required. As illustrated in the NHP model, delivery of CRISPR/Cas9 by AAV-vector was insufficient in eliminating all intact proviruses following single infusion, although CRISPR/Cas9 was successfully delivered to important anatomical reservoirs such as the CNS and gastrointestinal tract [31▪]. Presence of preexisting neutralizing antibodies to AAVs also poses risk of inactivation of the AAV vector, potentially resulting in loss of activity [55]. Anti-AAV neutralizing antibodies have been detected in as many as 74% of people, depending on the serotype [56], and levels were observed to increase following infusion of the EBT-001 product in NHPs [31▪], suggesting potential for reduced effectiveness with subsequent dosing.

LNPs could provide an alternative modality for in vivo delivery of CRISPR/Cas9; however, this would require modifications to ensure delivery of LNP cargo to all HIV anatomical reservoirs, including lymphoid organs and the CNS. Although LNP formulations can be modified for directed cell or tissue tropism [21,22,57,58], it is unlikely that any single formulation would facilitate delivery to all tissue reservoir sites, increasing the complexity in development of a single effective product.

ATTACK: IMMUNE CELL ENGINEERING FOR ANTI-HIV ACTIVITY

Broadly neutralizing antibodies (bNAbs) have been evaluated in people with HIV and shown to have potent antiviral activity [59,60]; however, repeated and potentially life-long administration would not classify this as a curative approach under the current TPP. Therefore, genetic engineering of B cells has been explored, with the aim of generating cells that can continuously produce bNAbs in vivo [61–63,64▪]. CRISPR/Cas is typically used to edit activated

primary B cells by inserting a bNAb-encoding cassette into the immunoglublin heavy (IgH) or kappa (Igκ) gene loci. This has been demonstrated ex vivo [61,64▪] and in vivo using AAV vectors to deliver CRISPR/Cas9 and bNAb templates to B cells [62]. A recent study demonstrated feasibility of encoding nonantibody biologics into the heavy chain locus of murine B cells, using CRISPR/Cas12a and a DNA template for the HIV entry inhibitor, CD4 immunoadhesin (CD4-Ig), delivered ex vivo using an AAV vector [63]. Expansion of engineered B cells in vivo for both bNAb and CD4-Ig-edited cells was achieved by immunization with HIV antigens to drive clonal expansion of the engineered cells [63].

A major benefit of B cell engineering to express bNAbs or HIV-targeting biologics is the ability of engineered B cells to undergo somatic hypermutation, class switching and affinity maturation in vivo. This may afford greater long-term ART-free viral control through the ability of B cells to continuously adapt to rebounding viral variants and reduces the risk of viral escape. Successful affinity maturation has been demonstrated for both bNAbs and CD4-Ig in vivo, with bNAb and CD4-Ig variants obtained from mice post immunization demonstrating higher neutralization activity of a panel of HIV isolates compared to wild-type variants [63,64▪]. These data demonstrate successful engraftment of engineered B cells. However, it remains unknown whether the durability and adaptability of engineered B cells in vivo will be sufficient to provide long-term protection, and whether secretion of bNAbs from engineered B cells would induce similar antidrug-antibody responses to those described following delivery of HIV bNAbs using AAV vectors for in vivo expression [65,66]. Further studies, including evaluation in NHP models, are required to assess efficacy of in vivo antiviral control with engineered B cells in the setting of antiretroviral treatment interruption.

Engineering of T cells to express a chimeric antigen receptor (CAR) may be a strategy to enhance recognition and direct killing of HIV-infected cells expressing Env [67]. Generating CAR T cells typically involves ex vivo lentiviral transduction of T cells to express the desired receptor [68]. Newer generation HIV-CAR T cells have been designed to express two CARs (duoCAR), allowing targeting of multiple distinct HIV Env-binding domains to prevent viral escape [69]. HIV-duoCAR T cells have been generated using lentiviral transduction and shown to have HIV-specific cell killing capacity in humanized mice [69,70] and are currently being evaluated in an open-label clinical trial (NCT04648046).

One challenge with HIV-CAR T cells is the long-term maintenance of functional CAR-T cells in vivo. In addition, HIV-CAR T cells are potentially vulnerable to HIV infection in vivo. CRISPR/Cas9 has been used to enhance CAR T cell function through excision of immune exhaustion markers, including PD-1 [71,72], although this has not yet been evaluated specifically for HIV-CAR T cells. Targeted insertion of the CAR transgene into the CCR5 locus using CRISPR/Cas9 and homology-directed repair has also been evaluated to assess the potential of combining CAR T activity with CCR5 knock-out for protection from rebounding HIV-infection with R5-tropic virus [73].

COMBINATION GENE THERAPY APPROACHES

In an effort to maximize potential for durable HIV-control, several studies have evaluated combination gene editing approaches, including CCR5-editing in HIV-chimeric antigen receptor (CAR) T cells [73] discussed above, CCR5-editing with proviral excision [74], and CCR5-editing with B cell engineering [75▪].

In a recent study, CRISPR/Cas9 knock-out of CCR5 was combined with knock-in of DNA coding for neutralizing antibodies or the HIV entry-blocking antibody, ibalizumab [75▪]. Human primary B cells or cord blood CD34+ HSPCs were edited ex vivo using electroporation of CRISPR/Cas9 followed by transduction with an AAV-vector containing the DNA donor template. Edited HSPCs demonstrated successful engraftment and reconstitution of cell lineages including B cells carrying the knock-in allele in transplanted mice. However, significant reduction in bone marrow human chimerism was observed, suggesting a detrimental effect of ex vivo editing using electroporation and AAV-vectors on engraftment capacity. Nevertheless, this work highlights the potential of combining CCR5 knock-out with antibody-secretion to confer additional protection from re-infection.

SAFETY CONSIDERATIONS

A major concern with gene therapies and CRISPR/Cas9 is the potential for off-target activity or genotoxicity. Off-target activity, including insertions, deletions and structural variants (e.g. translocations) have been described using CRISPR/Cas systems in both cell lines and primary cells in vitro [76]. A study using CRISPR/Cas9 editing of fertilized zebrafish eggs identified off-target mutations in 26% of offspring using long-read whole genome sequencing, including presence of large insertions and deletions of several kilobases [77]. However, CRISPR/Cas9 editing in several NHP studies have identified minimal or no off-target editing events by whole genome sequencing [78–80]. In addition, clinical trials for

the FDA-approved CRISPR/Cas9 therapeutic, Casgevy, demonstrated no concerning data related to genotoxicity [81,82]. Longer-term safety data are needed, which is the subject of a 15-year follow-up extension study (NCT04208529) for extended monitoring to assess late adverse effects including malignancy. A 15-year extension study is also planned for participants who received the HIV-targeting CRISPR/Cas9 therapy EBT-101 (NCT05143307).

Given the safety and tolerability of HIV antiretroviral therapy, a therapeutic aiming to achieve post intervention ART-free viral control should meet minimum safety targets, as described in the consensus TPP [12▪▪]. This includes <5% frequency of grade 3 or 4 treatment-emergent adverse events requiring hospitalization and <1% risk of insertional mutagenesis leading to leukaemia or tumours. With over 20 clinical trials using CRISPR/Cas9-based therapeutics currently listed as completed or actively recruiting (clinicaltrials.gov), additional safety data will soon be available to better inform clinical safety of CRISPR/Cas9 therapies for use in HIV.

CONCLUSION

Several proof-of-concept studies have demonstrated feasibility of gene therapy in obtaining postintervention HIV control. A combined approach will likely be required to maintain durable control off ART, including reduction in the HIV reservoir through direct proviral targeting and enhanced immune control through immune cell engineering. Further advances are required to improve both ex vivo and in vivo delivery methods of CRISPR/Cas9 and other gene editing tools to facilitate progression of these therapeutics to the clinic.

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Conflicts of interest

S.R.L. has received honoraria unrelated to the content of this manuscript for participation in advisory boards for Gilead, Viiv Healthcare, Merck, Abbvie and Esfam. She has received funding from Gilead and Merck for investigator-initiated research projects unrelated to the content of this manuscript. M.A.M., M.R., P.M.C. and S.R.L. are co-inventors of a patent for a novel LNP (PCT/AU2024/050506). P.M.C. and S.R.L. are co-inventors of a patent for targeting LNPs to T cells (PCT/AU2025/050194).

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