Beyond Antiretrovirals: Exploring Gene Therapy Approaches in HIV Treatment

Arshiya Bhasin

Abstract

Human Immunodeficiency Virus (HIV) is considered a major public health issue, having claimed the lives of 40.4 million people since the beginning of the epidemic in 1981, with ongoing transmission in all countries globally. Gene therapy has emerged as a promising avenue for combating HIV infection, offering potential advantages over conventional antiretroviral therapies. Currently, the most widely used therapy for the treatment of HIV is highly active antiretroviral therapy (HAART). Though this has significantly improved the quality of life for the people living with this virus, it comes with many challenges such as resistance mutations that allow for viral escape and adverse side effects. Gene therapy treatments for HIV aim to reconstitute the immune system with HIV-resistant cells and inhibit formation of HIV proteins that are vital for the survival of the virus, providing a potential functional cure for the disease with minimal adverse side effects for the patient. These strategies include the use of RNA and protein-based agents that target specific viral and host genes. There has been significant progress in the study of these therapies, some of which have even progressed to the clinical trial phase. This paper will review the different gene therapy approaches for HIV treatment as well as their advantages and disadvantages.

Introduction

Human immunodeficiency virus (HIV) is a lentivirus that targets the human immune system leading to acquired immunodeficiency syndrome (AIDS) in later stages of infection[1]. A lentivirus is a type of retrovirus containing an RNA genome that is converted to DNA with the help of reverse transcriptase enzyme and integrated into the genome of the host cell[2]. The virus mainly attacks and depletes CD4 T cells[3] which play an important role in protecting the body from pathogens[4], so infected individuals have weaker immune systems and are significantly more susceptible to opportunistic diseases than uninfected individuals[5]. The CD4 T cells count in healthy individuals ranges from 500 to 1500 cells/mm^3, but this count drops to below 200 cells/mm^3 in AIDS patients[6]. Despite significant progress in the study of this virus as well as the development of treatment options, HIV continues to be a topic of great concern worldwide. According to the World Health Organisation, an estimated 39 million people were living with HIV at the end of 2022. The complexity of the virus and its ability to persist in host cells poses a formidable challenge to scientists, and there is yet to be a functional cure for this disease.

HIV can enter the human body through various routes including sexual transmission, from mother to child during breastfeeding or childbirth and through blood contact wherein infected blood from one person enters the bloodstream of another individual[7]. Once inside the body, the

virus attacks CD4 T lymphocytes and establishes a reservoir of latent HIV, a group of cells wherein the virus is not actively replicating and causing an immediate infection, but it remains inside the cell in a dormant or latent state[8]. The attack on CD4 T cells by HIV virus is a multi-step process **(Figure 1).** First, viral attachment occurs, where the virus binds to a primary cell receptor (CD4) and a coreceptor (CCR5 or CXCR4). CD4, CCR5 and CXCR4 are receptors present on immune cells that facilitate viral attachment of HIV. In early stages of infection, the virus tends to use CCR5 cell receptors as coreceptors for viral attachment, while in later stages the virus uses CXCR4 cell receptors[9]. The viral and host membranes fuse together, allowing the viral RNA and enzymes into the cell cytoplasm[10]. The virus then undergoes a complex lifecycle including reverse transcription, integration, replication, assembly and budding[10], leading to an increase in viral load and progressive decline in CD4 T lymphocytes. Understanding of this life cycle has significantly contributed to the development of HIV therapy treatments. Through extensive research, multiple protein targets essential to various stages of the viral life cycle have been identified. Leveraging this knowledge, researchers have been able to develop innovative therapeutic strategies aimed at disrupting key viral processes, thereby inhibiting the virus's ability to propagate within the host.

In most patients, symptoms of HIV infection are currently being managed by the use of Highly active Antiretroviral therapy (HAART), a treatment regimen typically consisting of two or three antiretroviral drugs used in combination. Antiretroviral drugs work by temporarily inhibiting certain vital processes in the HIV life cycle[11]. The antiretroviral drugs used in HAART can be classified into six groups based on their targets: Nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), integrase strand transfer inhibitors (INSTIs), fusion inhibitors (FIs) and chemokine receptor antagonists (CCR5 antagonists)[12]. Each of these drug groups have their own adverse side effects and a combination of drugs is chosen specifically based on patient details and history[12]. In the years 1994 to 1997, the proportion of patients to whom a combination of antiretroviral drugs were prescribed increased significantly from 72% to 95%[13]. Mortality rates due to AIDS declined markedly from 29.4 per 100 persons-years in 1995 to 8.8 per 100 person-years in 1997[13]. Though the introduction of this treatment has significantly reduced morbidity and mortality among patients[13,14], it does not eliminate the reservoir of latent HIV in the affected person[15]. As a result, HAART treatment is only a temporary treatment, and HIV will rebound when this treatment is stopped[8]. The most common reasons for patients to discontinue HAART treatment is actual side-effects and fear of side-effects[16]. HAART is commonly associated with several adverse effects, long term toxicity and development of viral resistance[17].

Scientists are currently working towards developing alternate treatments for HIV, and one of their most promising avenues is gene therapy. Gene therapy treatments for HIV involve targeted silencing or editing of viral or host genes coding for proteins that are vital for the survival of the virus, leading to long term eradication. These treatments have been used to target genes that

code for crucial proteins in the HIV life cycle. They aim to inhibit viral replication and, in some cases, even to achieve systemic elimination of latent viral reservoirs[18,19]. Some of these strategies include the use of Zinc Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/cas9) and RNA interference (RNAi). Advancements in gene therapy delivery and ongoing clinical trials provide hope for a future where HIV can be effectively controlled or even eradicated. This review paper will provide an overview of the gene therapy strategies that are currently being studied and developed for the treatment of HIV.

Zinc Finger Nucleases

Zinc Finger Nucleases (ZFNs) are programmable DNA binding protein systems[20]. They are used to edit genomes by inducing site-specific double strand breaks, provoking cellular repair processes that in turn mediate modification of the targeted site. The double stranded break can be repaired via non-homologous end joining which results in small deletions or insertions in the target gene, leading to gene knockout. ZFNs may also facilitate targeted repair using a donor DNA template with the desired genetic sequence. This pathway can be used to introduce specific genetic modifications including gene knockouts, gene replacements and gene insertions[20]. ZFNs are small in size, making delivery of the therapy relatively easy[21]. A ZFN consists of two main parts: the zinc finger protein, which binds the DNA, [22] and the FokI restriction enzyme, which cuts the DNA[23,24].

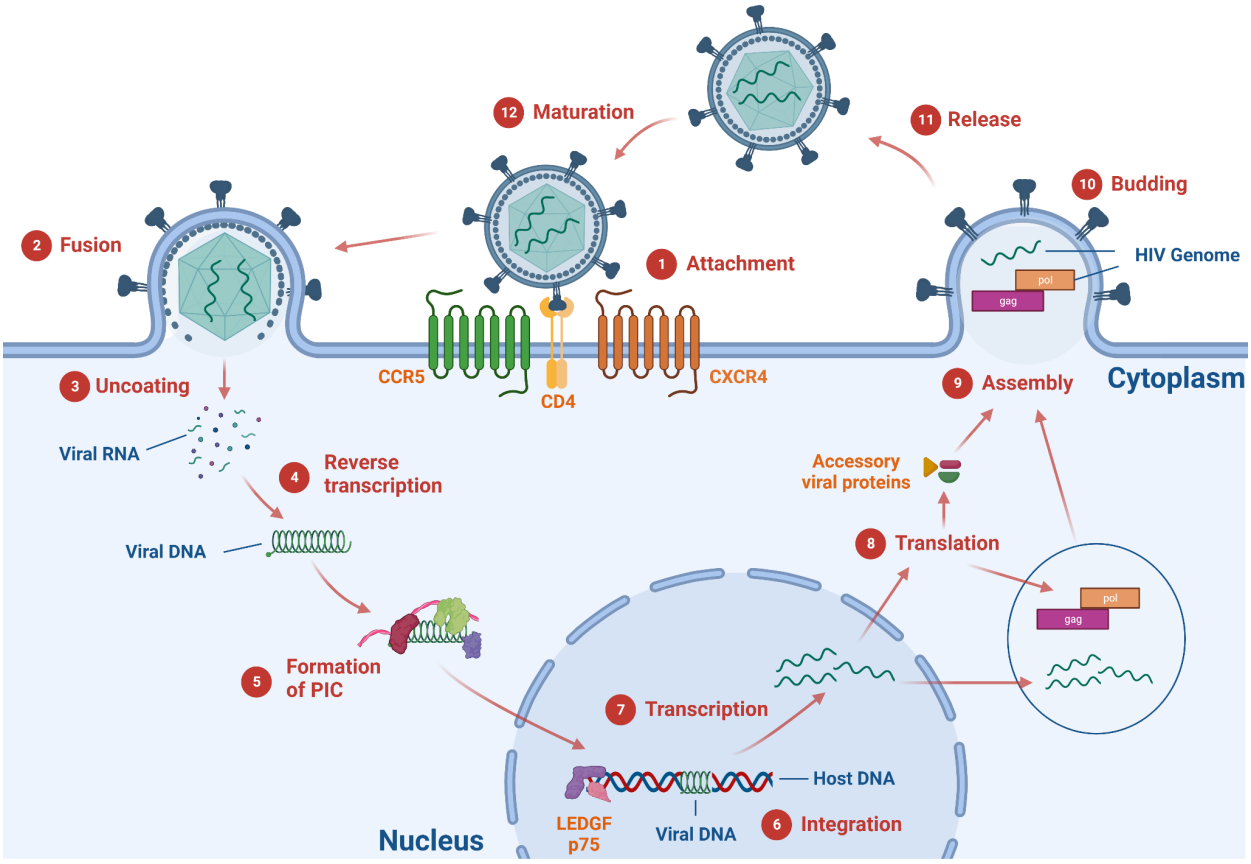

Figure 1. The HIV replication cycle in host immune cells. *The first step,* ***Viral Attachment (1),*** *occurs when the HIV Env protein binds to receptor (CD4) and coreceptor (CCR5 or CXCR4) on the surface of the infected cell. After attachment, the viral and host membranes fuse together in* ***Fusion (2)****, allowing the viral particles to enter intracellular space in capsid. Once entered the intracellular space,The next step is* ***Uncoating (3)*** *wherein the capsid is broken down* ***(3)****, releasing the viral genome and proteins into the host cell cytoplasm. In the cytoplasm, single stranded viral RNA molecules are transcribed into linear double stranded DNA molecules by* ***Reverse Transcription (4)****, leading to the* ***formation of the Pre-integration Complex (PIC) (5)****. This PIC is imported into the nucleus and* ***Integration (6)*** *occurs, where the viral DNA is inserted into the host genome with the help of the proteins HIV integrase and lens epithelium-derived growth factor/transcription co-activator (LEDGF/p75).* ***Transcription (7)*** *of this integrated genome results in the formation of viral mRNAs that code for viral proteins. The mRNA molecules are exported out of the nucleus and undergo* ***Translation (8)****, synthesizing multiple viral proteins such as gag and pol. The viral proteins synthesized move to the surface of the cell along with the new HIV mRNA and assemble into immature (non-infectious) HIV, this step is called* ***Assembly (9)****. The immature HIV pushes itself out of the host cell during* ***Budding (10)*** *and is eventually released outside the host cell* ***(11)****. Once released, the immature virus produces HIV protease, an enzyme that breaks down long protein chains into smaller ones causing the* ***Maturation (12)*** *of the virus, producing mature (infectious) HIV.*

These protein systems are designed in pairs, with one binding to the forward strand and another binding to the reverse strand[25]. Each zinc finger domain recognizes a 3-4 base pair DNA sequence, allowing scientists to target specific genes by selection of ZFNs that have high binding affinity to the target DNA sequence[20]. Once the ZFNs bind to specific sites, the FokI restriction enzyme can dimerize and cleave the DNA[26]. Following this double stranded break, the DNA is repaired by homologous end joining, non-homologous end joining or with a DNA template[27]. Homologous end joining retains the original DNA sequence[28], but the molecule is susceptible to re-cutting by ZFNs, whereas non-homologous end joining causes random changes in the nucleotide sequence leading to functional knockout of the gene[20].

This type of genome editing has been used to target multiple genes of the viral and host genomes. In human CD4 T cells it has been used to permanently knockout the *CCR5* and *CXCR4* genes which code for the major HIV receptors, rendering the cells immune to the virus due to inhibition of viral entry[20,21]. ZFNs have been shown to disrupt 30-50% of target alleles (*CCR5* or *CXCR4*) in a pool of CD4 T cells[21,22]. A study by Juan et. al. showed that when challenged with HIV *in vitro*, *CXCR4* modified CD4 T cells remained <1% viral positive for up to 42 days post challenge[21]. ZFN modified cells have also been tested in mice models. In a study conducted by Didigu et. al., ZFNs were used to simultaneously inhibit *CCR5* and *CXCR4* gene expression in CD4 T cells of humanized mice before exposure to HIV[23]. Mice that were modified by both ZFNswith disruptions to both *CCR5* and *CXCR4*, showed a much slower rate of decline in CD4 T cells. CD4 T cell counts in mice infused with modified cells were 200 fold higher than mice in the control group 55 days post infection[23]. It can be inferred through these observations that modified CD4 T cells have a significant survival advantage as compared to unmodified CD4 T cells.[23]. Though further research must be conducted to make this method more efficient, the results indicate that dual ZFN treatment modifying *CCR5* and *CXCR4* genes can be used to confer long-term protection against HIV as the decline in CD4 T cells is significantly slowed down. Clinical trials have also shown promising results for the infusion of ZFN *CCR5* modified CD4 T cells, with overall increases in CD4 T cell counts, decreases in viral loads, and no recurring adverse effects[24,25]. A clinical trial conducted by Tebas et. al. took HIV positive patients on HAART treatment and interrupted it. The patients were infused with *CCR5* modified CD4 T cells. The study found that though patients exhibited an initial peak in viral load, the viral load eventually began to decrease indicating that the treatment is effective in protecting CD4 T cells from HIV infection. One patient's viral load even decreased below the level of detection during the HAART interruption period, as the patient was heterozygous for the *CCR5* allele[25].

There are currently two types of cures being studied for HIV, functional cures and sterilizing cures. A functional cure refers to long term control of HIV replication without treatment whereas a sterilizing cure refers to complete eradication of the virus in the host. Disruption of *CCR5* and

CXCR4 can stop the initial viral infection process which could eventually result in a functional cure, but it does not completely eradicate the virus from the body as it has no impact on the reservoir of latent HIV. To overcome this challenge and eradicate the HIV reservoir as well, ZFNs have been developed to target HIV pro-viral DNA segments in infected cells. ZFN technology has been used *in vitro* to target multiple genes of the pro-viral DNA, excising over 80% of the pro-viral DNA in latently infected cells[26]. They can be used to target specific genes such as *TAT*[27] and *POL*[26], as well as the full length HIV pro-viral DNA[28].

The establishment of the latent reservoir of HIV is a complex process. Latent HIV can exist in a variety of cells and tissues throughout the patient's body[8,29,30]. Due to this, targeting and elimination of the reservoir is quite challenging, so clinical trials that involve the targeting of pro-viral DNA in latently infected cells using ZFNs are yet to be conducted. In the future, delivery systems for ZFNs targeting latently infected cells could be evaluated in humanized mice models which have been used to test various other gene therapy treatments for HIV[31]. Another limitation of using ZFN technology is the high occurrence of off-target toxicity, which is caused by unwanted disruption of vital genes resulting in malignancy and cytotoxicity[32] . Although ongoing clinical trials using ZFN mediated genetic modification have not shown regularly occurring adverse events, the long-term effects are yet to be assessed[33].

Transcription Activator-like Effector Nucleases

Transcription Activator-like effector Nucleases (TALENs) are artificially designed restriction enzymes that work similarly to ZFNs. They consist of Transcription Activator-like effectors, which act as DNA binding domains, and FokI restriction enzymes, which act as the nuclease domain, fused together[34]. Transcription Activator-like effectors (TALEs) are a class of naturally occurring DNA binding proteins found in the bacteria genus *Xanthomonas*[35]. Each TALE is made up of 34 amino acid repeat modules[35] that can be engineered to target specific 17-20 base pair DNA sequences[36]. Like ZFNs, TALENs are also designed in pairs and activate the FokI enzyme to cut DNA when they bind to their respective sites on the target DNA sequence[34,37]. This is followed by homologous DNA-repair, non-homologous DNA repair or directed repair with a template DNA sequence[38,39]. TALENs have higher specificity than ZFNs, as the target DNA sequence length is much greater[37,40], but this larger size also limits their delivery efficiency[31].

TALENs have been used to target the *CCR5* gene[41–43]. An experimental study conducted by Schwarze et. al. showed that TALE nucleases can efficiently knockout the *CCR5* gene. 44% of cells subjected to treatment with TALENs showed biallelic disruption and 16% showed monoallelic disruption[43]. A study conducted in 2014 by Mussolino et. al. compared the use of ZFNs to TALENs in functionally knocking out the *CCR5* gene. Compared to ZFNs, TALENs were

better tolerated in both cell lines and rat models, which could indicate that they have greater specificity, lower off-target cleavage events and lower cytotoxicity[44]. When 23 predicted off-target sites were analyzed after genome editing by ZFNs, 5 of them showed up to 4.4% off-target activity[44]. The same off-target sites were analyzed after genome editing by TALENs, and only 3 of them showed any off-target activity, and in all three cases it was very minor (0.12%)[44].

TALENs have also been used to target the host gene *PSIP1,* which codes for Lens epithelium derived growth factor (LEDGF/p75)[45]. The LEDGF/p75 protein acts as a chromatin tethering factor for the HIV integrase protein, and also maintains the stability of HIV integrase[46,47]. In the absence of LEDGF/p75, HIV integrase gets degraded and the virus is unable to integrate into the host genome[46]. When tested *in vitro*, different designs of TALENs showed 40-50% inhibition of the gene *PSIP1*[45]. Though the PSIP1 seems like a viable gene target for HIV therapy due to its vital function in the integration of the viral genome, it has not reached the clinical trial phase. This is because the functional knockout of the *PSIP1* gene has resulted in prenatal mortality or phenotypic abnormalities in humanized mouse models, raising safety concerns for the use of this approach in humans[48].

As compared to ZFNs, TALENs are harder to deliver due to their large size and repeating characteristics[49]. These characteristics make *in vivo* delivery of TALENs into target cells challenging[49]. Another limitation of using TALENs for gene knockout is that they cannot be used for all target DNA sites, such as methylated sites[50].

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/cas9)

CRISPR/cas9 is the most recently discovered method of genome editing and is widely considered as the most effective, efficient and accurate method of genome editing[51]. This editing system consists of two essential components, the single guide RNA (sgRNA) and CRISPR associated (cas9) proteins. Guide RNAs are responsible for recognition and are specifically designed to have a base sequence complementary to their target DNA sequence[51]. Cas9 proteins are large multi-domain endonucleases responsible for creating a double stranded break in the target DNA sequence[52]. The sgRNA directs the Cas9 protein to the complementary target DNA sequence and Cas9 finds an appropriate site to bind. This triggers local disruption of the target DNA sequence causing a double stranded break.[51]. Following this, the body repairs the double stranded break through homologous end joining, non-homologous end joining or with a template DNA sequence of a specific genetic sequence resulting in desired mutations or functional knockout of the target gene[38,39].

Gene editing has been used in HIV treatment to target host genes such as *CCR5* and *CXCR4*[53–55]. A study conducted by Hou et. al. showed high on-target efficiency with

approximately 45% knockout of the *CXCR4* gene *in vitro*[53]. To test whether this alteration confers protection against HIV, the cell line was infected with HIV-1 *in vitro* and tested for p24, an important HIV protein. The p24 level decreased by over 64% in modified cells as compared to control cells 7 days post infection, indicating that this genetic modification confers partial protection against HIV[53]. When used to edit the *CCR5* gene, the CRISPR/cas9 system showed higher efficiency of approximately 50-70%[54,55]. Two separate studies conducted by Yu et. al. and Liu et. al. analyzed the use of CRISPR/cas9 technology to simultaneously knockout *CCR5* and *CXCR4* genes[55,56]. Modified cell lines were challenged with HIV along with a control cell line. Modified cells showed some resistance to the infection as compared to unmodified cells indicating major selective advantage *in vitro*. Scientists also observed undetectable off-target activity, affirming the precision and specificity of this mode of gene editing[55,56].

CRISPR/cas9 has also been used to target HIV integrated proviral DNA, which is a fundamental source of viral protein production[57]. A study by Hu et. al. showed that dual gRNAs designed to target specific regions of the LTR, a part of the pro-viral DNA that drives HIV gene expression[58], can be used to excise a 9,709 base pair sequence in latently infected cells[59]. There was no off-target activity or genotoxicity, and the results showed this method efficiently prevented HIV infection in the modified cells[59]. *In vivo* testing of CRISPR/cas9 systems that target pro-viral DNA has shown great promise in a number of studies[60,61]. In a study by Kaminski et. al., the CRISPR/Cas9 system was used to target and delete a span of the HIV LTR Retrotransposon *gag* gene[60]. They used Adeno-Associated viral vectors (AAVs) to deliver the therapy in mice. Results showed significant reduction of viral load in circulating lymphocytes, indicating HIV eradication to a large extent[60]. In 2017, Yin et. al. demonstrated effective HIV pro-viral excision using Cas9 proteins and multiplex gRNAs delivered by an AAV vector in different animal models, including a transgenic mouse model and humanized mouse model[61]. In a transgenic mouse model with HIV, the treatment significantly reduced expression of the HIV proteins Env, Gag and Tat, demonstrating its efficacy[61]. There was approximately 50-60% reduction in Env, 80-90% reduction in Gag and 60-70% reduction in Tat[61]. In a humanized mouse model with HIV, effective *in vivo* excision of HIV provirus from latently infected human cells was detected. Humanized mice were treated with the AAV vector and then exposed to the HIV virus. Though there was an initial spread of the virus, the mice showed complete diminishment of the infection 60 days post treatment[61].

While these results show promise in inhibiting propagation of HIV in a host, there are many challenges that still need to be addressed and overcome. One important reason why CRISPR/Cas9 has not been used to target pro-viral DNA in clinical trials so far is because scientists have not yet developed an efficient delivery method. It has been especially challenging designing a delivery method for the CRISPR/Cas9 system due to the large size of gRNAs and Cas9 proteins[62]. Another major limitation of this system is the production of escape variants. Studies show that while the use of CRISPR/Cas9 can inhibit viral replication, it can

also facilitate viral escape due to occurrence of resistance mutations caused by DNA repair through non-homologous end joining[63,64].

RNA Interference

RNA interference (RNAi) is the process by which small RNA molecules perform post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS)[31]. RNAi can work via various pathways that involve complex sets of intracellular reactions leading to the formation of an RNA-induced silencing complex (RISC), which induces site-specific cleavage of RNA[65]. RISCs can be designed to target and silence almost any gene, since the mechanisms they may use are diverse. There are two components that are common to all RISCs: a small regulatory RNA, which functions as a guide that leads the complex to the target sequence, and a member of the Argonaute protein family, which binds to the small RNA, facilitates target recognition and induces target RNA cleavage[65]. RNAi was first induced in mammalian cells through the use of double stranded short interfering RNA (siRNA)[66]. It has since become a standard method of gene silencing in mammalian cells. The strength of the silencing response depends on the amount of siRNA successfully delivered and the potency of that particular siRNA[67]. Another method of inducing RNAi is the introduction of short hairpin RNA (shRNA). These RNA molecules mimic the structure and function of naturally occurring microRNAs (miRNAs) which endogenously cause gene silencing in most eukaryotes[68]. They integrate into the genome and can be used to produce siRNA in cells, causing long term gene knockdown of the target gene[68].

Both siRNAs and shRNAs have been used to simultaneously inhibit *CCR5* and *CXCR4* gene expression in cultured cell lines as well as stem cells[69,70]. These studies showed 80-85% transduction of cells and 70-80% reduction in *CCR5* and *CXCR4* expression[69,70]. When cultured cell lines were treated with siRNA and challenged with the HIV-1 virus, initially there was over a 10-fold decrease in viral p24 antigen levels followed by a slight increase on days 5 to 7 post challenge, which could be associated with the proliferation of non-transduced or low siRNA expressing cells[69]. Use of this technology in macrophages showed above 90% reduction in viral p24 antigen levels for up to 20 days post challenge, thus it was inferred that coreceptor downregulation by siRNAs results in effective protection of macrophages against viral challenge[70]. A study by Kim et. al. analyzed the use of liposome nanoparticles as a delivery method for *CCR5* targeted siRNAs (siCCR5) in humanized mice[71]. The siRNA potently reduced *CCR5* mRNA levels in cells with gene silencing lasting for up to 10 days, plasma viral load was almost 97% lesser in siCCR5 treated mice as compared to the control group[71]. These results demonstrate that siCCR5 delivered through a liposomal system is a promising approach for *in*

vivo treatment of HIV, however further study is required to reduce immunogenicity and off-target effects.

RNAi has also been used to target viral genes that code for proteins like Gag, Pol, Env, Tat, Rev, Nef and the LTR (long terminal repeat). A study by Yamamoto et. al. demonstrated the effective inhibition of Nef expression in macrophages using shRNA delivered by a lentiviral vector[72]. They targeted macrophages instead of CD4 T cells as macrophages play a critical role in the establishment and persistence of HIV in a host. In addition, macrophages are able to survive long after the infection and can spread the virus to healthy T cells[72,73]. Two variations of siRNA targeting *Nef* mRNA were used, one of which showed up to 90% suppression of *Nef* mRNA. Modified cells were challenged with HIV and showed sustained inhibition of HIV replication for at least 1 week[72]. Another study conducted by Singh et. al. analyzed the use of siRNA targeting the LTR region *in vitro*[74]. Modified cells when challenged with HIV showed 60-70% reduction in p24 levels on day 7 post challenge, and almost 90% suppression of productive infection for up to 24 days post challenge[74]. The use of RNAi technology for HIV treatment has been tested *in vivo* in humanized mice models. A study by Zhou et. al. used siRNA nanoparticles targeting cellular transcripts of CD4 and Transportin-3 as well as viral transcripts of Tat and Rev and injected these into mice intravenously[75]. Reduced expression of all target mRNA sequences was observed and mice treated with siRNA showed stable CD4 T cell levels for up to 22 weeks post infection[75]. ShRNAs targeting *Tat*, *Rev* and *CCR5* have been used in combination with ribozymes and decoys in multiple clinical trials. One particular trial conducted by DiGiusto et. al. showed persistence of Tat/Rev targeted shRNA in cells and protection from infection to some extent[76].

There are three main limitations of using RNAi technology for HIV treatment. One is that there is high potential for viral escape, the HIV virus rapidly develops resistance to treatments due to its high mutation rate. The development of viral escape mutants has been observed in studies by Das et. al. and Boden et. al. wherein viral loads increased after a significant initial decrease post treatment[83][84]. Another is it results in off-target toxicity. Finally, there remains challenges in the development of an efficient *in vivo* delivery system for RNAi technology. RNA molecules are hydrophilic so they can not easily pass the cell membrane, making the delivery of siRNA and shRNA into cells challenging[77]. A wide range of viral and non-viral delivery systems for RNAi are currently being studied, but further research and development is still required to proceed with this treatment in clinical settings[78].

Discussion

As explored in earlier sections, Highly Active Antiretroviral Therapy (HAART) is a treatment regimen that combines two or three antiretroviral drugs to combat HIV. This therapy includes antiretroviral drugs of six distinct classes, each associated with its unique array of adverse side

effects. Among the severe and potentially life-threatening consequences of HAART are mitochondrial toxicity, hepatitis, and Steven-Johnson's syndrome[12]. Beyond these critical risks, numerous other side effects significantly affect the quality of life of HIV patients, including symptoms such as dizziness, depression, anemia, and behavior resembling psychosis[12]. HAART necessitates a strict and rigid treatment schedule, including a lifelong commitment to daily medication intake and regular viral load assessments, typically conducted every four to eight weeks until viral levels drop below 20-50 copies/mL[12]. These factors render HAART less than optimal, particularly for individuals with limited resources, often serving as the cause for discontinuation or even refusal of treatment[16]. Gene therapy has evolved as an increasingly promising alternative, providing hope for a functional or even complete cure for this devastating disease. It has not only been used to interfere with the HIV life cycle, resembling the aim of HAART, but also to eradicate the integrated viral genome from host cells. Though there is need for further studies and investigation, gene therapy holds the promise of conferring long-term protection from the virus, reducing the need for drug management and significantly improving quality of life.

Due to the extremely high mutation rate of the HIV virus, viral escape is one of the major challenges faced while trying to establish a treatment or cure[79]. Combinatorial approaches for HIV gene therapy are currently being tested to overcome the challenge of development of viral escape mutants. These treatments target multiple regions of the host and viral genome, inhibiting multiple different stages of the HIV life cycle in an attempt to maximize treatment efficacy[80]. Combinatorial gene therapy treatments for HIV include the use of ZFNs, TALENs, CRISPR/Cas9 and RNAi as well as other modes of gene therapy such as RNA Decoys, Aptamers and Ribozymes. This type of gene therapy treatment has shown success in *in vitro* studies using cell lines; it has been observed that combinatorial gene therapy treatments can confer long term resistance to HIV[81][82]. These results prove that combinatorial gene therapy is a promising treatment option and one that is worth exploring. Though many *in vivo* and *in vitro* studies have shown success in efficiently immunizing cells from HIV infection using gene therapy, only a handful of treatments have moved to the clinical trial phase. Even so, the majority of the clinical trials that have been conducted are only in the earliest stages of development, aimed at evaluating only the safety of these treatments, not their efficacy in comparison to the current standard treatment, HAART[31]. Besides improving the treatment efficacy itself, researchers also face challenges such as the lack of efficient gene therapy delivery systems and the complex ethical considerations that need to be taken into account. Some challenges that arise while trying to develop a safe and efficient delivery system include immunogenicity, limited transgene capacity, stability and toxicity. Targeting the HIV latent reservoir has also proven to be challenging as these cells are few in number and circulate randomly all over the body[8,29]. A number of viral and non-viral delivery systems are currently being studied to improve delivery efficiency, these include: Adenoviral vectors, Lentiviral vectors, Adeno-Associated vectors, Polymeric Nanoparticles and physical methods such as

Micro-injection, Electroporation etc. Ethical considerations for clinical trial design are complex, and a risk-benefit analysis must be conducted to determine which patients will receive trial treatment[83]. For ethical reasons, the population most at risk should be receiving trial therapies, but in the case of HIV, this group is not optimal for gene therapy. At-risk patients receive front-line therapy which, though helpful in reducing viral loads and putting patients in remission, could cause bias in the observations needed to confirm safety and efficacy of the treatment being tested[84].

Conclusion

The exploratory gene therapy treatments for HIV detailed in this paper highlight the potential for groundbreaking advancements in the fight against this persistent viral infection. Gene therapy treatments aim to provide long-term immunization of cells against HIV as well as eradication of the latent viral reservoir, making this form of treatment much more convenient and effective as compared to HAART, the current standard treatment for HIV. With a large number of positive *in vitro* and *in vivo* experimental outcomes, gene therapy treatments have shown great promise in posing as functional cures for the disease. However, further research must be conducted to use them in clinical settings as there are some challenges that scientists have yet to overcome.

References

1. Fanales-Belasio, E., Raimondo, M., Suligoi, B., & Buttò, S. (2010). HIV virology and pathogenetic mechanisms of infection: A brief overview. *Annali dellIstituto Superiore Di Sanità*, *46*(1). https://doi.org/10.1590/S0021-25712010000100002
2. Yaniz-Galende, E., & Hajjar, R. J. (2014). Stem cell and gene therapy for cardiac regeneration. In *Cardiac Regeneration and Repair* (pp. 347–379). Elsevier. https://doi.org/10.1533/9780857096708.4.347
3. Doitsh, G., & Greene, W. C. (2016). Dissecting How CD4 T Cells Are Lost During HIV Infection. *Cell Host & Microbe*, *19*(3), 280–291. https://doi.org/10.1016/j.chom.2016.02.012
4. Zhu, J., & Paul, W. E. (2008). CD4 T cells: Fates, functions, and faults. *Blood*, *112*(5), 1557–1569. https://doi.org/10.1182/blood-2008-05-078154
5. Justiz Vaillant, A. A., & Naik, R. (2023). HIV-1 Associated Opportunistic Infections. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK539787/
6. Battistini Garcia, S. A., & Guzman, N. (2023). Acquired Immune Deficiency Syndrome CD4+ Count. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK513289/
7. Shaw, G. M., & Hunter, E. (2012). HIV Transmission. *Cold Spring Harbor Perspectives in Medicine*, *2*(11), a006965–a006965. https://doi.org/10.1101/cshperspect.a006965
8. Chen, J., Zhou, T., Zhang, Y., Luo, S., Chen, H., Chen, D., Li, C., & Li, W. (2022). The reservoir of latent HIV. *Frontiers in Cellular and Infection Microbiology*, *12*, 945956. https://doi.org/10.3389/fcimb.2022.945956
9. Wilen, C. B., Tilton, J. C., & Doms, R. W. (2012). HIV: Cell Binding and Entry. *Cold Spring*

Harbor Perspectives in Medicine, *2*(8), a006866–a006866. https://doi.org/10.1101/cshperspect.a006866

10. Kirchhoff, F. (2013). HIV Life Cycle: Overview. In T. J. Hope, M. Stevenson, & D. Richman (Eds.), *Encyclopedia of AIDS* (pp. 1–9). Springer New York. https://doi.org/10.1007/978-1-4614-9610-6_60-1
11. Arts, E. J., & Hazuda, D. J. (2012). HIV-1 antiretroviral drug therapy. *Cold Spring Harbor Perspectives in Medicine*, *2*(4), a007161. https://doi.org/10.1101/cshperspect.a007161
12. Eggleton, J. S., & Nagalli, S. (2023). Highly Active Antiretroviral Therapy (HAART). In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK554533/
13. Palella, F. J., Delaney, K. M., Moorman, A. C., Loveless, M. O., Fuhrer, J., Satten, G. A., Aschman, D. J., & Holmberg, S. D. (1998). Declining Morbidity and Mortality among Patients with Advanced Human Immunodeficiency Virus Infection. *New England Journal of Medicine*, *338*(13), 853–860. https://doi.org/10.1056/NEJM199803263381301
14. Hogg, R. S., Yip, B., Kully, C., Craib, K. J., O'Shaughnessy, M. V., Schechter, M. T., & Montaner, J. S. (1999). Improved survival among HIV-infected patients after initiation of triple-drug antiretroviral regimens. *CMAJ: Canadian Medical Association Journal = Journal de l'Association Medicale Canadienne*, *160*(5), 659–665.
15. Mellberg, T., Gonzalez, V. D., Lindkvist, A., Edén, A., Sönnerborg, A., Sandberg, J. K., Svennerholm, B., & Gisslén, M. (2011). Rebound of residual plasma viremia after initial decrease following addition of intravenous immunoglobulin to effective antiretroviral treatment of HIV. *AIDS Research and Therapy*, *8*(1), 21. https://doi.org/10.1186/1742-6405-8-21
16. Bassetti, S., Battegay, M., Furrer, H., Rickenbach, M., Flepp, M., Kaiser, L., Telenti, A.,

Vernazza, P. L., Bernasconi, E., & Sudre, P. (1999). Why is highly active antiretroviral therapy (HAART) not prescribed or discontinued? Swiss HIV Cohort Study. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *21*(2), 114–119.

17. Valentina Montessori, Natasha Press, Marianne Harris, Linda Akagi, & Julio S.G. Montaner. (2004). Adverse effects of antiretroviral therapy for HIV infection. *Canadian Medical Association Journal*, *170*(2), 229.
18. Bobbin, M. L., Burnett, J. C., & Rossi, J. J. (2015). RNA interference approaches for treatment of HIV-1 infection. *Genome Medicine*, *7*(1), 50. https://doi.org/10.1186/s13073-015-0174-y
19. Kunze, C., Börner, K., Kienle, E., Orschmann, T., Rusha, E., Schneider, M., Radivojkov-Blagojevic, M., Drukker, M., Desbordes, S., Grimm, D., & Brack-Werner, R. (2018). Synthetic AAV/CRISPR vectors for blocking HIV-1 expression in persistently infected astrocytes. *Glia*, *66*(2), 413–427. https://doi.org/10.1002/glia.23254
20. Perez, E. E., Wang, J., Miller, J. C., Jouvenot, Y., Kim, K. A., Liu, O., Wang, N., Lee, G., Bartsevich, V. V., Lee, Y.-L., Guschin, D. Y., Rupniewski, I., Waite, A. J., Carpenito, C., Carroll, R. G., S Orange, J., Urnov, F. D., Rebar, E. J., Ando, D., … June, C. H. (2008). Establishment of HIV-1 resistance in CD4+ T cells by genome editing using zinc-finger nucleases. *Nature Biotechnology*, *26*(7), 808–816. https://doi.org/10.1038/nbt1410
21. Yuan, J., Wang, J., Crain, K., Fearns, C., Kim, K. A., Hua, K. L., Gregory, P. D., Holmes, M. C., & Torbett, B. E. (2012). Zinc-finger Nuclease Editing of Human cxcr4 Promotes HIV-1 CD4+ T Cell Resistance and Enrichment. *Molecular Therapy*, *20*(4), 849–859. https://doi.org/10.1038/mt.2011.310
22. DiGiusto, D. L., Cannon, P. M., Holmes, M. C., Li, L., Rao, A., Wang, J., Lee, G., Gregory, P.

D., Kim, K. A., Hayward, S. B., Meyer, K., Exline, C., Lopez, E., Henley, J., Gonzalez, N., Bedell, V., Stan, R., & Zaia, J. A. (2016). Preclinical development and qualification of ZFN-mediated CCR5 disruption in human hematopoietic stem/progenitor cells. *Molecular Therapy - Methods & Clinical Development*, *3*, 16067. https://doi.org/10.1038/mtm.2016.67

23. Didigu, C. A., Wilen, C. B., Wang, J., Duong, J., Secreto, A. J., Danet-Desnoyers, G. A., Riley, J. L., Gregory, P. D., June, C. H., Holmes, M. C., & Doms, R. W. (2014). Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection. *Blood*, *123*(1), 61–69. https://doi.org/10.1182/blood-2013-08-521229
24. Tebas, P., Jadlowsky, J. K., Shaw, P. A., Tian, L., Esparza, E., Brennan, A. L., Kim, S., Naing, S. Y., Richardson, M. W., Vogel, A. N., Maldini, C. R., Kong, H., Liu, X., Lacey, S. F., Bauer, A. M., Mampe, F., Richman, L. P., Lee, G., Ando, D., … Riley, J. L. (2021). CCR5-edited CD4+ T cells augment HIV-specific immunity to enable post-rebound control of HIV replication. *Journal of Clinical Investigation*, *131*(7), e144486. https://doi.org/10.1172/JCI144486
25. Tebas, P., Stein, D., Tang, W. W., Frank, I., Wang, S. Q., Lee, G., Spratt, S. K., Surosky, R. T., Giedlin, M. A., Nichol, G., Holmes, M. C., Gregory, P. D., Ando, D. G., Kalos, M., Collman, R. G., Binder-Scholl, G., Plesa, G., Hwang, W.-T., Levine, B. L., & June, C. H. (2014). Gene Editing of *CCR5* in Autologous CD4 T Cells of Persons Infected with HIV. *New England Journal of Medicine*, *370*(10), 901–910. https://doi.org/10.1056/NEJMoa1300662
26. Wayengera, M. (2011). Proviral HIV-genome-wide and pol-gene specific Zinc Finger

Nucleases: Usability for targeted HIV gene therapy. *Theoretical Biology and Medical Modelling*, *8*(1), 26. https://doi.org/10.1186/1742-4682-8-26

27. Ji, H., Lu, P., Liu, B., Qu, X., Wang, Y., Jiang, Z., Yang, X., Zhong, Y., Yang, H., Pan, H., Zhao, L., Xu, J., Lu, H., & Zhu, H. (2018). Zinc-Finger Nucleases Induced by HIV-1 Tat Excise HIV-1 from the Host Genome in Infected and Latently Infected Cells. *Molecular Therapy - Nucleic Acids*, *12*, 67–74. https://doi.org/10.1016/j.omtn.2018.04.014
28. Qu, X., Wang, P., Ding, D., Wang, X., Zhang, G., Zhou, X., Liu, L., Zhu, X., Zeng, H., & Zhu, H. (2014). Zinc finger nuclease: A new approach for excising HIV-1 proviral DNA from infected human T cells. *Molecular Biology Reports*, *41*(9), 5819–5827. https://doi.org/10.1007/s11033-014-3456-3
29. Wayengera, M. (2011). Targeting persistent HIV infection: Where and how, if possible? *HIV & AIDS Review*, *10*(1), 1–8. https://doi.org/10.1016/j.hivar.2011.01.002
30. Onafuwa-Nuga, A., McNamara, L. A., & Collins, K. L. (2010). Towards a cure for HIV: The identification and characterization of HIV reservoirs in optimally treated people. *Cell Research*, *20*(11), 1185–1187. https://doi.org/10.1038/cr.2010.140
31. Bennett, M., & Akkina, R. (2013). Gene Therapy Strategies for HIV/AIDS: Preclinical Modeling in Humanized Mice. *Viruses*, *5*(12), 3119–3141. https://doi.org/10.3390/v5123119
32. Pattanayak, V., Ramirez, C. L., Joung, J. K., & Liu, D. R. (2011). Revealing off-target cleavage specificities of zinc-finger nucleases by in vitro selection. *Nature Methods*, *8*(9), 765–770. https://doi.org/10.1038/nmeth.1670
33. Maier, D. A., Brennan, A. L., Jiang, S., Binder-Scholl, G. K., Lee, G., Plesa, G., Zheng, Z., Cotte, J., Carpenito, C., Wood, T., Spratt, S. K., Ando, D., Gregory, P., Holmes, M. C.,

Perez, Elena. E., Riley, J. L., Carroll, R. G., June, C. H., & Levine, B. L. (2013). Efficient Clinical Scale Gene Modification via Zinc Finger Nuclease–Targeted Disruption of the HIV Co-receptor CCR5. *Human Gene Therapy*, *24*(3), 245–258. https://doi.org/10.1089/hum.2012.172

34. Joung, J. K., & Sander, J. D. (2013). TALENs: A widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology*, *14*(1), 49–55. https://doi.org/10.1038/nrm3486

35. Sanjana, N. E., Cong, L., Zhou, Y., Cunniff, M. M., Feng, G., & Zhang, F. (2012). A transcription activator-like effector toolbox for genome engineering. *Nature Protocols*, *7*(1), 171–192. https://doi.org/10.1038/nprot.2011.431

36. Boissel, S., Jarjour, J., Astrakhan, A., Adey, A., Gouble, A., Duchateau, P., Shendure, J., Stoddard, B. L., Certo, M. T., Baker, D., & Scharenberg, A. M. (2014). megaTALs: A rare-cleaving nuclease architecture for therapeutic genome engineering. *Nucleic Acids Research*, *42*(4), 2591–2601. https://doi.org/10.1093/nar/gkt1224

37. Yee, J.-K. (2016). Off-target effects of engineered nucleases. *The FEBS Journal*, *283*(17), 3239–3248. https://doi.org/10.1111/febs.13760

38. Haber, J. E. (2000). Partners and pathways. *Trends in Genetics*, *16*(6), 259–264. https://doi.org/10.1016/S0168-9525(00)02022-9

39. Thompson, L. H., & Schild, D. (2001). Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *477*(1–2), 131–153. https://doi.org/10.1016/S0027-5107(01)00115-4

40. Deng, P., Carter, S., & Fink, K. (2019). Design, Construction, and Application of

Transcription Activation-Like Effectors. In F. P. Manfredsson & M. J. Benskey (Eds.), *Viral Vectors for Gene Therapy* (Vol. 1937, pp. 47–58). Springer New York. https://doi.org/10.1007/978-1-4939-9065-8_3

41. Shi, B., Li, J., Shi, X., Jia, W., Wen, Y., Hu, X., Zhuang, F., Xi, J., & Zhang, L. (2017). TALEN-Mediated Knockout of CCR5 Confers Protection Against Infection of Human Immunodeficiency Virus: *JAIDS Journal of Acquired Immune Deficiency Syndromes*, *74*(2), 229–241. https://doi.org/10.1097/QAI.0000000000001190
42. Romito, M., Juillerat, A., Kok, Y. L., Hildenbeutel, M., Rhiel, M., Andrieux, G., Geiger, J., Rudolph, C., Mussolino, C., Duclert, A., Metzner, K. J., Duchateau, P., Cathomen, T., & Cornu, T. I. (2021). Preclinical Evaluation of a Novel TALEN Targeting *CCR5* Confirms Efficacy and Safety in Conferring Resistance to HIV-1 Infection. *Biotechnology Journal*, *16*(1), 2000023. https://doi.org/10.1002/biot.202000023
43. Schwarze, L. I., Głów, D., Sonntag, T., Uhde, A., & Fehse, B. (2021). Optimisation of a TALE nuclease targeting the HIV co-receptor CCR5 for clinical application. *Gene Therapy*, *28*(9), 588–601. https://doi.org/10.1038/s41434-021-00271-9
44. Mussolino, C., Alzubi, J., Fine, E. J., Morbitzer, R., Cradick, T. J., Lahaye, T., Bao, G., & Cathomen, T. (2014). TALENs facilitate targeted genome editing in human cells with high specificity and low cytotoxicity. *Nucleic Acids Research*, *42*(10), 6762–6773. https://doi.org/10.1093/nar/gku305
45. Fadel, H. J., Morrison, J. H., Saenz, D. T., Fuchs, J. R., Kvaratskhelia, M., Ekker, S. C., & Poeschla, E. M. (2014). TALEN Knockout of the *PSIP1* Gene in Human Cells: Analyses of HIV-1 Replication and Allosteric Integrase Inhibitor Mechanism. *Journal of Virology*, *88*(17), 9704–9717. https://doi.org/10.1128/JVI.01397-14

46. Llano, M., Delgado, S., Vanegas, M., & Poeschla, E. M. (2004). Lens Epithelium-derived Growth Factor/p75 Prevents Proteasomal Degradation of HIV-1 Integrase. *Journal of Biological Chemistry*, *279*(53), 55570–55577. https://doi.org/10.1074/jbc.M408508200

47. Maertens, G., Cherepanov, P., Pluymers, W., Busschots, K., De Clercq, E., Debyser, Z., & Engelborghs, Y. (2003). LEDGF/p75 Is Essential for Nuclear and Chromosomal Targeting of HIV-1 Integrase in Human Cells. *Journal of Biological Chemistry*, *278*(35), 33528–33539. https://doi.org/10.1074/jbc.M303594200

48. Sutherland, H. G., Newton, K., Brownstein, D. G., Holmes, M. C., Kress, C., Semple, C. A., & Bickmore, W. A. (2006). Disruption of Ledgf/Psip1 Results in Perinatal Mortality and Homeotic Skeletal Transformations. *Molecular and Cellular Biology*, *26*(19), 7201–7210. https://doi.org/10.1128/MCB.00459-06

49. Wang, L., Li, F., Dang, L., Liang, C., Wang, C., He, B., Liu, J., Li, D., Wu, X., Xu, X., Lu, A., & Zhang, G. (2016). In Vivo Delivery Systems for Therapeutic Genome Editing. *International Journal of Molecular Sciences*, *17*(5), 626. https://doi.org/10.3390/ijms17050626

50. Ansari, W. A., Chandanshive, S. U., Bhatt, V., Nadaf, A. B., Vats, S., Katara, J. L., Sonah, H., & Deshmukh, R. (2020). Genome Editing in Cereals: Approaches, Applications and Challenges. *International Journal of Molecular Sciences*, *21*(11), 4040. https://doi.org/10.3390/ijms21114040

51. Mengstie, M. A., & Wondimu, B. Z. (2021). Mechanism and Applications of CRISPR/Cas-9-Mediated Genome Editing. *Biologics: Targets and Therapy*, *Volume 15*, 353–361. https://doi.org/10.2147/BTT.S326422

52. Mei, Y., Wang, Y., Chen, H., Sun, Z. S., & Ju, X.-D. (2016). Recent Progress in

CRISPR/Cas9 Technology. *Journal of Genetics and Genomics*, *43*(2), 63–75. https://doi.org/10.1016/j.jgg.2016.01.001

53. Hou, P., Chen, S., Wang, S., Yu, X., Chen, Y., Jiang, M., Zhuang, K., Ho, W., Hou, W., Huang, J., & Guo, D. (2015). Genome editing of CXCR4 by CRISPR/cas9 confers cells resistant to HIV-1 infection. *Scientific Reports*, *5*(1), 15577. https://doi.org/10.1038/srep15577

54. Li, C., Guan, X., Du, T., Jin, W., Wu, B., Liu, Y., Wang, P., Hu, B., Griffin, G. E., Shattock, R. J., & Hu, Q. (2015). Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirus-delivered CRISPR/Cas9. *Journal of General Virology*, *96*(8), 2381–2393. https://doi.org/10.1099/vir.0.000139

55. Yu, S., Yao, Y., Xiao, H., Li, J., Liu, Q., Yang, Y., Adah, D., Lu, J., Zhao, S., Qin, L., & Chen, X. (2018). Simultaneous Knockout of *CXCR4* and *CCR5* Genes in CD4+ T Cells via CRISPR/Cas9 Confers Resistance to Both X4- and R5-Tropic Human Immunodeficiency Virus Type 1 Infection. *Human Gene Therapy*, *29*(1), 51–67. https://doi.org/10.1089/hum.2017.032

56. Liu, Z., Chen, S., Jin, X., Wang, Q., Yang, K., Li, C., Xiao, Q., Hou, P., Liu, S., Wu, S., Hou, W., Xiong, Y., Kong, C., Zhao, X., Wu, L., Li, C., Sun, G., & Guo, D. (2017). Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4+ T cells from HIV-1 infection. *Cell & Bioscience*, *7*(1), 47. https://doi.org/10.1186/s13578-017-0174-2

57. Wu, Y. (2004). HIV-1 gene expression: Lessons from provirus and non-integrated DNA. *Retrovirology*, *1*(1), 13. https://doi.org/10.1186/1742-4690-1-13

58. Shah, S., Alexaki, A., Pirrone, V., Dahiya, S., Nonnemacher, M. R., & Wigdahl, B. (2014).

Functional properties of the HIV-1 long terminal repeat containing single-nucleotide polymorphisms in Sp site III and CCAAT/enhancer binding protein site I. *Virology Journal*, *11*(1), 92. https://doi.org/10.1186/1743-422X-11-92

59. Hu, W., Kaminski, R., Yang, F., Zhang, Y., Cosentino, L., Li, F., Luo, B., Alvarez-Carbonell, D., Garcia-Mesa, Y., Karn, J., Mo, X., & Khalili, K. (2014). RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. *Proceedings of the National Academy of Sciences*, *111*(31), 11461–11466. https://doi.org/10.1073/pnas.1405186111
60. Kaminski, R., Bella, R., Yin, C., Otte, J., Ferrante, P., Gendelman, H. E., Li, H., Booze, R., Gordon, J., Hu, W., & Khalili, K. (2016). Excision of HIV-1 DNA by gene editing: A proof-of-concept in vivo study. *Gene Therapy*, *23*(8–9), 690–695. https://doi.org/10.1038/gt.2016.41
61. Yin, C., Zhang, T., Qu, X., Zhang, Y., Putatunda, R., Xiao, X., Li, F., Xiao, W., Zhao, H., Dai, S., Qin, X., Mo, X., Young, W.-B., Khalili, K., & Hu, W. (2017). In Vivo Excision of HIV-1 Provirus by saCas9 and Multiplex Single-Guide RNAs in Animal Models. *Molecular Therapy*, *25*(5), 1168–1186. https://doi.org/10.1016/j.ymthe.2017.03.012
62. Behr, M., Zhou, J., Xu, B., & Zhang, H. (2021). In vivo delivery of CRISPR-Cas9 therapeutics: Progress and challenges. *Acta Pharmaceutica Sinica B*, *11*(8), 2150–2171. https://doi.org/10.1016/j.apsb.2021.05.020
63. Wang, G., Zhao, N., Berkhout, B., & Das, A. T. (2016). CRISPR-Cas9 Can Inhibit HIV-1 Replication but NHEJ Repair Facilitates Virus Escape. *Molecular Therapy*, *24*(3), 522–526. https://doi.org/10.1038/mt.2016.24
64. Wang, Z., Pan, Q., Gendron, P., Zhu, W., Guo, F., Cen, S., Wainberg, M. A., & Liang, C.

(2016). CRISPR/Cas9-Derived Mutations Both Inhibit HIV-1 Replication and Accelerate Viral Escape. *Cell Reports*, *15*(3), 481–489. https://doi.org/10.1016/j.celrep.2016.03.042

65. Pratt, A. J., & MacRae, I. J. (2009). The RNA-induced Silencing Complex: A Versatile Gene-silencing Machine. *Journal of Biological Chemistry*, *284*(27), 17897–17901. https://doi.org/10.1074/jbc.R900012200
66. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., & Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, *411*(6836), 494–498. https://doi.org/10.1038/35078107
67. Rossi, J. J. (2006). RNAi as a treatment for HIV-1 infection. *BioTechniques*, *40*(4S), S25–S29. https://doi.org/10.2144/000112167
68. Taxman, D. J., Moore, C. B., Guthrie, E. H., & Huang, M. T.-H. (2010). Short Hairpin RNA (shRNA): Design, Delivery, and Assessment of Gene Knockdown. In M. Sioud (Ed.), *RNA Therapeutics* (Vol. 629, pp. 139–156). Humana Press. https://doi.org/10.1007/978-1-60761-657-3_10
69. Anderson, J., & Akkina, R. (2005). HIV-1 resistance conferred by siRNA cosuppression of CXCR4 and CCR5 coreceptors by a bispecific lentiviral vector. *AIDS Research and Therapy*, *2*(1), 1. https://doi.org/10.1186/1742-6405-2-1
70. Anderson, J., & Akkina, R. (2005). CXCR4 and CCR5 shRNA transgenic CD34+ cell derived macrophages are functionally normal and resist HIV-1 infection. *Retrovirology*, *2*(1), 53. https://doi.org/10.1186/1742-4690-2-53
71. Kim, S.-S., Peer, D., Kumar, P., Subramanya, S., Wu, H., Asthana, D., Habiro, K., Yang, Y.-G., Manjunath, N., Shimaoka, M., & Shankar, P. (2010). RNAi-mediated CCR5 Silencing by LFA-1-targeted Nanoparticles Prevents HIV Infection in BLT Mice. *Molecular*

Therapy, *18*(2), 370–376. https://doi.org/10.1038/mt.2009.271

72. Yamamoto, T., Miyoshi, H., Yamamoto, N., Yamamoto, N., Inoue, J. -i., & Tsunetsugu-Yokota, Y. (2006). Lentivirus vectors expressing short hairpin RNAs against the U3-overlapping region of HIV nef inhibit HIV replication and infectivity in primary macrophages. *Blood*, *108*(10), 3305–3312. https://doi.org/10.1182/blood-2006-04-014829
73. Kruize, Z., & Kootstra, N. A. (2019). The Role of Macrophages in HIV-1 Persistence and Pathogenesis. *Frontiers in Microbiology*, *10*, 2828. https://doi.org/10.3389/fmicb.2019.02828
74. Singh, A., Palanichamy, J. K., Ramalingam, P., Kassab, M. A., Bhagat, M., Andrabi, R., Luthra, K., Sinha, S., & Chattopadhyay, P. (2014). Long-term suppression of HIV-1C virus production in human peripheral blood mononuclear cells by LTR heterochromatization with a short double-stranded RNA. *Journal of Antimicrobial Chemotherapy*, *69*(2), 404–415. https://doi.org/10.1093/jac/dkt348
75. Zhou, J., Neff, C. P., Liu, X., Zhang, J., Li, H., Smith, D. D., Swiderski, P., Aboellail, T., Huang, Y., Du, Q., Liang, Z., Peng, L., Akkina, R., & Rossi, J. J. (2011). Systemic Administration of Combinatorial dsiRNAs via Nanoparticles Efficiently Suppresses HIV-1 Infection in Humanized Mice. *Molecular Therapy*, *19*(12), 2228–2238. https://doi.org/10.1038/mt.2011.207
76. DiGiusto, D. L., Krishnan, A., Li, L., Li, H., Li, S., Rao, A., Mi, S., Yam, P., Stinson, S., Kalos, M., Alvarnas, J., Lacey, S. F., Yee, J.-K., Li, M., Couture, L., Hsu, D., Forman, S. J., Rossi, J. J., & Zaia, J. A. (2010). RNA-Based Gene Therapy for HIV with Lentiviral Vector–Modified CD34 $^{+}$ Cells in Patients Undergoing Transplantation for AIDS-Related Lymphoma. *Science Translational Medicine*, *2*(36).

https://doi.org/10.1126/scitranslmed.3000931

77. Castanotto, D., & Rossi, J. J. (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature*, *457*(7228), 426–433. https://doi.org/10.1038/nature07758

78. Martínez, M. A. (2009). Progress in the Therapeutic Applications of siRNAs Against HIV-1. In M. Sioud (Ed.), *siRNA and miRNA Gene Silencing* (Vol. 487, pp. 1–26). Humana Press. https://doi.org/10.1007/978-1-60327-547-7_17

79. Cuevas, J. M., Geller, R., Garijo, R., López-Aldeguer, J., & Sanjuán, R. (2015). Extremely High Mutation Rate of HIV-1 In Vivo. *PLOS Biology*, *13*(9), e1002251. https://doi.org/10.1371/journal.pbio.1002251

80. Strayer, D. S., Branco, F., Landré, J., BouHamdan, M., Shaheen, F., & Pomerantz, R. J. (2002). Combination Genetic Therapy to Inhibit HIV-1. *Molecular Therapy*, *5*(1), 33–41. https://doi.org/10.1006/mthe.2001.0513

81. Li, M.-J., Kim, J., Li, S., Zaia, J., Yee, J.-K., Anderson, J., Akkina, R., & Rossi, J. J. (2005). Long-Term Inhibition of HIV-1 Infection in Primary Hematopoietic Cells by Lentiviral Vector Delivery of a Triple Combination of Anti-HIV shRNA, Anti-CCR5 Ribozyme, and a Nucleolar-Localizing TAR Decoy. *Molecular Therapy*, *12*(5), 900–909. https://doi.org/10.1016/j.ymthe.2005.07.524

82. Anderson, J., Li, M.-J., Palmer, B., Remling, L., Li, S., Yam, P., Yee, J.-K., Rossi, J., Zaia, J., & Akkina, R. (2007). Safety and Efficacy of a Lentiviral Vector Containing Three Anti-HIV Genes—CCR5 Ribozyme, Tat-rev siRNA, and TAR Decoy—In SCID-hu Mouse–Derived T Cells. *Molecular Therapy*, *15*(6), 1182–1188. https://doi.org/10.1038/sj.mt.6300157

83. Muthuswamy, V. (2013). Ethical issues in clinical research. *Perspectives in Clinical Research*, *4*(1), 9. https://doi.org/10.4103/2229-3485.106369

84. Stan, R., & Zaia, J. A. (2014). Practical Considerations in Gene Therapy for HIV Cure. *Current HIV/AIDS Reports*, *11*(1), 11–19. https://doi.org/10.1007/s11904-013-0197-1