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NOVEL APPROACHES IN HIV-1 CURE: GENETIC EDITING AND GENE REPRESSION TECHNIQUES. TOPIC REVIEW

Nuevos Enfoques en la Cura del VIH-1: Técnicas de Edición Genética y Represión Génica. Revisión de Tema

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Artículo de revisión

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ABSTRACT

Introduction: The worldwide challenge presented by human immunodeficiency virus type 1, affecting 39 million individuals, endures despite the highly active antiretroviral therapy. Limitations of it, including insufficiently addressing viral reservoirs leading to prolonged drug toxicity, resistance concerns, and economic burdens, have fueled exploration into alternative approaches. Gene editing and gene repression techniques have emerged as potential solutions. Objective: Conduct a comprehensive analysis of recent research following Preferred Reporting Items for Systematic Reviews and Meta-Analyses guidelines, focusing on genetic modification and gene repression in HIV-1-infected samples. **Methods:** A meticulous search identified 413 articles, of which 76 met strict criteria. Inclusion criteria involved studies on gene editing and HIV-1 expression (2019–2023), emphasizing CCR5 repression. Exclusions targeted weak evidence, lacking practical efficacy, and non-relevance to HIV-1 or genetic tools. Duplicates and non-open-access articles were also excluded. **Results:** The identified strategies show promise in their ability to mitigate the chronicity of the infection. However, challenges such as off-target effects, ethical considerations surrounding in vivo, ex vivo, and germ line editing and variable efficacy across studies are evident. The imperative for continued evaluation, refinement of techniques, and the development of effective administration methods is underscored. **Conclusions:** This topic review provides a nuanced exploration of recent advancements in gene editing and gene repression techniques for managing HIV-1. While the findings suggest promising avenues, the identified challenges necessitate ongoing research and innovation in order to harness the full potential of these strategies in the quest for an effective HIV-1 solution.

K**EYWORDS:** HIV-1, RNA interference, miRNA, siRNA, Gene silencing, CRISPR-Cas Systems, Receptor CCR5

RESUMEN

Introducción: El reto global del virus de la inmunodeficiencia humana tipo 1, afectando a 39 millones de personas, persiste pese al éxito de la terapia antirretroviral de gran actividad. La resistencia viral, toxicidad a largo plazo y cargas económicas han impulsado la exploración de técnicas de edición genética y represión de expresión génica como soluciones potenciales.

Objetivo: Realizar un análisis exhaustivo de investigaciones recientes siguiendo las pautas de Elementos de Informes Preferidos para Revisiones Sistemáticas y Metaanálisis, centrándose en la modificación genética y la represión genética en muestras infectadas por VIH-1. **Métodos:** Se identificaron 413 artículos, de los cuales 76 cumplían criterios. Los criterios de inclusión incluyeron estudios sobre edición de genes relacionados con expresión del VIH-1 (2019-2023), enfatizando la represión de CCR5/CXCR4. Las exclusiones se aplicaron en pruebas débiles, sin eficacia práctica y sin relevancia al VIH-1 o las herramientas genéticas, así como los duplicados y los artículos que no eran de acceso libre. **Resultados:** Hay estrategias que muestran promisorias capacidades para reducir la cronicidad de la infección, pero se enfrentan a desafíos notables como los efectos fuera del objetivo, dilemas éticos en la edición in vivo, ex vivo y de la línea germinal, y variabilidad en la eficacia entre estudios. Remarca la necesidad de evaluación, mejorar técnicas y desarrollar métodos de administración más eficaces. **Conclusiones:** Esta revisión de tema destaca avances recientes en edición genética para VIH-1. Aunque prometedores, los desafíos demandan investigación continua para aprovechar plenamente estas estrategias en la búsqueda de una cura eficaz.

PALABRAS CLAVE: VIH-1, Interferencia de ARN, miARN, siARN, Silenciador del Gen, Sistemas CRISPR-Cas, Receptor CCR5

INTRODUCCIÓN

HIV in public health

In 2022, the prevalence of human immunodeficiency virus infections persisted as a substantial and enduring global public health concern. In 2022, the global population of individuals living with human immunodeficiency virus (HIV) amounted to 39.0 million, comprising 37.5 million adults and 1.5 million children. Notably, 86% of these individuals were aware of their diagnosis, with a predominant concentration of cases (25.7 million) in Africa [1, 2]. While HIV infection is no longer a death sentence, the success of these therapies has led to decreased public awareness and increasing infection rates, particularly in Africa, Asia, disadvantaged communities in North America, and Europe. Despite the progress, HIV/Acquired

immunodeficiency syndrome (AIDS) remains a significant infectious disease with a large number of people living with HIV, and a cure has not yet been developed, even though millions have died from HIV/AIDS-related diseases [3]. Around 1.3 million individuals acquired new HIV infections, with ninety-five percent of the cases predominantly concentrated in developing countries [4]; down from the peak in 1995 when there were 3.2 million new infections, with a significant portion of these new cases occurring among women and girls. In 2022, AIDS-related illnesses claimed the lives of 630,000 individuals, with a range of 480,000 to 880,000. Additionally, there were variations in access to antiretroviral therapy among different demographics. HIV prevalence rates were notably higher among key populations, including sex workers, men who have sex with men (MSM), individuals who inject drugs, transgender persons, and those in prison. Progress was made toward the 95-95-95 targets in 2022; a considerable portion, 86%, of individuals with HIV were aware of their status, while 89% of them had access to treatment, and an impressive 93% of those on treatment achieved viral suppression. Investments reacting to the therapeutic intervention of acquired immunodeficiency syndrome (AIDS) reached a substantial level, approximately US$20.8 billion in 2022, with around 60% coming from domestic sources, but Joint United Nations Programme on HIV and AIDS estimates that US$29 billion will be required in 2025 to eradicate AIDS as a menace to public health [1].

Highly active antiretroviral therapy (HAART) has been a transformative development in the treatment of AIDS, changing it transforming it from a perilous and difficult-to-treat illness into a controllable medical condition. However, the need for lifelong treatment and the absence of a definitive cure continues to be significant challenges for many individuals living with AIDS [5-8]. Effectiveness of HAART against HIV is hindered by several factors, including low oral bioavailability, challenges in adhering to drug regimens, elevated side effects because of the need for elevated and frequent doses, coupled with the substantial expenses linked to the therapy [9-11]. Additionally, funding for antiretroviral therapy (ART) initiatives in nations with lower and moderate incomes encounter challenges related to long-term viability. To address these issues, a

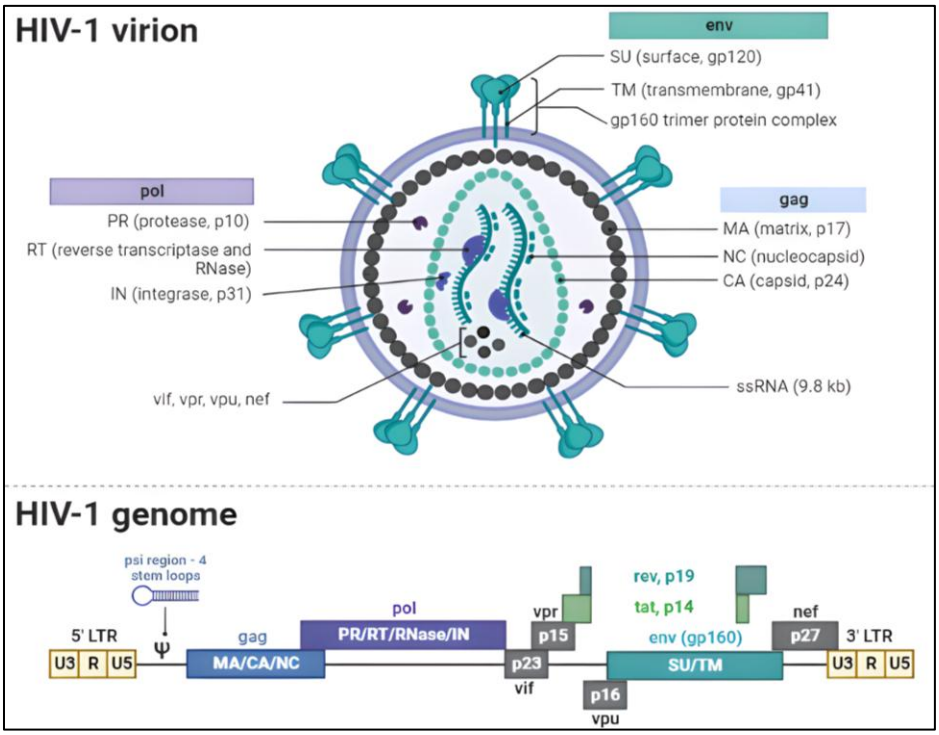
curative intervention for HIV is being explored, with complete virus eradication as the ultimate goal. Assessment of therapeutic options through clinical examination often involves treatment interruption, posing risks not only to individuals but also to their sexual partners. Hence, prioritizing the discovery of non-viral biomarkers that forecast the recurrence of viral activity is of utmost importance. Even with suppressive ART, HIV may persist due to minimal viral replication in specific regions. Ultimately, the cost-effectiveness and ease of administration of any curative intervention will be essential factors in determining its global feasibility, especially in resource-limited settings [12]. The "Undetectable = Untransmissible" message is important for reducing the stigma associated with HIV and promoting the understanding that individuals living with HIV who are on effective treatment can lead healthy lives and do not pose a significant risk of transmitting the virus to their partners [13].

HIV Life Cycle

HIV-1, (Figure 1), originated from chimpanzees and gorillas, has a genome of 9.8kb of information. Within the HIV genome, there are two extended terminal repeat (LTR) regions, bounding nine genes. These genes encompass structural elements (gag, pol, env), regulatory components (tat, rev), and additional genes (vif, vpr, vpu, nef) [14]. HIV-1 invades cells through the affinity that gp120 has with the chemokine receptor type 5 (CCR5) and C-X-C chemokine receptor type 4 (CXCR4) [15]. It gains entry into cells attaching to gp120 surface protein to the cellular receptor CD4. CCR5 functions as the primary co-receptor for CCR5-tropic HIV-1 (R5), while CXCR4 serves as the co-receptor for CXCR4-tropic HIV-1 (X4), which is prevalent in late-stage infections [4, 16]. It's worth noting that CCR5, while a potential target for HIV-1 inhibition, also assumes pivotal functions in inflammatory signaling and is ubiquitously expressed across diverse immune cell types, extending beyond CD4+ T cells, which serve as the main host cells for HIV-1 [10, 17, 18]. Once HIV-1 enters the host, it traverses the mucosal barrier and binds to CD4+ receptors found on diverse immune cells, such as monocytes and CD4+ T lymphocytes. The virus utilizes its surface

glycoprotein gp120 to bind to these receptors, triggering alterations in the structure of gp120, enabling its attachment to chemokine coreceptors CCR5 or CXCR4. CCR5 is primarily found on CD4+ T cells, while CXCR4 is found on other immune cells. This binding leads to fusion between viral gp41 and the cell membrane, enabling the capsids to access into the cytoplasm of the cell. Once there, the capsids use microtubules motors to be transported to the nuclear region, where the reverse transcription starts its process until the significance of CCR5 as a potential target for interventions against the virus [21]. capsid arrives at the nucleus, through the nuclear pore complex [19, 20]. The env gene, responsible for encoding the viral glycoprotein gp160, is pivotal in determining the virus's tropism and binding to host cell receptors. Specifically, HIV-1 requires binding to the CD4 molecule in conjunction with the coreceptors mentioned, for host cell entry. Notably, individuals with a specific mutation in the CCR5 gene, causing reduced CCR5 expression, show resistance to HIV-1 infection. This genetic variation underscores the

Figure 1. HIV-1 virion and genome. ***Source:*** Prepared by the author.



The HIV-1 virus possesses a structured genome comprising essential genes, including Long Terminal Repeats (LTR) for gene expression regulation and integration, Group-Specific Antigen (gag) for core structure formation, DNA Polymerase (pol) for replication enzymes, Viral Infectivity Factor (vif) for infectivity enhancement, Viral Protein U (vpu) for viral release, Envelope (env) for entry glycoproteins, and Negative Regulatory Factor (nef) for replication and immune regulation. These genes collectively define the genetic makeup of HIV-1, playing critical roles in its life cycle. The virion, representing the complete viral particle, incorporates these genetic elements along with other necessary viral and host cell components for assembly and infectivity.

Inside the host cell's nuclei, reverse transcriptase is activated, converting the HIV single RNA genome into complementary DNA (cDNA). Subsequently, this proviral DNA is conveyed into the host cell genome, guided by integrase and host factors, and incorporated into the genomic material of the host cell. Following integration, viral transcription is facilitated by factors like tat and tar, leading to translation, post-translational modifications, and protease-mediated processing and packaging of viral proteins into new viral particles [4].

Antiretroviral Therapy

HIV-1 infection has been effectively managed through the administration of HAART, involving combinations of three or more drugs [23]. However, long-term use of this therapy is associated with certain side effects, including accelerated aging with detrimental impacts on various organs, as well as elevated susceptibility to mental health issues, including major depressive disorder [24]. This is also linked to persistent immune activation, particularly monocyte activation, and enduring toxicity. There exist over 25 antiretroviral medications accessible for the treatment of HIV-1, and various combinations have proven successful in diminishing mortality and morbidity in infected individuals. However, these medications don't address the integrated proviral genome within host cell chromosomes, leading to the persistence of viral infection, and viremia rebounds upon therapy cessation. Consequently, lifelong administration of these drugs is required, resulting in diverse drug-

induced toxicities and comorbidities, especially linked to the aging process.

Providing HAART to all HIV-positive individuals, especially those in resource-limited settings, remains challenging due to various factors, such as the imperative for strict adherence, potential drug toxicity, drug interactions, and enduring immune dysfunction. [25]. As a result, there is an increasing focus on seeking a cure for HIV rather than solely managing its replication. Chronic kidney disease risk is heightened, especially with drugs like Tenofovir disoproxil fumarate, which can lead to tubulopathy in a small percentage of recipients. Factors like diabetes, immunodeficiency, and prolonged exposure to ritonavir-boosted protease inhibitors can further increase this risk. Ritonavir initiates cytotoxic impacts, resulting in stress on the endoplasmic reticulum and dysfunction of the mitochondria. Moreover, liver diseases stand out as a significant comorbidity linked to antiretroviral therapy, contributing to 13% of fatalities in individuals with HIV [6, 26]. Moreover, prevalence of drug resistance in individuals receiving lifelong ART rises, resulting in the dissemination of HIV strains resistant to drugs [23, 27].

LTR is a critical component of the HIV-1 genome because it contains various cis-regulatory elements that play a crucial role in controlling viral transcription [28]. It has been observed that HIV-1 possesses the capability to resume its replication activity subsequent to the discontinuation of HAART, leading to the infection of new host cells, particularly targeting CD4+ T lymphocytes [15].

Neurological compromise due to HIV infection

In individuals with HIV infection, a significant proportion, ranging from 18% to 50%, will experience neurological and cognitive changes. These changes can manifest as various symptoms, including memory loss, behavioral alterations, declining mental sharpness, difficulties in concentration, and motor dysfunction [29]. The progression of acute HIV infection commonly transpires within the initial four weeks subsequent to the transmission of the viral agent. During this period, HIV undergoes replication and it disseminates from the point of entry to diverse tissues. Simultaneously, the mounting of a defense mechanism against the virus promptly initiates,

resulting in the synthesis of anti-HIV antibodies.

The entry of HIV into the central nervous system (CNS) is postulated that the process takes place via infected lymphocytes and monocytes that traverse the blood-brain barrier (BBB). The HIV proteins and the consequent immune reactions have the capacity to disturb the integrity of the intercellular junctions within the BBB, thereby facilitating the ingress of immune cells harboring the virus into the CNS. Proinflammatory cytokines and chemokines function to augment the migration of immune cells to the brain, amplifying the viral recount within the CNS and immune responses are elicited from resident macrophages and microglia. Changes in the condition of the brain's blood vessels, as indicated by the presence of cerebral small vessel disease, have been noticed in individuals undergoing ART. Additionally, research suggests a potential connection between emtricitabine and the emergence of this bran vessel disease in the white matter of the brain [30]. Once HIV gains entry into the CNS, it infects resident cells, including microglia and astrocytes. Despite the initial belief that astrocytes lacked the capacity for productive replication of viral DNA, contemporary research has revealed that human astrocytes can indeed undergo infection through a CD4-independent, CXCR4-dependent mechanism. This results in the generation of infectious viral particles. Furthermore, the viral protein Tat plays a crucial role in causing disruption to the BBB and triggering inflammation within the CNS. This includes the activation of the complement system within the CNS. The complex interplay between HIV infection, the CNS, and the immune response underscores the challenges in managing HIV-associated neurological complications [31].

Infection of the CNS by HIV can give rise to neurological complications, including cognitive impairment and neurodegeneration. HIV-related neurocognitive disorders span a spectrum, encompassing asymptomatic neurocognitive impairment to the more advanced condition known as HIV-associated dementia [7]. The brain poses a considerable obstacle to both drug penetration and immune surveillance, primarily attributed to the formidable barrier presented by the BBB, resulting in limited genetic information exchange between brain-confined virus populations

and those in the bloodstream. Despite patients achieving undetectable viral levels in the blood under HAART, up to 50% may experience milder forms of HIV-Associated neurocognitive disorder, providing evidence for the presence of cellular reservoirs within the CNS. There, various types of infected cells have been identified: astrocytes, cells of the monocyte lineage, and microglial cells, which form reservoirs shortly after HIV-1 infection. Among these, microglial cells are acknowledged as a principal reservoir within the brain, with astrocytes' status as true reservoirs still debated. Understanding the molecular processes dictating HIV-1 latency within microglial cells is crucial for developing innovative strategies to target these reservoirs effectively [32].

Inability to eradicate HIV-1

In the past, efforts in HIV-1 cure research have predominantly focused on the complete eradication of the latent reservoir of cells containing transcriptionally silent yet functionally proviruses, the viral reservoirs. When we talk about viral reservoirs, it is referring to those cells or locations that harbor HIV proviral DNA even under ART. It has been demonstrated that HIV-1 can infect neurons and microglia cells [24]. However, a challenge in evaluating the efficacy of these curative approaches lies in accurately quantifying the magnitude of the viral reservoir in the human body of an infected person. Frequently, the size of the reservoir has been overestimated in measurements due to the presence of defective proviruses. This presents a challenge in developing effective HIV-1 eradication approaches [32]. Patients with HIV infection face an elevated risk of experiencing AIDS-related syndromes, encompassing diverse hematological malignancies such as lymphomas, multiple myeloma and leukemia [22]. As of now, effective vaccines or a definitive cure for HIV infections are currently unavailable. Cells within the latent reservoir have the potential to undergo activation at any given moment, leading to virus production and the potential for rekindling viral replication if therapy is discontinued [23, 33]. This highlights the ongoing challenges in managing HIV and the need for continued research and innovation in the field. Latent reservoirs of HIV are formed in the initial phases of HIV infection, and the virus's genetic material becomes integrated into the

genome of infected cells. Subsequently, it remains transcriptionally silent, rendering it imperceptible to the immune system. Despite the administration of appropriate ART, latent HIV can endure within the quiescent memory CD4+ T cells of individuals who are infected [34]. If ART is discontinued, HIV can reactivate and resume replication, leading to disease progression [15, 22]. The median duration until the reappearance of the virus at a concentration equal to or exceeding 1000 HIV RNA copies per milliliter was 22 days [35]. Notably, microglias, a type of cell in the brain, have been identified as key contributors to these viral reservoirs. Additionally, epithelial cells, such as renal tubular epithelial cells, have shown susceptibility to HIV infection. This complex network of latent reservoirs presents a formidable challenge for efforts aimed at HIV eradication. Understanding the intricacies of these reservoirs is critical for developing therapies that can reactivate latent HIV, making it vulnerable to the host immune system or other interventions, ultimately attaining a state of functional cure for HIV. Moreover uncovering the mechanisms that underlie the establishment of these reservoirs [37].

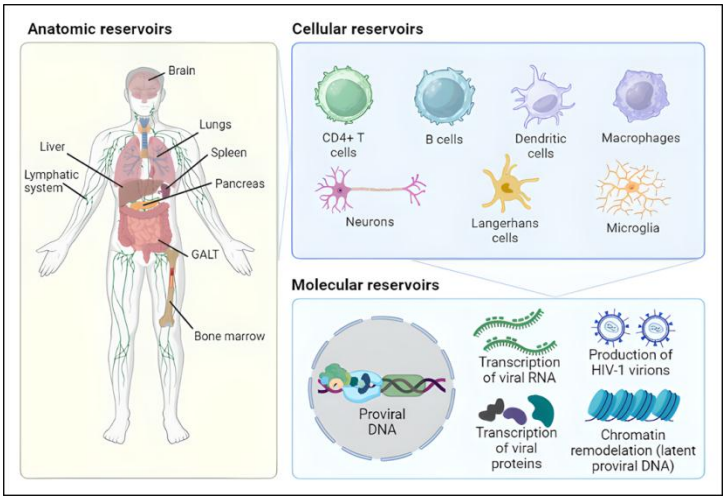
To achieve a cure, it is essential to eliminate viral reservoirs that establish themselves in specific body locations since HAART cannot eradicate them. These reservoirs, largely found in CD4+ T cells like naive and Rho (memory) effector CD4+ T cells, persist in a latent state during treatment, with a frequency of approximately 10^-7 per resting CD4+ T cells. Eradicating these reservoirs is a pivotal challenge in curing HIV infection [34, 35]. The primary cellular component of the HIV reservoir consists of resting memory CD4+ T cells. These cells contain HIV genomes capable of replication, and they are impervious to both ART and immune responses. Additionally, HIV reservoirs are found in various tissues, including lymphoid organs and tissues, as well as sites that have distinctive immunological and pharmacological attributes that contribute to the persistence of HIV, and where certain studies propose that continuous HIV replication and evolution take place in it, like the CNS, lungs, liver, bone marrow, and reproductive tissues [8, 25, 38]. The formation of viral reservoirs, along with HIV-1's strong affinity for CD4+ T cell lymphocytes, constitutes the principal obstacle in the quest for a

functional cure [7, 39-41]. Lymph nodes are particularly challenging for an HIV cure due to three reasons: they house follicular T helper cells, a key contributor to latency persistence; they facilitate these cells infection by keeping them close to follicular dendritic cells in B-cell follicles; these cellular entities exhibit a comparatively diminished susceptibility to pharmacological agents than other tissues. Other HIV special reservoirs are the genital tract and the brain, with reservoirs occurs primarily during the initial phases of HIV-1 infection. Notably, the predominant reservoirs are known to persist in the lymphatic system and the GALT [43].

instances where activated HIV-infected cells come into contact with resting T cells, there is a potential for the generation of latent viruses exhibiting diminished reactivation capabilities [42]. There are various cell types that can serve as reservoirs of viral particles (see Figure 2), with CD4+ T cells, dendritic cells, B cells, macrophages, and certain Langerhans cells representing prominent instances. The establishment of these

Emerging research has shed light on the existence of latent reservoirs in hematopoietic progenitor cells and CNS.

Figure 2. Viral reservoirs. Source: Prepared by the author.



At the molecular level, HIV-1 viral reservoirs may contribute to latent infection by actively producing functional or non-functional virions (viral RNA or protein transcript only) that maintain a chronic inflammatory state.

As a result, the attention has shifted to achieving a functional cure, characterized by inhibiting the virus without the ongoing need for HAART or the complete eradication of latent reservoirs [6]. The primary focus of HIV-1 research is to discover methods for eliminating or permanently deactivating the latent reservoir of integrated proviral DNA, responsible for viral rebound upon discontinuation of ART [44]. In terms of HIV persistence, despite ART, the HIV reservoir remains stable after around 4 years [45].

To be considered a true cellular reservoir, a cell must meet certain criteria, including integration of viral DNA into the host cell genome, the capacity to host the virus in a quiescent state, and the potential to generate active viral particles upon activation. Commencing ART during this early stage of infection does not preclude the formation of latent infected cells. While early treatment can diminish the size and distribution of the HIV reservoir, it cannot completely forestall its establishment [46]. The elucidation of mechanisms responsible for the persistence of HIV remains a contentious subject among researchers.

Certain investigations propose that sustained HIV replication and evolutionary processes take place in sanctuary sites, contributing to the replenishment of the viral reservoir. The genetic diversity of HIV subtypes can also influence drug resistance mutation fitness, challenging previous assumptions based on analyses of a single subtype (B subtype) of HIV-1 sequences [38]. HIV-1 employs the viral protein Tat to boost its transcription and regulate cellular gene expression, acting as a switch between latent and active transcription states. Nascent pre-mRNAs, including those of HIV-1, acquire a 7-methylguanylate cap at their 5' end after reaching a specific length. HIV-1 pre-mRNA can undergo multiple splicing events, producing numerous proteins from a single transcript, with over half of full-length transcripts undergoing this process. The hyperphosphorylated form of RNA polymerase II plays a role in coordinating transcription and splicing by interacting with splice factors [11, 32].

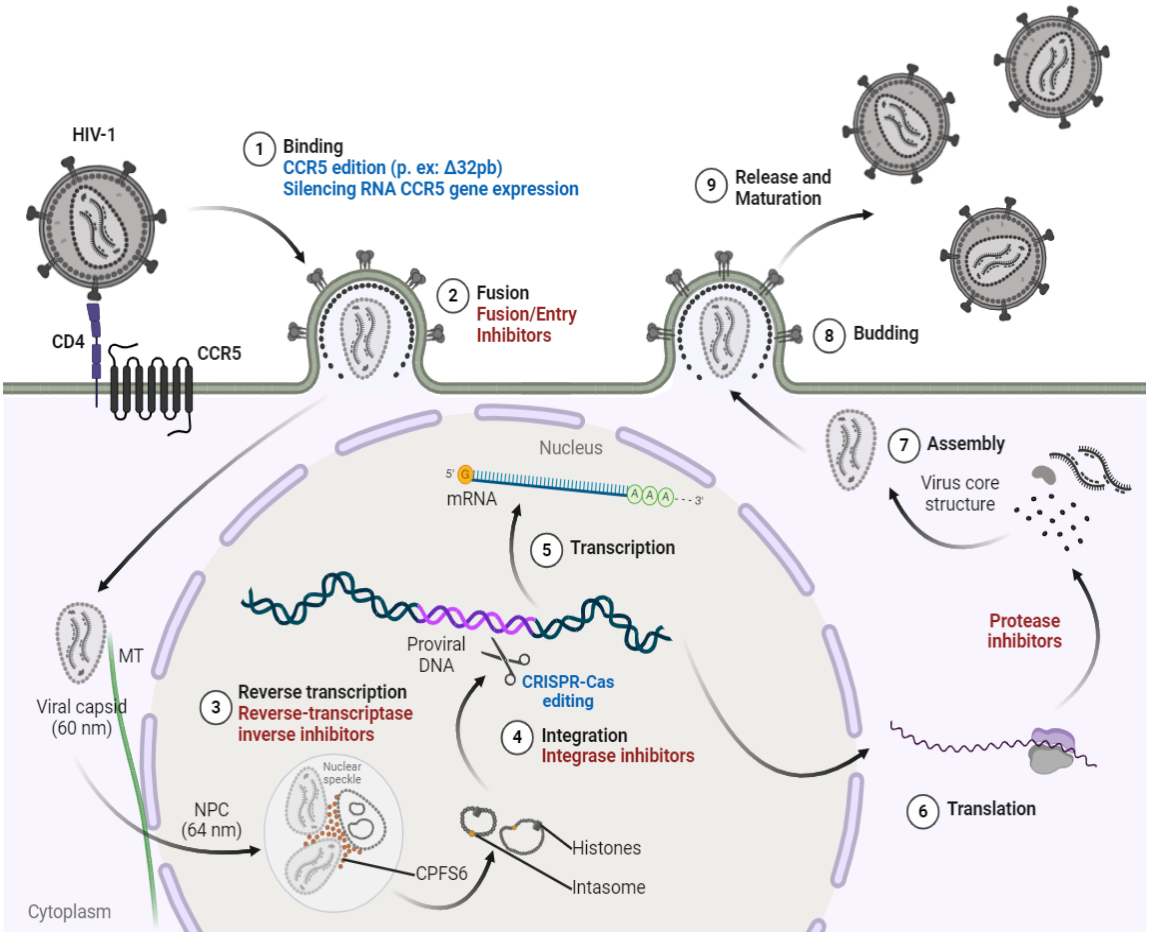
Looking towards the definitive cure for HIV-1: genetic editing and mechanisms of gene expression repression

The endeavor to achieve an HIV cure is closely linked to strategies aimed at eliminating viral reservoirs. In addition to traditional antiviral drugs, gene editing methods, notably the Clustered regularly interspaced palindromic repeat-Cas system (CRISPR-Cas), which was originally discovered as a defense mechanism in prokaryotes against viral infections, is a powerful tool in genetic editing [33, 47]. CRISPR-Cas9 offers the capability to target both the host cell genome and the genomic material of proviral DNA [43]. In the context of CRISPR-Cas, two highly effective strategies for genome editing are "deletion" and "knock-out". A minority of individuals demonstrate innate resistance to HIV-1 infection attributed to a specific genetic mutation called CCR5Δ32, wherein they are homozygous for a deletion of 32 base pairs in the CCR5 gene, experience a reduction or inhibition in the expression of the CCR5 receptor on their cell surfaces. The CCR5 deletion mutation renders the receptor non-functional, making it difficult for the virus to enter and infect cells. Importantly, this genetic mutation is not associated with major health risks [48]. Challenges associated with HIV-1 infection, have spurred the advancement of novel molecular approaches aimed at eradicating the proviral reservoir. Gene therapy is perceived as a supplementary strategy to HAART, contingent upon the establishment of a secure protocol devoid of risks for HIV-infected individuals. The formulated molecular strategies are directed towards precisely editing the viral genome, ultimately seeking the complete eradication of the virus from infected cells [49].

Notably, the CCR5 knock-out strategy has shown promising results as it does not harm lymphocytes and demonstrates a high level of efficiency (see Figure 3). This approach holds significant potential for targeted genome editing to combat HIV infection and presents a valuable avenue for further research and development in the quest for effective treatments. The sequences that are preferred to be targeted with CRISPR-Cas are those that are the most conserved. Two sequences are in the single guide RNA (sgRNA) which are the spacer's transcript CRISPR RNA (crRNA) and the trans activating CRISPR RNA

(tracrRNA), The sgRNA can guide CRISPR-Cas9 to cleave DNA double strands with a high precision. The mending of this double-strand break can occur through two mechanisms: non-homologous end joining (NHEJ), which may result in DNA deletion, replacement, or insertion; and homologous-dependent repair aided by a donor template. [11, 50]. It has been searched to inflict damage to the proviral DNA sequence through CRISPR/Cas9, targeting to the LTR that the viral genome has for improving the connection of transcription factors that may allow the transcription of the genome, and also to signal the poli (A) tail to protect the transcript genes. It is necessary to have in mind that there is a potential off-target modification risk and the triggering of an innate immune response, causing toxicity in cells and viral escape [15]. CRISPR/Cas9 emerges as a potent gene therapy instrument for the eradication of HIV-1 infection, but it faces several obstacles, including viral DNA sequence diversity due to the high rate of mutations (1 in 1,700 nucleotides). Hence, CRISPR can concomitantly cleave crucial rev and env genes, transcribed from the genomic segment but within distinct open reading frames. CRISPR has undergone testing for its effectiveness in targeting latent HIV-1 by manipulating proviral transcription, facilitated by a specialized Cas9 mutant. However, viral escape, immunogenicity, off-target effects, and effective delivery of the CRISPR/Cas9 system to the target cells continues to pose substantial challenges [51]. Research endeavors have explored the efficacy of CRISPR/Cas9 in the removal of integrated HIV-1 DNA and its potential synergistic action with ART for virus elimination. Promising results were seen in humanized mice subjected to a combination of ART and the genetic editing tool [7].

Figure 3. HIV infection cycle, current treatments (red), and potential cure approaches (blue). ***Source:*** Prepared by the author.



The HIV-1 replication cycle is a multifaceted process encompassing numerous steps. The HIV-1 replication cycle is a multifaceted process encompassing numerous steps. It begins with the virus binding to host receptors and introducing viral RNA into host cells. Subsequently, reverse transcriptase is utilized to synthesize viral DNA, which integrates into the nucleus of the host cell and undergoes transcription. Post-translational modifications occur within the ribosome, resulting in the formation of a functional virion. This assembly is formed and released from the cell, ultimately compromising the body's immune response and culminating in the establishment of AIDS. MT: Microtubules. CPSF6: Polyadenylation specificity factor 6. NPC: Nuclear pore complex.

Gene therapy is an attractive approach that delivers antiviral gene administering substances to cellular entities susceptible to HIV infection. Approaches involving gene expression modulation technologies, such as interference of RNA (RNAi) or CRISPR-Cas-mediated genome editing, present novel avenues for inhibiting HIV [52]. The formulation of a CRISPR-based therapeutic strategy against HIV-1 within these requirements will need a comprehensive methodology that seamlessly integrates bioinformatics, genetics, and functional studies. These components will work together to design highly specific molecules targeting conserved segments within the genomic structure of HIV-1, predict and mitigate off-target effects, and assess the functional impact of CRISPR-Cas9-mediated genome editing on the virus and host cells [26].

In the year 2020, the Nobel Prize in Chemistry was conferred upon Drs. Jennifer Doudna and Emmanuelle Charpentier in recognition of their pioneering contributions to the advancement of gene editing through the development of CRISPR-Cas guide RNAs (gRNAs). Their discoveries have revolutionized our ability to make precise changes to DNA in mammals, plants, and microorganisms, offering hope for innovative strategies in HIV cure research [14].

Noting that the landscape of proviral reservoirs might be predominantly characterized by proviruses situated in heterochromatin regions, these proviruses may have limited ability to rebound in vivo. This phenomenon could elucidate the exceptional ability of elite controllers to sustain a prolonged, medication-free remission of HIV-1 infection. Formerly, the estimation suggested that a substantial reduction in HIV-1 reservoir cells of clinical significance might necessitate 70 or more years of uninterrupted ART. Nevertheless, these computations were derived from theoretical extrapolations utilizing in vitro outgrowth assays, and their applicability to the genuine behavior of HIV-1 reservoir cells in vivo may be incomplete. The growing population of individuals living with HIV who commenced treatment roughly two decades ago, coinciding with the advent of triple-drug ART, provides an opportunity to empirically examine the progression and enduring presence of HIV-1 over a span of two decades under sustained suppressive therapy [53]. This

review explores innovative strategies for achieving a cure in HIV-1 infection, focusing on genetic editing and techniques for repressing gene expression.

Objectives

This research seeks to conduct a comprehensive exploration of the applications of CRISPR-Cas technology in targeted genetic editing. Our objective is to understand, in great detail, the precision and efficacy of various genetic editing techniques, particularly focusing on their ability to remove integrated proviral DNA from host cells. This investigation will extend to the potential of CRISPR-Cas in the pursuit of a remedy for HIV-1 infection. We aim to explore its effectiveness at eradicating the virus at crucial stages, thereby potentially addressing the chronicity of HIV-1.

Another central goal of this research is to delve into gene expression silencing strategies. This includes an in-depth analysis of RNA interference and related methodologies applied to HIV-1 infection. The objective is to identify and develop approaches that can effectively maintain the HIV-1 virus in a latent state within host cells, consequently preventing viral reactivation. We intend to contribute to the body of knowledge surrounding these techniques and their potential in mitigating the impact of HIV-1.

We aim to explore both viral and non-viral nanovectors as potential delivery mechanisms. Understanding the safety, efficiency, and viability of these delivery methods is a core focus. This aspect of our research aims to comprehensively explore and advance the methods by which genetic treatments, particularly CRISPR-Cas based therapies and gene silencing strategies, are delivered to target cells effectively and safely. One key objective is to take evidence of the potential development of advanced viral nanovectors. These vectors, based on modified viral particles, hold immense potential for delivering genetic editing components precisely to target cells. An equally important goal is to explore non-viral nanovectors for delivering genetic treatments.

Rationale

This research is driven by the urgent need to confront the persistent challenges posed by HIV-1, despite notable progress in highly active antiretroviral therapy (HAART). With millions still afflicted

and no definitive cure in sight, lifelong treatment presents various obstacles, including toxicity, chronic inflammation, and economic and access barriers. Genetic editing and gene repression offer a promising avenue, targeting pivotal stages of the HIV-1 lifecycle to redefine treatment paradigms. CRISPR-Cas technology, in particular, holds potential in this pursuit, necessitating a comprehensive understanding facilitated by systematic review (SR) methodologies. Focusing on CCR5 as a strategic target aligns with its critical role in viral pathogenesis, especially with CCR5-tropic strains predominant in early infection. This emphasis on early intervention distinguishes the approach from targeting CXCR4, which poses safety and efficacy concerns, particularly in advanced disease stages. By synthesizing diverse evidence and exploring the intersection of CRISPR-Cas technology and HIV eradication, this research aims to contribute a rigorous, up-to-date resource to the scientific community, fostering advancements towards a sustainable solution for HIV-1 and ultimately enhancing healthcare and quality of life for affected individuals.

SEARCH METHODOLOGY

Search Approach and Inclusion Criteria

This topic review adheres to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. The following search equations were used for the search: (PubMed: (HIV-1 OR CCR5) AND (RNA interference OR Gene silencing) AND (siRNA OR CRISPR-Cas OR shRNA)); (Scopus: TITLE-ABS-KEY (HIV-1 OR CCR5) AND (RNA interference OR Gene silencing) AND (siRNA OR CRISPR-Cas OR shRNA)); and (Web of Science: TS= (HIV-1 OR CCR5) AND TS= (RNA interference OR Gene silencing) AND TS= (siRNA OR CRISPR-Cas or shRNA)).

Inclusion Criteria

For this review, studies had to fulfill the subsequent criteria to be considered, based on screening by scientific articles, experimental techniques and language:

- Focus on gene editing and gene expression in HIV-1 infection.
- Published within the past five years (2019–2023).

- Investigate the application of genetic editing or gene repression mechanisms in the context of HIV-1, with a specific emphasis on the repression of the CCR5 gene as a therapeutic target.
- Investigate the use of nanovectors in the application of genetic tools.
- Grounded in vitro, animal, or human studies.
- Present results from diverse experimental techniques, such as in vitro, ex vivo, or in vivo studies.
- Utilize RNA interference or CRISPR-Cas strategies to target viral or host components crucial for the viral implantation and chronicity process.
- Articles in English.

Information Collection and Exclusion Criteria

The collection and evaluation of the gathered information were independently performed by the author, following a search methodology additionally, articles incomplete documents or those with unexplained methodologies, were excluded. An initial screening was conducted to remove duplicate articles from the collected literature. After this first screening, the articles were filtered since they were related to the main topic, according to its content in title, abstract and keywords. To ensure the rigorous selection of articles, the following stringent exclusion criteria were applied:

- Articles or book chapters were excluded if they solely provided a theoretical foundation for CRISPR-Cas or RNA silencing without demonstrating or showing a based-on evidence relevance or application to the research objective. Emphasis was placed on practical applications and tangible outcomes.
- Studies that did not present or show strong evidence findings as a basis were excluded. The focus was particularly on the ability of these techniques to remove integrated proviral DNA from host cells. This criterion aimed to ensure that the included studies demonstrated practical efficacy in a controlled laboratory environment.
- The review specifically included articles that comprehensively assessed the effectiveness, safety, clinical outcomes, and technological advancements related to gene editing and gene repression in HIV infection. There was no distinction based on study design or type to encompass a broad spectrum of evidence.
- Articles that were not related directly with HIV-1 infection or genetic

editing tools nor gene repression techniques applied to HIV-1 infection context.

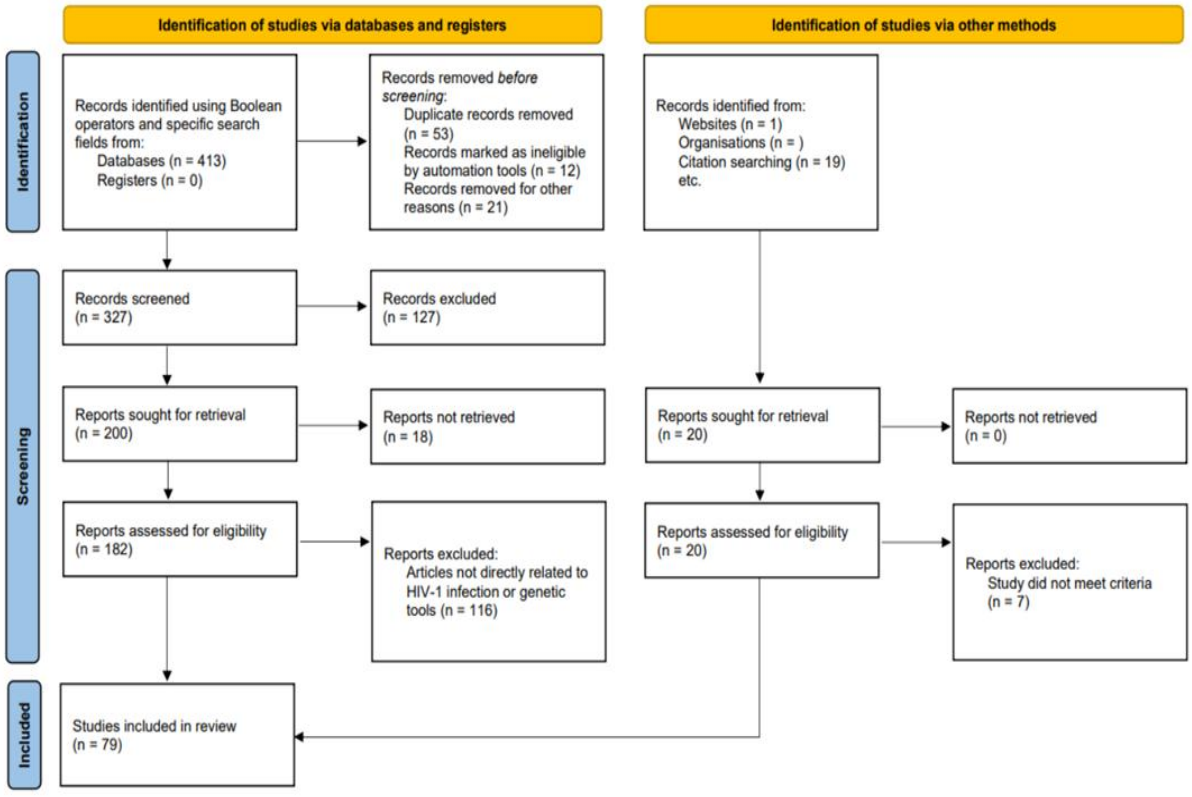
- Articles duplicated.

RESULTS

Article Selection and Characteristics

The flowchart depicting the process of study selection (see Figure 4) shows the process where a total of 413 articles were identified by its compatibility in Title, Abstract and Key Words containing information of the research equations made with the key words selected. From these articles, 347 did not meet the inclusion criteria and were subsequently excluded, resulting in 66 articles included in the final review. The details about the selection process can be seen in the figure in mention. Those were also filtered systematically giving 13 articles accepted.

Figure 4. PRISMA 2020. Flowchart for new systematic reviews which included searches of databases, registers and other sources. Source: Page MJ 2020. McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021; 372:n71. doi: 10.1136/bmj.n



This figure illustrates the systematic review process, which entailed comprehensive searches across databases, registers, and other sources. At the initial stage, certain articles were excluded due to duplication in search term combinations, articles that were not written in English or a lack of relevance to the research topic, as assessed through a primary screening of Title, Abstract, and Key Words. This led to 327 articles proceeding to the next stage. In the subsequent filtering process, these 327 articles were further assessed to ensure a direct alignment with the research objectives. Simultaneously, some articles proved inaccessible for review, necessitating a new screening. This resulted in the evaluation of 182 articles for eligibility, out of which 96 found not to be substantially related to the primary research objectives in their results and 18 of them lacking access.

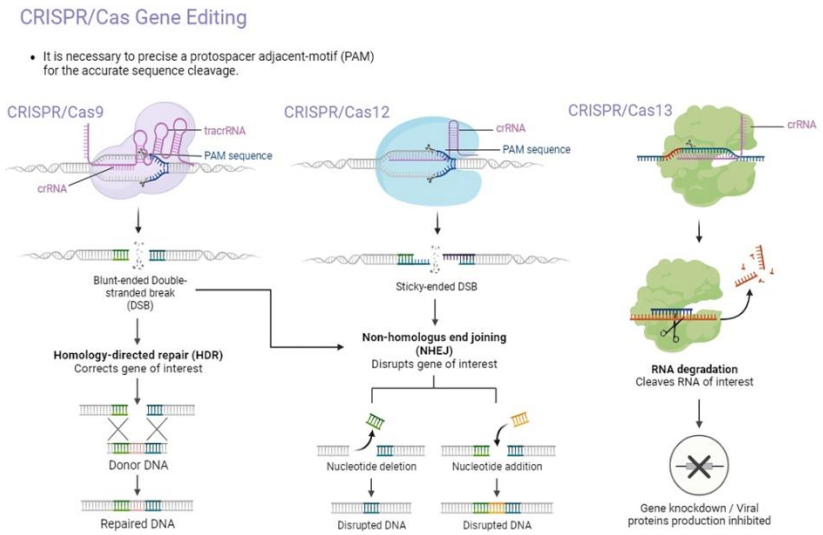
CRISPR-Cas as an editing tool: immunity or elimination

CRISPR-Cas contains a nuclease with the capacity to enzymatically cleave either DNA or RNA, contingent upon the particular methodology employed. This nuclease is guided by a short crRNA sequence that is complementary to the target gene, providing instructions for precise gene editing. CRISPR-Cas methodologies encompass both singular and multiplexed targeting of viral genetic elements. Single approaches use a single crRNA to target viral genes but have a higher potential for viral evasion due to the DNA repair mechanism NHEJ induced mutations. In contrast, multiplex strategies employ two or more crRNAs directed at either a solitary gene or a multitude of genes. This approach yields sustained effects and diminishes the probability of viral escape. Combining viral and host gene targeting and incorporating additional strategies, such as introducing genes encoding virus-neutralizing antibodies can further enhance the effectiveness of CRISPR-Cas-based therapies.

Cas proteins, including Cas9, Cas12 and Cas13, exhibit distinctive mechanisms for genome editing. Cas9 employs a sgRNA composed of two RNA molecules, recognizes specific DNA sequences, and makes precise cuts near a protospacer adjacent motif (PAM) sequence. Cas12, like Cas14, utilizes a gRNA to target

double-stranded DNA, causing staggered cuts away from the PAM. Moreover, Cas12 can activate nonspecific DNAse activity, cleaving single-stranded DNA indiscriminately. Cas13 establishes a molecular complex with its gRNA to identify complementary RNA sequences and activates a nonspecific ribonuclease, leading to the nonspecific cleavage of surrounding single-stranded RNA. These differences in mechanisms and target molecules offer diverse genome editing capabilities, from precise DNA modification with Cas9 to RNA degradation with Cas13 (54). Additionally, choosing the appropriate Cas system, like Cas12a, can minimize viral escape by performing multiple rounds of editing, introducing substantial mutations that hinder the virus's ability to evade treatment. show promise in achieving a functional HIV cure by deactivating integrated HIV DNA. This system employs nucleases like Cas9 and Cas12 to cleave DNA, activating cellular repair mechanisms that can lead to mutations in the viral DNA. The homology-directed repair pathway is used for precise gene editing with donor templates. Cas13 endonucleases can inhibit HIV replication by targeting RNA degradation; their impact does not extend to the integrated HIV DNA within the viral reservoir.

Figure 5. Types of CRISPR-Cas. Cas12 differs from the others in its simplicity of use. Cas13 degenerates the target RNA, functioning as a gene repression strategy. Source: Prepared by the author.



Recently, modified "dead Cas" or "deactivated Cas" (dCas) variants have been developed, allowing sequence-specific regulation of gene expression without DNA cleavage by linking dCas to regulatory domains [33]. CRISPR/Cas9 system is composed of a single-guide RNA and a CRISPR-associated endonuclease (Cas protein). The sgRNA is a concise synthetic RNA molecule featuring an intricately devised 20-nucleotide sequence, purposefully crafted to align with and complement the target DNA. This sequence is positioned proximal to a short DNA motif [17, 18]. PAM sequences are short and species-specific facilitated by the 17–20 nucleotides located at the 5' terminus of the gRNA, this sequence specificity serves to mitigate inadvertent off-target interactions. [14, 26]. S. pyogenes Cas9, commonly used in gene editing, recognizes the NGG PAM sequence. Studies have demonstrated the potential of CRISPR/Cas9 in treating HIV infection by cleaving the viral genome at LTR regions, inducing mutations, and even eliminating integrated HIV genes from the host cell genome. Additionally, this system has the ability to induce immunization to counteract HIV infection, as demonstrated by integrating Cas9 and gRNA sequences into T cells' genomes, resulting in reduced viral expression in infected cells compared to non-manipulated T cells [55]. The dCas9 fusion protein, in conjunction with a sequence-specific sgRNA, forms a complex that binds to the target DNA and make the deletion/insertion in the nucleotide specific sequence. However, the pivotal stage entails transitioning to clinical trials involving human subjects, wherein paramount consideration is given to ethical considerations and preservation of human genome integrity. CRISPR/Cas9 is poised to emerge as the primary genetic tool for rectifying a myriad of genetic disorders in the human population [23].

The transcription of HIV-1 is facilitated by RNA polymerase II. The Tat protein, encoded by the virus, plays a key function in promoting transcription through interaction with the positive transcription elongation factor b. The virus tends to integrate within genomic regions demonstrating transcriptional activity within the host cell's genetic material, particularly in sites associated with clonal expansion in CD4+ T cells [36]. This site-specific transgene integration can be

targeted employing the CRISPR/Cas methodology to instigate or induce a specified outcome, which is a break in both strands, potentially affecting its integration and aiding in therapeutic interventions. The design of crRNAs is a crucial aspect to consider when using CRISPR/Cas systems, as guide RNAs must exhibit elevated on-target efficacy while minimizing the likelihood of unintended effects recognition. Incidental cleavage away from the intended target poses significant and unpredictable risks, presenting a substantial safety concern for CRISPR-based therapies [56].

Gene therapy for HIV has focused on various gene targets, primarily host genes and HIV genes encoding structural proteins. Host genes like CXCR4 and CCR5 have been widely used as targets. Additionally, genes within HIV itself have been common targets in clinical trials. The genetic editing technique CRISPR/Cas has been extended to cut crucial HIV host dependency factors, CCR5 and CXCR4, resulting in resistance to its tropic HIV-1 infections. This represents a promising avenue for preventing HIV infection through genetic modifications [5]. In vivo investigations have shown the complete removal of HIV-1 proviral DNA through the implementation of CRISPR strategies in humanized mice. The use of CRISPR/Cas9 has resulted in a significant decrease in viral DNA copy numbers, which has shown a reduction in replication-competent viruses without inducing off-target effects [22].

This technological advancement has spawned two strategies for eradicating the latent HIV-1 reservoir:

- **Ex Vivo Eradication of Coreceptor Genes:** This approach involves the extraction of hematopoietic stem cells from individuals infected with HIV, followed by the utilization of CRISPR/Cas to excise the genes responsible for expressing the CCR5 and CXCR4 coreceptors. As a result of this procedure, the aforementioned cells acquire resistance to HIV-1 infection, and if they proliferate successfully, they can replace susceptible cells. This approach aims to recreate the protective effects observed in individuals with the CCR5Δ32 mutation, such as the Berlin and London patients [30];
- **Directing Focus Towards the HIV-1 Proviral DNA:** This strategy centers on the utilization of CRISPR/Cas to specifically target the integrated

proviral DNA within cells that are latently infected. The goal is to either excise the provirus or specific portions of it; achieving this involves either orchestrating concurrent CRISPR/Cas cleavage events or obliterating viral promoters to induce transcriptional silencing. Unlike coreceptor ablation, this strategy addresses the latent reservoir at the genetic level [30].

It is improbable that CCR5 editions alone would be sufficient to bestow resistance against HIV-1, given that the virus has the ability to evolve and utilize the alternate coreceptor, CXCR4. In approximately 50% of individuals with chronic HIV infection, the virus is shown to switch to using CXCR4 in the advanced phase of R5-tropic HIV-1 infection or in the absence of CCR5. Unfortunately, CXCR4 serves a function in maintaining hematopoietic regulatory processes and properties of stem cells, and its absence may lead to potential malignancies [57]. The application of CRISPR-Cas-mediated gene editing has been investigated for the disruption of the CCR5 coreceptor, aiming to impart resistance to HIV-1 infection. Effective introduction of the CRISPR-Cas system into susceptible cells expressing CCR5 has yielded immunity against R5 tropic HIV-1 strains. However, it's worth noting that this approach may not protect against viruses with different tropisms, such as X4 tropic strains. To achieve more comprehensive protection, researchers have considered concurrent disruption of both CCR5 and CXCR4 coreceptors. This strategy has demonstrated reduced expression of both coreceptors, resulting in cells that are impervious to infection by both R5 and X4 tropic HIV-1 strains. This approach shows promise in mitigating HIV-1 infection, but further research is needed to assess its safety and efficacy in clinical settings [58].

Disrupting these co-receptor genes has been shown to render cells, particularly immune cells, resistant to HIV infection, with no significant adverse impact on cell viability [56]. Furthermore, it is crucial that both alleles of the CCR5 gene are successfully cleaved or targeted to produce an effective resistance [24]. The efficiency of genetic editing in the context of HIV depends on the specific target site and its susceptibility to mutations. The ability of a target site to withstand mutations varies depending on the biological function that the sequence represents. Notably, studies have

demonstrated that mutations in the amino acids of the capsid protein can render the virus inactive, achieving a success rate of approximately 70% [39]. This highlights the importance of understanding the target sites and their mutation tolerance when employing genetic editing techniques to combat HIV.

One critical aspect of using CRISPR-Cas for gene editing is the importance of the PAM. It serves as a kind of recognition signal for Cas9. When designing CRISPR-Cas experiments, choosing a target site with a unique PAM sequence is crucial to ensure that Cas9 accurately binds to the intended genomic location and minimizes off-target effects [48]. Previous research suggests that mutations directed at non-coding regulatory sequences are less efficacious in attaining prolonged control of viral replication. However, it's important to note that focusing on regions with lower conservation, non-critical genes, regulatory sequences, or simple mutations that do not lead to deletions may create opportunities for the virus to evade control. Additionally, when utilizing multiplex gRNA targets, there is a risk of unintended cleavage events due to the continuous expression of CRISPR/Cas, potentially leading to changes in the host cell genome. Enhancements in CRISPR-Cas9 efficiency can be achieved through modifications to the gRNA module, including but not limited to mutation, extension, or truncation. The CRISPR-Cas9 system exhibits a degree of tolerance to variations in the RNA-DNA duplex, leading to potential cleavage at unintended sites [50].

The utilization of the LTR promoter has been instrumental in appraising HIV-1 infection within cell lines susceptible to a range of viral isolates. Researchers have guided RNAs to various positions in the HIV-1 genome, with significant emphasis on the R and U3 regions situated within the LTR. These studies have consistently shown a sustained decrease in viral expression in cells containing gRNA specifically designed to target HIV-1. Importantly, this strategy acts independently of HIV-1 strain variations, as it targets viral genomic sequences before integration into the host cell's genetic material [49].

Another strategy involves inhibiting viral replication by directing attention to various locations within the HIV-1 genome, encompassing gag, LTR, rev, tat and pol. In one approach, Cas9,

augmented with a nuclear location signal, was employed in HEK293T cells concurrently with multiplexed gRNAs designed to target the LTR region and additional structural genes such as gag and pol. Results demonstrated high efficiency in reducing viral protein expression, particularly when amalgamating gRNAs aimed at the LTR region with those targeting structural genes [21]. The rate of viral escape has been faster when targeting non-essential viral sequences, being capable of accommodating the customary indel mutations introduced during the process of DNA repair. On the contrary, targeting essential protein-encoding HIV sequences, which are highly conserved, delayed viral escape. Dual-gRNA combinations exhibited prolonged and more effective inhibition. Notably, some combinations achieved complete virus inactivation for certain HIV-1 strains. Despite the utilization of dual gRNAs targeting diverse domains of HIV-1, the occurrence of viral escape persisted in the majority of combinations. This phenomenon was ascribed to mutations introduced by NHEJ repair within both targeted regions [18].

The CRISPR system is categorized into two primary classes, Class I and Class II are distinguished by the composition of their effector proteins, from which Class II CRISPR/Cas system comprises a Cas nuclease intricately associated with a crRNA, facilitating the cleavage of a complementary target nucleic acid, whether it be DNA or RNA. In bacterial systems, the CRISPR/Cas mechanism operates through the acquisition of spacer sequences derived from viral DNA. These sequences are subsequently integrated into the bacterial CRISPR cassette, facilitating a defensive response against viral intrusion. This process encompasses distinct phases, namely adaptation, expression, and interference. However, for genome editing purposes, the interference phase is the most relevant. The frequently utilized effector nuclease is Cas9 derived from Streptococcus pyogenes, representing a Class II type II CRISPR/Cas system. This system necessitates the presence of both tracrRNA and crRNA for the binding to target DNA. For the simplification of Cas9-mediated DNA targeting, tracrRNA and crRNA have been amalgamated into a sgRNA, expressed under the U6 promoter. This arrangement facilitates the

efficient and specific targeting of diverse genes within various human cell types [56, 58]. The precision of this system can be readily adjusted by altering the crRNA sequence, thereby allowing controlled modifications of DNA/RNA targets.

Specifically, within the Class II, type V CRISPR/Cas system, a subgroup known as Cas12 (including Cas12a and Cas12b) predominates, characterized by their proficiency in guiding single RNA molecules to target double-stranded DNA effectors. The experiments developed with Cas12e enzymes from Planctomycetes showed different efficacy levels, being preferent to sequences with purines in the fourth space of its PAM. Active Cas12 then induces site-specific dsDNA cleavage, resulting in the generation of a double strand breaking with uneven termini [60]. Although this experiment was conducted in a free-cell in vitro environment, the findings emphasize the requisite for a systematic examination of Cas target selection and gRNA design, aimed at augmenting the efficacy and precision in the utilization of these emerging CRISPR enzymes within therapeutic frameworks [58]. CRISPR-Cas12a systems are more appealing for genome editing applications due to their heightened specificity, and may alleviate off-target issues. Additionally, the relatively smaller size of the cassette encoding the nuclease and corresponding crRNA could streamline and enhance the efficiency of delivery. Nevertheless, the Cas12a system has demonstrated diminished efficiency in gene editing when compared to Cas9.

While the well-known CRISPR-Cas9 system targets DNA, a recently discovered system called CRISPR-Cas13 has demonstrated considerable promise in the selective silencing of genes at the RNA level without impacting the entirety of the genome. The Cas13a system belongs to the type VI CRISPR-Cas13 systems, distinguished following the classification of Cas12a. Cas13 proteins exhibit distinctive properties in their ability to cleave single-strand RNA (ssRNA) rather than DNA, and notably, lack a DNA catalytic domain within their structure. [47, 56, 61]. In vivo experiments have demonstrated that the CRISPR-Cas13 system demonstrates heightened efficacy in the suppression of RNA compared to traditional RNAi technology [60]. Utilizing CRISPR-Cas9 to target the tat and rev genes has demonstrated encouraging outcomes in

safeguarding T cells from HIV-1 infection. Earlier investigations have established that the gRNA-Cas9 constructs targeting tat and rev effectively eradicate the expression and functionality of the Tat and Rev proteins, thereby contributing to the suppression of HIV-1. Specifically, the targeting of tat using CRISPR-Cas9 inhibited HIV-1 escape, a phenomenon observed with other gRNAs. An analysis of the mutational pattern induced by Cas9 revealed an unexpected increase in base substitutions, especially in instances involving a single gRNA targeting the tat and rev genes. This suggests that patterns of escape involving substitutions might potentially exert a reduced influence on the replicative capacity of HIV-1. To enhance the effectiveness of this approach, multiplexed tat and rev-targeting CRISPR-Cas9 constructions were formulated within a comprehensive vector. Multiplexed tatABC effectively shielded T cells from acute HIV-1 infection for a duration of up to 45 days, enduring subsequent infection assays or co-culture with unprotected T cells. These findings highlight the potential of CRISPR-Cas9-based strategies, particularly those targeting tat and rev genes, in protecting T cells from HIV-1 infection and suppressing the virus over extended periods [63].

In pursuit of attaining a sustainable solution for HIV-1 infection, mathematical models propose that reaching a minimum level of CCR5-edited cells is imperative. This involves transplanting hematopoietic stem cells with the CCR5Δ32 allele into a patient to reach a certain frequency of edited cells [62]. Cas9 displays limited tolerance in the presence of two mismatches in the target sequence, even if these mismatches are located far from the PAM. Virus strains that matched perfectly consistently showed delayed replication. Remarkably, Cas9 exhibits greater tolerance to mismatches in the PAM-distal region than in proximal regions. Combining effective gRNAs like gGag1 and gTatRev led to a lasting blockade of replication in certain HIV-1 strains. This dual gRNA strategy delayed replication more than individual gRNAs, demonstrating a synergistic effect. Consequently, dual-gRNA protection resulted in the complete deactivation of HIV-1 genomes, eliminating infected cell cultures by inducing mutations through Cas9/NHEJs and subsequently removing viral genetic

information integrated [17]. The precision and specificity of cleavage at the specified locations in the LTR sequences were verified through DNA sequencing. These results propose the utilization of long-acting, slow-effective release ART; by reducing the number of integrated HIV-1 copies, it augments the capability of CRISPR-Cas9 to modify integrated virus DNA inside of the host cell genome [8].

The utilization of the CRISPR-Cas12a system has demonstrated remarkable precision in targeting specific cleavage sites, including those within the proviral DNA or sequences encoding the CCR5 or CXCR4 receptors. This precision can disrupt the production of these receptors, inducing cellular resistance against HIV-1 infection. Alternatively, the system may achieve a cure by specifically disrupting key genes essential for virus production. Existing literature consistently supports the idea that employing a combination of CRISPR-Cas techniques, rather than relying on a single CRISPR-Cas therapy, significantly enhances viral suppression and reduces the risk of viral escape, as seen with some single therapies. These genetic mutations exhibited variability in regions with lower conservation, such as the LTR promoter region. Primarily, these mutations entailed substitutions and 3-nucleotide insertions within highly conserved targets, specifically in protein-coding domains. This mutational profile diverged from the patterns observed in the presence of other inhibitors, such as antiretroviral drugs or RNA interference therapeutics [38]. While the likelihood of unintended effects on non-target elements with CRISPR/Cas12a has been noted, its effectiveness in targeting HIV reservoirs is promising, especially when used in combination therapies targeting specific regions like LTR-1+LTR-3 and Gag1+Tat1, resulting in functional cures in T cell cultures [63]. Delivery in vivo can be accomplished through viral vectors; however, notable challenges persist, including off-target effects, immunogenicity, viral escape, and the efficient delivery of the CRISPR/Cas9 system to the intended target cells. Nonetheless, following lentiviral treatments with CRISPR Tat transfection, reductions of 82% to 94% were observed in all transmitted founder strains. The application of electroporation for TatDE ribonucleoprotein, along with the delivery of LNP Tat gRNA and Cas9 mRNA to latently infected cells, yielded viral

excision rates of up to 100%. Double accomplishments were realized in terms of protection against HIV-1 challenge and the induction of virus during latent infection. These achievements were observed in both primary and transformed CD4+ T cells or monocytes [49]. The findings illustrated that synthetic HIV gRNA/Cas9 ribonucleoproteins, directed towards genes such as HIV DNA polymerase and vpr/tat, proficiently impeded HIV reactivation and replication while avoiding cytotoxic effects. These findings suggest that these synthetic HIV gRNA/Cas9 ribonucleoproteins have the potential for further investigation as therapeutic agents. The investigation underscores the significance of investigating innovative delivery strategies, including engineered nanoparticles or exosomes, for the in vivo administration of potential gRNA/Cas9 ribonucleoprotein drugs (64). A study realized an analysis of proviral DNA following multiple rounds of dual gRNA-Cas9 protein treatment unveiled genetic alterations at the gGag1 target locus in 50% of proviral DNA instances. Additionally, in 32% of cases, the segment located between the designated target sites was either removed or flipped, while 18% showed inversion. Similar frequencies of these different outcomes were observed in previous CRISPR-Cas9 experiments targeting HIV in cells with stable Cas9 expression. Conversely, when cells underwent repeated dual crRNA-Cas12a protein treatment, 66% of proviral DNA displayed mutations at the crGag1 site. The incidences of excision (5%) and inversion (1%) were markedly reduced. This discrepancy suggests that Cas9 cleavage tends to lead to excision or inversion more frequently when contrasted with Cas12a cleavage. This distinction can be ascribed to distinct target sequences and PAM requirements for Cas9 and Cas12a (65). Another study showed that Cas12a outperformed Cas9 in extended HIV challenge experiments involving stably transduced T cells, even though only modest HIV inhibition was observed in transient transfections. They attribute this difference to variations in the DNA editing processes driven by Cas9 and Cas12a endonucleases. Notably, the research unveiled a remarkable dissimilarity within the patterns of genetic mutations induced by Cas9 and Cas12a editing. While Cas9 typically leads to small indel mutations at its target sites, Cas12a-edited sites showed a distinct

mutation class referred to as "delin" characterized by sequence deletions rather than insertions (66).

Cas13a is a type VI-A ribonuclease that can target and cleave ssRNA and catalyze both crRNA maturation and RNA-guided ssRNA degradation through an interdependent process that involves two distinct catalytic sites. Differing from type II Cas9, which functions as an endonuclease (see Figure 5), Cas13a functions as a ribonuclease with dual roles, playing a role in both crRNA maturation facilitation and executing RNA-guided single-stranded RNA degradation. These activities are interdependent and involve two separate catalytic sites within Cas13a. It's worth noting that while non-specific RNA degradation by Cas13a has been observed in vitro and in prokaryotic cells, there have been no reports of such activity in eukaryotic cellular environments. Instead, the collateral cleavage activity of Cas13a has been harnessed for specific RNA transcript detection, even in cases where the RNA copies are present at very low levels [61]. The expression of Cas13a in cells has been utilized to achieve the knockdown of both reporter and endogenous RNA, demonstrating efficiency comparable to RNAi and superior specificity. This establishes Cas13a as an alternative technological approach for the potential development of novel therapeutics. Cas13a can operate within a versatile CRISPR/Cas system guided by RNA, demonstrating significant promise for accurate, robust, and scalable applications in targeting RNA with RNA guidance [45]. The Cas13a system has been tested for targeting RNA viruses; various strategies can be employed to enhance the targeting precision and interference efficacy of this system. The Cas13a system has distinctive attributes: Cas13 proteins exhibit a propensity for cleaving ssRNA rather than DNA, lacking a DNA catalytic domain in their composition. The Cas13b system represents another variant within the type VI CRISPR-Cas13 systems, delineated subsequent to the characterization of Cas13a. It has been tested for targeting HIV-1 RNA, crRNAs directed at the LTR and rev gene exhibited a notably heightened inhibition of HIV-1 infection compared to other tested crRNAs.

Nucleases like Cas13a and Cas12a have shown efficacy in degrading viral RNA and causing distinct mutations, offering

diverse approaches for HIV therapy. HIV treatment may pave the way for the clinical application of CRISPR/Cas antivirals, representing a significant step towards eliminating viral reservoirs in patients [45]. Precisely, Cas13a is intricately engineered to recognize and cleave RNA molecules. This design imparts the effector with the capability to provide defense against RNA phages through targeted degradation of the offending RNA entities. Cas13a has been effectively delivered into cellular systems, demonstrating its capacity to selectively target and degrade both exogenous reporter RNA and endogenous RNA. Its efficacy rivals that of RNA interference, yet it exhibits superior specificity in its target recognition and cleavage activities. The distinctive attribute of Cas13a renders it a promising technological platform for the advancement of innovative therapeutic interventions. In experiments targeting HIV-1, crRNAs directed at the LTR and rev gene exhibited stronger inhibition of Y-box-binding protein 1 expression compared to other crRNAs tested. This suppressive impact was additionally substantiated by a significant reduction in the expression of viral Gag protein in cells that expressed the Cas13a/crRNA combination specifically designed to target HIV-1. These findings collectively establish evidence that Cas13a functions to hinder the generation of HIV-1 by attenuating the expression of viral RNA and proteins [59].

It is of essential importance to meticulously confirm that the designed guide RNA is not complementary to any segment of the host cell genome, particularly in regions associated with growth and metabolism. This step is crucial to prevent unintended off-target effects that could potentially impact cellular functions and compromise the integrity of the genome. Thorough assessment and validation of gRNA specificity are imperative to ensure the safety and accuracy of genome-editing procedures; for optimal functionality, it is imperative that the target sequence of the gRNA be positioned in the 5' direction relative to the nucleotide sequence denoted as the PAM [67]. It employs a 20-nucleotide sequence within the gRNA to guide Cas9 to a complementary DNA target sequence [38, 48]. The RNA-guided Cas9 nuclease, in conjunction with LentiCRISPR/SaCas9, was employed for the purpose of disrupting

the CCR5 gene in human CD4+ T cells and Jurkat T cells. This intervention aimed to confer resistance against R5-tropic HIV-1 infection. The disruption of CCR5 in human T lymphocytes also enhanced survival benefits observed following exposure to challenges in humanized mice infected with HIV-1. Additionally, concomitant knockout of the CXCR4 and CCR5 genes in CD4+ T cells using CRISPR/Cas9 technology imparted resistance to infections caused by both X4- and R5-tropic strains of HIV-1 [16]. The CRISPR-Cas9 system was applied to selectively target multiple genes within the HIV genome. These encompassed structural genes, enzymatic genes, accessory genes, and the LTR responsible for facilitating the integration of HIV DNA. The effectiveness of CRISPR-Cas9 varied between 30% and 96.3%, with a predominant proportion of gRNAs exhibiting efficiency surpassing 80%, particularly when targeting the LTR sequence. Nevertheless, variations were identified in the effectiveness of CRISPR-Cas9 when directed towards structural genes, indicating nuanced disparities in the gene-editing outcomes. Certain investigations reported an efficiency range of 50-70%, whereas others observed efficiencies exceeding 80% [68]. Simultaneously employing two gRNAs to target the integrated genome of HIV-1 may lead to the accumulation of mutations at both targeted sites. The removal or inversion of the intervening sequence can occur as a result. Genotyping analysis of amniotic stem cells clones, which were transduced with two sgRNAs, demonstrated the successful biallelic targeting of the CCR5 gene [69]. This led to the generation of both substantial in-frame deletions and frameshift mutations. It shows potential in delaying viral escape, more efficiently blocking virus replication, and potentially achieving the eradication of all proviral DNAs within infected T cell cultures. Nonetheless, further research is needed to confirm its efficiency and assess off-target effects [7]. The frequencies exhibited variability across different gRNA combinations, without any apparent association with the proximity between the designated target sites. This suggests that factors beyond the spatial arrangement, including distinct gRNA and target DNA characteristics, play a role in influencing the processes of DNA cleavage and repair.

CRISPR/Cas9 holds the potential to remove the HIV provirus or essential host cell genes like CCR5, inhibiting HIV propagation. However, clinical implementation encounters difficulties concerning the extended safety implications and the possibility of unintended effects in the host cell genome [40]. It was found in a study that the CRIPSR-Cas attacks targeting HIV-1 integrated genetic information may affect close host cell genomic sequences and even surrounding chromosomic information [70]. Findings suggest that synthetic gRNA and Cas9 hold promise as innovative drugs targeting HIV, with the potential to facilitate the eradication of the virus. These entities have the capacity to induce swift DNA cleavage while minimizing off-target effects, exhibiting a low risk of insertional mutagenesis. However, further research is needed to evaluate the enduring inhibitory effects of gRNA/Cas9 ribonucleoproteins, host immune responses, and the efficiency of delivery represent critical considerations for sustained therapeutic outcomes. Combining multiple gRNAs may enhance viral elimination, and previous studies have indicated that vectors expressing gRNA and vectors expressing Cas with suitable delivery methods can efficiently impede or eliminate HIV replication [64]. Several studies have shown the efficacy of diverse CRISPR-Cas techniques.
(Table 1).

Given the essential role of the tat and rev genes, that undergo expression shortly following the viral integration and significantly influence the viral life cycle, it is imperative to consider strategies that effectively target these genes. Tat inhibition, for instance, can effectively impede the reactivation of proviruses from a latent state. One promising hypothesis is that multiple CRISPR attacks on tat and rev genes could induce hypermutation, making escape mutations unlikely. Any such mutations would likely impose a significant replicative fitness burden on the virus, rendering this approach particularly potent in disrupting HIV-1's life cycle [39, 61].

Table 1. Targets of different CRISPR-Cas techniques. LV: Lentivirus. LNP: Lipid nanoparticles. *: Viral escape detected. Source: Prepared by the author.

Applied to	Target	System used	Delivery	Effects/Findings	Reference
Viral DNA	Tat	Cas9, Cas12a	LV	Suppression of virions production, prevention of viral escape, and the elimination of HIV.	31
	Env	Cas9	LV	Suppression of viral production leading to the elimination of HIV.	33
	LTR1+LTR3	Cas12a	LV	Strongest antiviral activity*.	65
	Tat, Rev, Gag, Env	Cas9	LV	Effective mutations and excisions in genes targeted.	34
	Tat, Rev, gp41	Cas9	Electroporation, LV and LNP	The viral load exhibited an 82% to 94% reduction across all transmitted founder strains.	51
	NF-κB binding sites (LTR)	Cas9	Plasmid vector	Significantly reduced LTR-driven transcription in the culture cells experimented (HEK293T cells, TZM-bl cells, T cell latency J-Lat model).	41
	RNA HIV 1	Cas13a	Electroporation	Suppression of HIV replication observed in CD4+ T cells.	61
	gag, pol, protease, integrase, central polypurine tract, central termination sequence	Cas13d	LV	Effectively suppressed HIV-1 replication in cellular models.	6
	Gag, Pol, Tat, Env	Cas9	LV	In the regions targeted by the guide RNA, there was an occurrence of insertions and deletions surpassing 76%, and no reliable off-target sites were identified.	73
	Tat, Rev	Cas9	LV	Successfully shielded T cells from acute infection with replicative HIV. Inhibited viral escape.	63
	Tat	Cas12a	LV	Absence of viral replication up to the end of the experiment.	68
	LTR, Gag	Cas9	AAV_9	The transduction into cortical glial cells showed a HIV-1 mRNA reduction both *in vitro* and *in vivo* experiments, seen through *in situ hybridization* techniques.	79
Host genome	CCR5	Cas9	Neon Electroporation System	Medium efficacy in reducing CCR5 expression.	13
		Cas9	LV	Enhanced resistance to HIV-1 infection observed in human CD4+ T cells.	16
		Cas9	AAV	Integration of CAR cassette into CCR5 locus zone, generating resistant HIV infection CAR T cells.	78
		Cas12e	Cell-free in vitro assay	Cleavage reactions showed different efficacy levels of activity depending on spacer length.	60

Gene expression repression: RNA silencing

Approximately more than 1,200 human genes are believed to be associated with viral replication [27]. Diverse RNA molecules have the capacity to induce epigenetic silencing, including short interfering (si) and short hairpin (sh) RNA molecules. siRNA in vitro delivery can be achieved through methods such as transfection, employing reagents like lipofectamine, nucleofection, or calcium phosphate. Alternatively, delivery can be facilitated through cell-penetrating nanoparticles carrying siRNA. Once inside the cell's cytoplasm, siRNA moves to the nucleus and assembles the RNA-Induced Transcriptional Silencing Complex, resulting in the imposition of suppressive epigenetic modifications (histone methylation) on the promoter region. The shRNA can also induce epigenetic silencing through viral delivery. In this method, a cell is transduced with a viral vector harboring the shRNA of interest, which enters the cell facilitated by a viral envelope. Once inside the nucleus, shRNA is processed into siRNA, which then follows a similar path to induce epigenetic silencing [13, 21]. RNAi exhibits tolerance towards variations in the RNA duplex, which can cause off-target effects on non-related mRNAs. Si/shRNA can mediate efficient decay of HIV RNA, but there are limitations to this approach, including structural constraints, viral sequence diversity, and suppression of cellular RNAi machinery by viral proteins, and off-target effects. To address these challenges, an effective HIV RNA-targeting strategy should be independent of the endogenous RNAi machinery enabling the concurrent targeting of multiple conserved sites within the virus. Target sites were chosen in regions encoding key HIV proteins, and gRNAs were designed to target these sites. The strategy led to a noteworthy decrease in HIV replication and the generation of viable HIV particles. This demonstrates the potential of this strategy to inhibit HIV and potentially achieve a functional cure [6].

RNAi and newer gene editing tools like CRISPR-Cas9 show promise for HIV-1 treatment. To amplify its efficacy and mitigate the emergence of viral RNAi-escape mutants, the CCR5 coreceptor has been contemplated as a supplementary target. However, HIV-1 has the capacity to alter its preference for coreceptors,

transitioning to alternatives such as CXCR4. The synergistic application of RNA interference targeting both cellular and viral elements results in a substantial enhancement of antiviral effects. For instance, shRNAs targeting CCR5 result in a substantial decrease in HIV-1 infection, even a non-total repression in CCR5 proves highly detrimental to the infection process. ShRNAs targeting CCR5 demonstrate high protective effects, better performance in mixed cell populations, and sustained functionality, while shRNAs targeting the Rev transcripts show nearly 100% inhibition of viral replication, though with less sustained effects. This research highlights the potential of RNAi-based therapies for HIV-1 treatment [71]. The utilization of miRNAs in quiescent CD4 T cells demonstrated notable efficacy in suppressing HIV transcription through targeted interactions with specific regions of HIV-1 mRNA. Scientists have formulated RNA inhibitors, including siRNAs, antisense RNAs, and ribozymes, with the aim of targeting Tat and impeding the viral replication across diverse cell types [11].

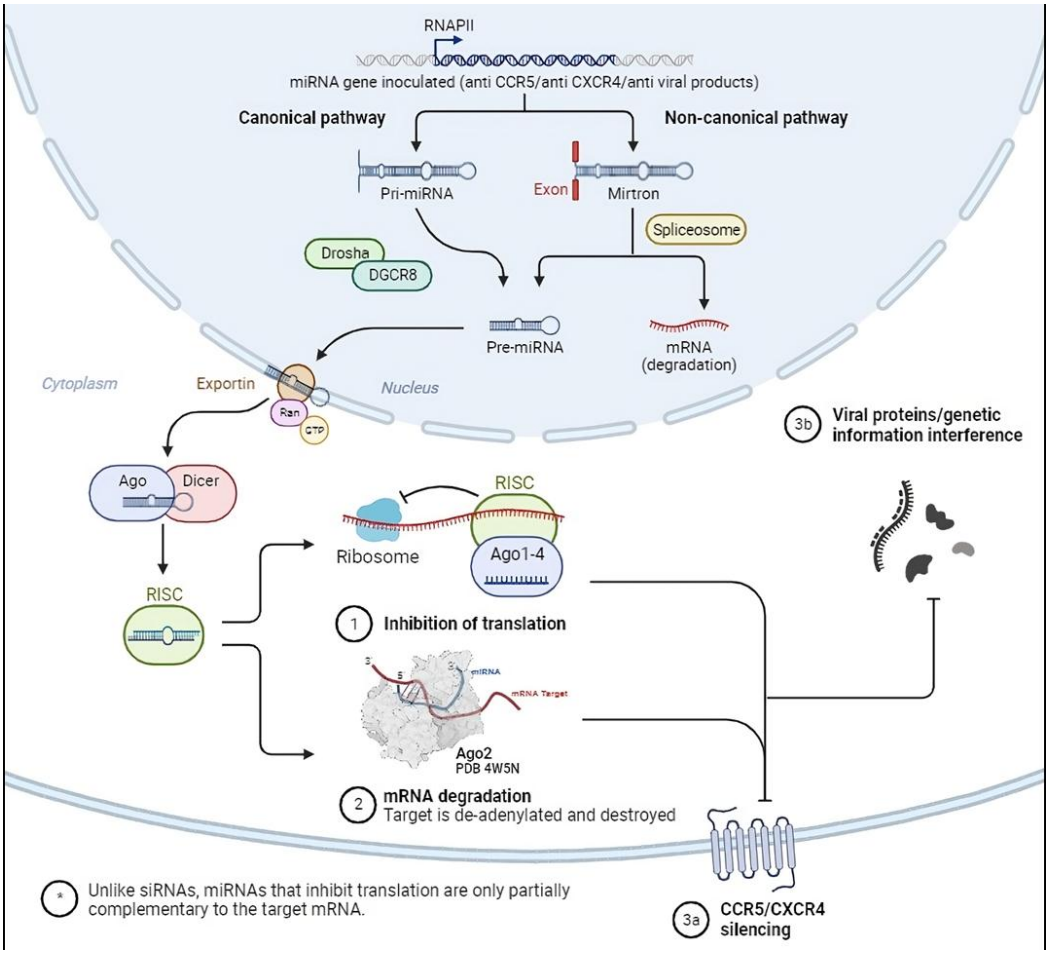
ShRNA, miRNA, siRNA

MicroRNAs (miRNAs), are small RNA molecules that can cause the mRNA degradation and the regulation of gene expression. Synthetic siRNAs and shRNAs have been used to target important HIV-1 RNA regions. Targeting conserved sequences like the polyadenylation sequence is crucial. Employing multiple sgRNAs to concurrently target various genes within HIV-1 (multiplex genome editing) holds the potential to impede viral escape, rendering it a promising strategy for the treatment of HIV-1 infection [25]. New therapeutics based on the use of nucleic-acid molecules, including aptamers, and RNAi, have arisen as auspicious alternatives to HAART for combating HIV. Among these, siRNA, typically 21-23 nucleotides in length, is widely utilized. Several siRNA therapeutics have gained approval for clinical use. siRNAs designed to silence key HIV-1 key genes like tat, rev, nef, and vif are under development, with dual-functionality genes encoding two proteins showing particular promise. Delivery methods include viral vectors, nanovectors, and electroporation, with lentiviral vectors being commonly used for ex-vivo

delivery to specific cell types like CD4+ T cells or CD34+ hematopoietic progenitor cells. However, the effectiveness of cellular membrane-based nanoscale vesicles in inhibiting HIV infection is constrained by the extensive population of host cells in vivo. Talking about its delivery, the inherent complexities associated with vesicular entities present formidable impediments, hindering their capacity to competently engage in competition with viruses freely circulating or infecting cells across a wide spectrum of host cells.

In response to this challenge, a delivery platform, denoted as the lymphocyte membrane and 12p1-dual functionalized siRNA delivery lipid nanoparticle, has been devised. This demonstrated the potential to neutralize the cytotoxicity of gp120, a protein associated with HIV-1, and significantly improved the viability of CD4+ T cells when combined with HIV-1MN gp120 recombinant proteins. Additionally, it displayed strong inhibition against R5-tropic HIV-1BaL gp120 recombinant proteins-induced CD4+ T-cell death, highlighting its therapeutic potential [9]. This mechanism can be harnessed for therapeutic purposes by introducing small RNA duplexes, such as miRNA, siRNA, and short-hairpin shRNA, which act as artificial RNAi agents. While siRNA and shRNA operate through the RNAi pathway, shRNA necessitates further processing and is often transcribed from viral vectors or plasmid DNA in the nucleus, whereas siRNA is typically synthesized. The RNAi route (Figure 6) involves the cleavage of double-stranded RNA or primary miRNAs into short RNA fragments (siRNA or miRNA), which are loaded onto the RNA-induced silencing complex. This complex, with either siRNA or miRNA as a guide strand, targets complementary mRNA sequences, leading to gene repression through mRNA inactivation or post-translational degradation.

Figure 6. Silencing mechanism. Either of its pathways involves the gene expression repression, which can be used to inhibit the HIV-1 proteins production, and/or to silence the coreceptors used by HIV-1 expression. Source: Prepared by the author.



While lentiviral vectors integrate their cargo into the host genome, non-viral approaches like liposomes and polymers are less efficient. Encapsulating siRNAs within biodegradable polymer nanocapsules can protect them from degradation and enhance gene knockout. RNAi therapy offers specificity, versatility, and survival in HIV-1-infected cells, making it a promising approach, especially when using combinations of shRNA-miRs targeting both host factors and viral genes. Clinical trials employing ex-vivo lentiviral vector delivery have shown promise, but further research is needed to develop effective in-vivo delivery methods for HIV-1 treatment [44]. While these techniques share the

common goal of regulating gene expression, they exhibit important differences. ShRNAs and siRNAs both target a single mRNA transcript but differ in their mechanisms. ShRNAs integrate their coding sequence within the cellular genome, incorporation occurs, facilitating enduring regulation of gene expression. In contradistinction, siRNAs operate fleetingly within the cytosol. MiRNAs, initially found naturally in cells, can now be artificially synthesized and have a unique structure that allows them to target multiple mRNAs.

All three approaches have been explored for their potential in reducing CCR5 expression as a therapeutic strategy in HIV-1 infection [10]. ShRNAs tailored to specifically target CXCR4 have been formulated as a viable alternative. Notably, targeting towards proviral DNA can induce the suppression of virus replication through the mechanism of transcriptional gene silencing [72]. Small non-coding RNAs, particularly miRNAs, play a crucial role in regulating gene expression by inducing mRNA degradation or translational repression. These regulatory mechanisms are governed through two primary pathways, namely the canonical and non-canonical routes, involving the processing of primary RNA into precursor miRNA subsequent cleavage by Dicer, followed by integration into the RNA-induced silencing complex. MiRNAs have been identified as potential targets for modulating various stages of the HIV life cycle. For instance, miR-107 can bind to the 3′UTR of CCR5, a key player in HIV internalization, potentially preventing HIV infection. Furthermore, genome-wide RNA interference has identified host factors involved in HIV replication that are regulated by miRNAs. Selectively blocking or enhancing miRNAs targeting specific antiviral proteins or pathways may offer therapeutic strategies against HIV [29, 73].

Delivery of tools to target cells

Nanocarriers offer various advantages, exhibiting the capability to encapsulate a variety of therapeutic agents, resulting in the extended circulation or prolonged retention time within tissues, maintaining controlled drug release over an extended period, enhancing solubility and bioavailability for improved pharmacological efficacy, mitigating toxicity and adverse effects while augmenting drug potency. Nanocarriers

have exhibited efficacy in facilitating the delivery of combinations of antibodies, different RNAi targets, or gene editing tools with a gene silencing molecule. Considering the intricate nature of HIV integration sites and the virus's propensity for rapid mutation, the implementation of combination therapies, even within the framework of singular gene therapies such as the simultaneous delivery of multiple siRNAs, has the potential to amplify efficacy. Various types of nanocarriers have been explored in the context of HIV cure research. These include dendrimer-siRNA nanoparticles, lipid nanoparticles, biodegradable nanoparticles, lactoferrin nanoparticles, magnetic nanoparticles, peptide carriers, liposomes, nanoemulsions, and carbosilane dendrimers. Each of these nanocarriers has its own unique properties and advantages, rendering them apt for diverse applications in the advancement of gene-based therapies for the cure of HIV. Understanding their biodistribution and targeting capabilities is crucial for optimizing their use in HIV cure strategies [74]. This approach has been combined with adeno-associated virus (AAV) delivery and long-acting slow effective release ART to target HIV genomic material integrated effectively [22]. A significant constraint in the therapeutic utilization of the CRISPR/Cas system lies in the delivery of its components, and the presence of residual non-edited infected cells may serve as viral reservoirs, impeding the efficacy of the treatment. The selection of optimal carriers is contingent upon the specific location and cell type under consideration.

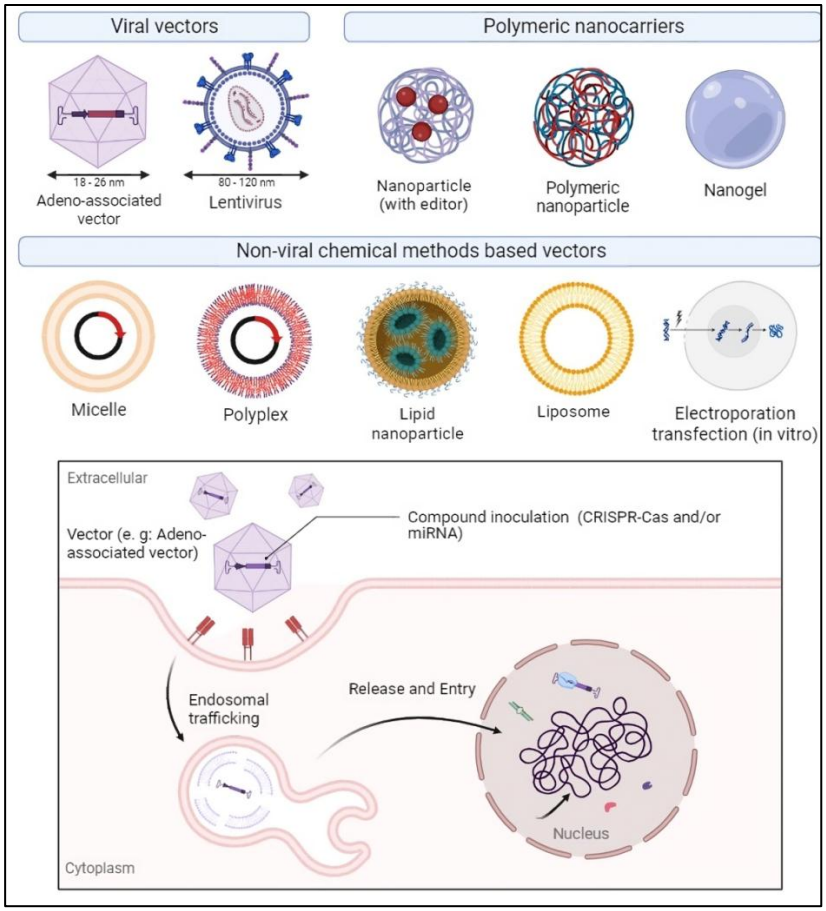
Nanotechnology represents a promising tool for the non-viral delivery of Cas-RNPs, given its potential to address the primary drawbacks associated with viral vectors, safety concerns and limited loading capacity [54]. In the context of in vivo applications, lipid nanoparticles or viral vectors such as lentiviral vectors or adeno-associated viruses may be employed. However, it is important to note that viral vectors possess a restricted packaging capacity, potentially impeding the efficiency of delivery. Small SaCas9 with AAVs are one potential solution, although it is less efficient than SpCas9. Recent investigations have indicated that gRNAs engineered to target specific sites, when delivered to latently infected cells via lipid nanoparticles, can achieve approximately 100% viral excision.

While these experiments started with T-cells, the HIV-1 reservoir is not limited to T-cells alone [24]. Although adenoviral vectors have demonstrated efficacy in delivering the CRISPR/Cas9 system, there are concerns about off-target effects. Lentiviral delivery, although promising, may concurrently elevate the risk of off-target effects. Additionally, challenges such as cytotoxicity and the potential for immune tolerance represent limitations in the application of CRISPR-based technology against HIV [2]. Adenoviruses are another option for non-integrating CRISPR transgene delivery, although they may have limitations in transduction efficiency [22]. Retroviral vectors offer permanent RNA expression, although they may generate new viruses in HIV+ cells. Different anti-HIV RNAs have unique advantages and limitations, with some potentially causing sequence-independent toxicity [75]. Although carrier-free delivery methods have shown effectiveness in some applications, they come with potential limitations, including unwanted immunostimulation, challenges in renal clearance, limited transport into cellular compartments, and susceptibility to degradation by cellular enzymes. To overcome these challenges, various viral vectors have been developed and utilized in preclinical settings for CRISPR-Cas9 delivery.

Lentiviral vectors are commonly used, and safety improvements have been achieved by introducing deletions in the promoter-enhancer region within the 3' LTR, resulting in self-inactivating vectors. Another popular choice is AAV vectors, known for their broad serotype specificity, adeptness in targeting both proliferating and non-proliferating cells, non-pathogenic characteristics, and minimal immunogenicity. The most commonly used viral platform for gene therapy is the AAV (see Figure 7), which is highly suitable for several reasons: (i) it is not associated with known diseases and typically triggers only a mild immune response in humans; (ii) similar to integrase-deficient lentiviral vectors, AAV can maintain its genome in episomal forms for extended periods, enabling prolonged expression of transgenes in non-dividing cells and tissues. To overcome the constraint imposed by the functional packaging capacity of AAV, which is approximately 4.7 kb, one strategic approach involves the partitioning of a CRISPR/Cas transgene into two segments.

Subsequently, each segment is independently packaged into separate AAV vectors. These vectors are then co-delivered, and the complete protein is reassembled in vivo using a split intein, a pair of domains that self-excise, reconnecting two peptides in an end-to-end configuration [76]. As an example, adeno-associated virus has been utilized for the targeted transport of reconstituted CRISPR-SaCas9 specifically to the liver employing a liver-specific promoter. Adenoviral vectors, derived from adenoviruses, are a third class of viral vectors with potential for CRISPR-Cas9 delivery. These carriers, while promising, have their limitations, including an increased risk of tumorigenesis and potential immunogenicity. Moreover, incorporating new sequences into viral vectors can be challenging, and the process of scaling up can be intricate and time-intensive. In contrast, non-viral vectors like nanoparticles offer an alternative capable of surmounting the limitations linked to viral-based delivery systems; these alternatives have exhibited notable efficacy in transporting diverse therapeutic agents to designated tissues [58]. Dissemination of CRISPR-Cas antivirals to every HIV-infected cell is a significant obstacle, and researchers have explored methods like lent viral vectors to achieve stable transduction of antivirals in cells[67].

Figure 7: Vectors and nanovectors. Adeno-associated vector (AAV) can be considered as an effective transport vector due to its attributes in inoculation to different cell types. Further research must be done due to its limited packaging (4.7 kb). Source: Prepared by the author.



Limitations

These promising findings indicate that addressing current limitations in facilitating the deployment of the CRISPR/Cas9 molecular toolset while concurrently mitigating the occurrence of unintended off-target genetic modifications may pave the way for a noteworthy paradigm shift in the investigation and management of infectious and autoimmune disorders [55]. Off-target editing refers to instances where CRISPR/Cas9 cleaves DNA at sites unintended by the investigator. One significant challenge in curing HIV-1 is the elevated frequency of genetic mutations, which results in viral diversity. Targeting regions that exhibit a high degree of conservation across various HIV-1 strains is a feasible approach; however, the emergence of escape variants remains a potential

challenge. Off-target activity is another concern, as CRISPR can tolerate variations in the RNA duplex, leading to unintended effects. Within the context of HIV-1, the CRISPR/Cas9 technology has been harnessed to specifically target the LTRs, thereby inducing disruption or elimination of the HIV-1 genome within latently infected cells. While this approach shows promise, it should be noted that CRISPR/Cas9 can induce double-stranded DNA breaks, leading to error-prone repair processes, including mutations and insertions, which necessitates further research to optimize its clinical potential as an HIV cure method [11].

Reducing off-target effects is crucial to avoid mutations in essential genes, tumor suppressor genes, or chromosome translocations. Bioinformatic tools have been developed to design gRNAs and predict their off-target activity, addressing this problem. Overall, CRISPR-Cas9 has shown promise in reducing viral copy numbers and excising proviral genomes in latently infected cells, but challenges related to viral diversity and off-target effects must be carefully considered [26]. Bioinformatic screening is typically used to identify potential off-target sites based on sequence complementarity between the gRNA and DNA. However, off-target cleavage can occur even with mismatches between the gRNA and the target DNA, particularly when the mismatches are distal to the PAM site. The efficiency of off-target cleavage varies depending on the position of the mismatch along the gRNA sequence. Strategies for minimizing off-target effects include refining gRNA design and employing ex vivo detection methods in human primary cells to screen for off-target editing [26]. Challenges include ensuring the gRNA's specificity against various HIV-1 strains, targeting the entire HIV-1 genome, and finding safe delivery methods. Despite these obstacles, CRISPR technology holds promise for eventually eradicating HIV-1 [7]. While targeting HIV genome sequences with CRISPR, it is essential to evaluate off-target effects on host gene sequences. Studies have shown that a small percentage of LTR-targeting gRNAs have potential recognition for the human genome, but most of these complementary sequences are in non-coding DNA or intronic regions. Research has generally failed to demonstrate significant off-target effects

[14]. Challenges in delivering the necessary gene-editing tools to all reservoir cells and the presence of proviruses in inaccessible chromatin structures remain significant obstacles to overcome in the quest for an HIV cure [77].

While the employment of two gRNAs directed towards distinct HIV-1 domains can decelerate viral escape, it is crucial to acknowledge that escape remains plausible, potentially arising from mutations introduced by NHEJ in both targets [17]. The widespread deployment of gene therapy, such as CRISPR/Cas9, in Africa or other regions, would depend on several key factors. The methods used, like autologous transplants involving modification of a patient's CD4+ T cells in the laboratory, may pose logistical challenges. However, in the event of a straightforward and economically viable CRISPR/Cas9 delivery is developed, it could potentially be deployed more widely for HIV treatment [2]. The effective clinical implementation of CRISPR/Cas9 therapy for HIV-1 depends on effective delivery to the target tissues and intracellular compartments hosting latent infections, such as the viral reservoirs and sanctuary tissues.

Recent advancements have illustrated the practicability of in vivo applications of delivery using methods like tail-vein injection of AAV and nano-formulations that can cross the blood-brain barrier [44]. Several strategies using CRISPR/Cas9 have been suggested for the treatment of HIV infection. They can be categorized into two groups: those targeting HIV DNA and those targeting host factors. In the first category, sgRNAs are designed utilizing the viral genome as a template to activate the sgRNA-Cas9 system, inducing targeted attacks on HIV-1 DNA. The second category targets HIV co-receptor genes like CXCR4 and CCR5 [72]. Targeting cellular elements like CCR5 reduces selective pressure but raises the risk of CXCR4 virus emergence [74]. To address the limitations of existing gene-editing approaches, novel methods like Prime Editing and RNA-guided, RNA-targeting CRISPR effectors, such as Cas13a and Cas13d, have emerged, offering promising strategies for targeting HIV-1 at the genetic and RNA levels, potentially advancing HIV/AIDS treatment [57, 78].

DISCUSSION

Efforts to address the complexities of HIV necessitate a multifaceted strategy, with research findings indicating promising paths towards an effective cure. The convergence of genetic editing and gene expression repression techniques offers an innovative approach, with the potential to revolutionize HIV therapeutics by concurrently targeting multiple facets of the viral lifecycle. However, ethical considerations surrounding precision, equitable access, and intergenerational responsibility must be carefully navigated. Personalized approaches tailored to individual patient profiles hold promise in optimizing treatment outcomes, while ongoing refinement of techniques is essential to mitigate unintended consequences.

The contemplation of employing genetic editing within targeted cellular populations signifies a paradigm shift in personalized medicine, offering unprecedented precision in addressing specific medical conditions. Challenges persist in optimizing techniques, ensuring long-term safety, ethics consideration about the genome manipulation and its potential/identified risks of off-target effects, and demonstrating clinical efficacy, yet the potential application of genetic editing to combat HIV exemplifies the transformative power of science and technology.

The results drawn from a range of research articles underscore the dynamic and intricate nature of employing genetic editing and gene expression repression strategies against HIV. Notable outcomes include sustained viral suppression, disruption of integrated proviral DNA sequences, and minimal impact on host genome DNA regions. However, variability in efficacy across studies highlights the complexity inherent in these approaches, underscoring the need for a comprehensive understanding of underlying mechanisms. While subject to limitations, this review contributes to the ongoing dialogue and advancements at the intersection of CRISPR-Cas technology and HIV research.

CONCLUSIONS

Despite the advancements of HAART, HIV-1 remains a formidable global public health challenge, with viral reservoirs hindering a definitive cure. The persistence of these reservoirs, established early in infection, leads to viral resurgence upon treatment cessation.

The review emphasizes the urgent need for scalable strategies capable of eliminating enduring HIV reservoirs. CRISPR-Cas genetic editing, particularly targeting the CCR5 gene, shows promise in countering HIV-1 tenacity. However, challenges such as off-target effects, ethical concerns, and variable efficacies demand meticulous navigation. The integration of genetic editing with gene expression repression mechanisms presents a multifaceted strategy, enhancing therapeutic efficacy and preventing resistance. The recent studies have shown the improvement in the neurological functions after the reduction of HIV-1 expression in cortical brain tissue [79], demonstrating the relevance that the pursuit of a cure currently has.

Advanced delivery modalities, including vectors and nanovectors, enhance precision and safety in genetic editing. Ethical considerations, especially regarding off-target effects and broader implications, underscore the need for a harmonious balance between scientific progress and ethical principles. The long-term benefits of genetic editing and gene expression repression techniques have the potential to reshape HIV-1 therapeutics, promising perpetual viral suppression. Collaboration across institutions and a commitment to knowledge dissemination are crucial. The insights provided by research findings collectively pave the way toward advanced strategies for a viable HIV-1 cure.

Addressing the complexities of HIV-1 requires a comprehensive approach, considering its impact on public health, challenges posed by viral reservoirs, the intricacies of CRISPR-Cas, innovative delivery methods, and the potential of RNA-mediated gene expression repression. These multifaceted solutions, driven by collaborative efforts and innovation, hold the potential to transform HIV-1 therapeutics, advancing patient care and bolstering public health.

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AVAILABILITY OF DATA AND MATERIALS

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DECLARATIONS

Ethics approval and consent to participate
The current manuscript does not involve the use of clinical patient or animal samples.

Conflict of interest statement

The author asserts that the research was conducted without any discernible commercial or financial affiliations that might be interpreted as potential conflicts of interest.

Consent for publication

The authors declare their authorization for the publication of this article.

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