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Antiretrovirals to CCR5 CRISPR/Cas9 gene editing - a paradigm shift chasing an HIV cure

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Abstract

The evolution of drug-resistant viral strains and anatomical and cellular reservoirs of HIV pose significant clinical challenges to antiretroviral therapy. CCR5 is a coreceptor critical for HIV host cell fusion, and a homozygous 32-bp gene deletion (Δ32) leads to its loss of function. Interestingly, an allogeneic HSCT from an HIV-negative Δ32 donor to an HIV-1-infected recipient demonstrated a curative approach by rendering the recipient's blood cells resistant to viral entry. *Ex vivo* gene editing tools, such as CRISPR/Cas9, hold tremendous promise in generating allogeneic HSC grafts that can potentially replace allogeneic Δ32 HSCTs. Here, we review antiretroviral therapeutic challenges, clinical successes, and failures of allogeneic and allogeneic Δ32 HSCTs, and newer exciting developments within CCR5 editing using CRISPR/Cas9 in the search to cure HIV.

Keywords

CCR5; Δ32; HIV; CRISPR/Cas9; antiretroviral therapy

1. Introduction

Human immunodeficiency virus (HIV) infection remains a significant global public health threat responsible for tens of millions of deaths [1]. Currently, the treatment of HIV includes oral and extended-release injectable drugs [2]. Since its inception, antiretroviral therapy (ART) has significantly reduced morbidity, mortality, and the global burden of the virus [1]. ART, started within the first six months after infection, reduces viral load to undetectable levels, improves CD4 T cell counts, inhibits HIV transmission, and significantly improves a patient's quality of life [3].

Forty-six FDA-approved antiretroviral (ARV) drugs administered in efficacious combinations target viral proteins and are effective [2]. ARVs are not very accessible in

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Declaration of Competing Interest

None.

resource-limited areas and, unfortunately, have inadequate reach to nations needing them most [4]. ARVs are generally not well-tolerated and are at constant risk of obsolescence due to the emergence of drug-resistant strains [5]. Most ARVs have a limited capacity to penetrate the blood-brain barrier (BBB); thus, their access to the CNS remains a significant ongoing challenge to treating HIV sequestered in several immune and drug-privileged sites [6]. Not surprisingly, a complete or even functional HIV cure via ART is currently impossible.

HIV infects different compartments of the host immune system. In the circulation, for example, where the virus is most infectious, HIV replicates fastest in proliferating CD4+ T cells [7]. In contrast, HIV usually remains latent in resting CD4+ T and phagocytic cells in dispersed tissues and organ systems [8]. Consequently, latently infected CD4+ cells act as sanctuaries for the integrated provirus to hide undetected for years or even decades before emergence [9].

HIV infection begins with the interaction of the viral surface protein, gp120, with a CD4 molecule expressed on several types of immune cells [10]. This interaction critically requires C-C Chemokine Receptor Type 5 (CCR5) coreceptor engagement, allowing membrane fusion of the HIV particle to the CD4+ host cell [11]. Intriguingly, a genetic mutation identified in a small set of individuals of European descent leads to a functional loss of CCR5 activity, resulting in significant HIV-1 resistance [12]. Individuals with the Δ32/Δ32 homozygous mutation (Δ32) of their *CCR5* genes are resistant to HIV-1 [13], and stem cell transplantation (SCT) from Δ32 healthy donors to HIV patients leads to presumed cures. To date, allogeneic hematopoietic stem cell transplantation (HSCT) from Δ32 donors has provided the only curative therapy for HIV [14–18].

Here, we will discuss HIV infection and treatment challenges, the *CCR5* gene and its Δ32 mutation, allogeneic Δ32 stem cell transplants, clinical successes, and potential clustered regularly interspaced short palindromic repeats (CRISPR)-based methods to reproduce this mutation in individuals living with HIV.

2. Infection

HIV is an infectious retrovirus that contains two copies of positive-sense single-stranded (ss) RNA. Encoded and equipped with many critical essential and regulatory enzymes, HIV exploits the host's cellular machinery to replicate itself, leaving infected CD4+ cells metabolically exhausted, after which infected cells scavenge their membranes to bud new virions and die [19].

HIV can be transmitted from infected individuals through sexual contact, during pregnancy or breastfeeding, or through exposure to infected materials, including blood products and contaminated needles [20]. Once HIV enters the individual, the virus infects CD4+ cells. This primary infection is associated with a fever and other common flu-like symptoms, which quickly resolve, and can be difficult to distinguish from a superficial influenza infection [21]. After this, the virus starts infecting other CD4+ cells, and subsequent symptoms appear, including skin rashes, fatigue, lymphadenopathy, sore throat, repeated

episodes of fever, and infection with opportunistic pathogens [21]. If left untreated, HIV leads to a collapse in CD4+ T cell numbers, with the host immune system deteriorating to a point where multiple infections or HIV-specific cancers arise, overwhelming the host, terminating in the virus's terminal end-stage, acquired immunodeficiency syndrome (AIDS) [21].

3. Treatment

ARVs are anti-HIV drugs administered in a combination that inhibits structural or functional targets critical for viral pathogenesis. ART is the only cost-effective treatment for HIV and is the current standard of care [22]. Approved ARVs have distinct targets, including viral reverse transcriptase (Non- Nucleoside Reverse Transcriptase Inhibitors and Nucleoside Reverse Transcriptase Inhibitors) [23], HIV protease (Protease Inhibitors) [24], HIV fusion (Fusion inhibitors) [25], CCR5 (CCR5 antagonists) [26], HIV integrase (Integrase Strand Transfer Inhibitor and Integrase inhibitors) [27], viral gp120 protein (Attachment inhibitors) [28] and host CD4 receptor (post-attachment inhibitors) [29] (Table 1). Other pharmacokinetic enhancers, such as ritonavir and cobicistat, can increase the effectiveness of some ARVs in an anti-HIV regimen [30].

HIV treatment relies on combinations of ARVs given for life. After testing positive for the virus, the treatment protocol is to start first-line ART immediately, which consists of two nucleoside reverse transcriptase inhibitors combined with a third ARV from one of three other classes, such as a protease inhibitor, an integrase strand transfer inhibitor, or a non-nucleoside reverse transcriptase inhibitor [22]. If the patient is non-compliant and/or the initial drugs are no longer effective, then second-line ART is considered [22]. Alternative ART commences if viral load shows no further improvement with second-line drugs, usually due to cross-drug resistance. ART administration is not benign and can lead to chronic adverse effects and toxicities with associated comorbidities [31]. ART administration can also induce lactic acidosis associated with fatigue, pulmonary complications, altered liver function, circulation complications, heart palpitations, and weight loss [32]. Patients on ART commonly complain of chronic nausea, vomiting, and abdominal discomfort.

3.1. Drug resistance

ARVs were initially designed to target HIV structures or viral enzymes, hoping that effective agents would be curative. However, viral drug resistance and other escape mechanisms challenged the continuing efficacy of ARVs. Retroviruses like HIV use reverse transcriptase enzymes and the infected host cell transcriptional machinery to synthesize cDNA based on their genomic (g) RNA template. HIV reverse transcriptase has limited proofreading activity and a reported error rate of 1/1,700 bp's [33], an exceptionally high error rate, even for a retrovirus. Thus, each time the virus synthesizes new DNA strands during replication, mistakes go uncorrected and are incorporated into its nucleic acid, producing new gRNA variant copies and, consequently, new virion variants [34]. The error-prone process usually leads to replicative incompetent virions or, less often and more importantly, allows the virus to escape the effect of the original ARVs. Conventional ARVs target native forms of HIV

enzymes, but newly emerging virion variants produce nonnative enzymes, and often ARVs may no longer be effective [35].

For this reason, individuals on long-term ART (~10 years) may suddenly show high viral loads despite being ART compliant and may need to switch to second or alternative-line drugs to manage the disease. Not surprisingly, ART non-compliance, for whatever reason, hastens this process by giving the virus time to replicate, mutate, and develop resistance [36]. Nevertheless, ART effectively suppresses viral load, improves CD4+ T cell numbers, dramatically reduces viral transmission, improves patient quality of life, and delays symptomatic disease, but it is not curative.

3.2. Inaccessible anatomical sites

Inaccessible HIV reservoirs in immune- and drug-privileged sites can act as sanctuaries for the provirus and pose ongoing clinical challenges to ART. For example, HIV enters the CNS early after infection, and in almost half of ART-treated patients, progresses to HIV-1-associated neurologic disorders (HAND) [37]. HAND is associated with dementia, cognitive motor disorders, and neurocognitive disorders, which can become severe and debilitating over time. For a cure, the principal HIV-infected cell subsets in the CNS that harbor the provirus must be eliminated, including CD4+ macrophages, CD4+ microglia, CD4+ T cells, and perhaps CD4− astrocytes [38]. HIV can infect resident CNS cells by cell-free (cis) mechanisms and direct cell-to-cell (trans) transmission [39–41]. HIV-infected cells, such as monocytes and CD4+ T cells, can transit from the bloodstream across the BBB and into the CNS, directly infecting microglia [41]. Astrocytes are presumed infected by a CD4-independent cell-to-cell contact mechanism in which immature HIV virions bud off lymphocytes, binding directly to CXCR4 and fusing with the astrocyte membrane [42].

Most ARVs cannot penetrate the BBB. However, some ARVs, such as non-nucleoside reverse transcriptase inhibitors, including efavirenz, etravirine, rilpivirine, and nevirapine, can disrupt BBB integrity and enter the CNS, leading to potentially deleterious consequences and increasing the likelihood and severity of a life-threatening stroke [43]. Newer methods to facilitate the bioavailability of other ARVs to the CNS can be envisioned, such as nasal delivery directly to the brain; however, these strategies need careful and thorough assessment [44].

The testes, fetus, and placenta are other immune-privileged sites most ARVs cannot reach [45]. Fortunately, mothers can dramatically lessen the risk of fetal HIV transmission when on ART due to very low or undetectable viral loads [46].

3.3. Reservoir cells

HIV preferentially binds CD4 receptors but can bind to other receptors, including DC-SIGN, Syndecan-3, and Siglec-1, to acquire cell membrane access [47–49]. Once within the cell, HIV integrase, a critical pathogenic enzyme, inserts the newly generated HIV dsDNA into the host genome [50]. The virus resides in resting and productive target cells, resulting in latent and active HIV-infected cells. Viral antigens, such as gp160, are not expressed on latently infected cells, which are immunologically inert and quiescent, with the provirus

remaining transcriptionally silent for years or decades [8]. However, virus replication can resume once the cognate antigen activates the host T cell.

It is still unclear how HIV infects so many different types of cells, but CCR5, expressed by many immune and epithelial cells, appears as a central receptor facilitating host cell-viral fusion [51]. In addition, cell-to-cell viral transmission by mechanisms including efferocytosis [52], tunneling nanotubes [53], virus-containing compartments (VCC) [54], cell-cell fusion [55], and virological synapses [56] are also responsible for expanding the pool of target cells. Monocytes and macrophages also display CD4 receptors and likely play an essential role in generating additional proviral latent reservoirs. Once in local tissues, infected peripheral monocytes become infected resident macrophages (monocyte-derived macrophages), which act as a long-term reservoir of HIV [57]. Translation induces intracellular immunologically inert VCC formation when these monocytes transcribe viral genes such as *gag*, which encodes the viral core's structural components. Each VCC contains numerous copies of HIV virions bound to the tetherin/BST-2 protein sheltered in monocytes [58]. During immunological synapse formation, viral transmission occurs by coreceptor and adhesion molecule engagement on the surface of infected antigen-presenting cells, infected CD4+ T cells, productively infected cells, or uninfected target cells [56]. Finally, CD14+CD11c+ monocyte-derived dendritic cells and langerin-expressing conventional dendritic cells can present HIV particles to CD4+ cells following vaginal or anal sex, leading to transmission [59].

HIV transmission primarily occurs by exchanging infected blood or bodily fluids harboring latently infected CD4+ T cells or infectious virus [8]. The virus then spreads to different compartments, including the gastrointestinal tract-associated lymphoid tissues, CNS, spleen, liver, lungs, and kidneys. HIV particles at these sites can be genetically distinct from those in the peripheral circulation. Surprisingly, local CD4+ T cell subsets are frequently unlike those observed in the peripheral circulation concerning phenotype and function. The stability of the CD4+ T cell reservoir is unique to each infected cell that resides in a unique environment of regulatory cytokines and chemokines [60]. In a constant back-and-forth battle, immune cells mount proinflammatory responses against HIV by cytokine and chemokine secretion. However, HIV hijacks many of these cytokines to establish and maintain latent reservoirs [61]. Many of these cytokines regulate HIV replication and innate and adaptive responses. For example, TNF-α, IL-2, and IL-6 break latency in resting CD4+ cells, promoting proviral emergence, and IL-18 boosts viral replication.

As the disease progresses from acute to chronic, a decline in healthy immune cells occurs, and the immune response shifts from attack to defense. In this phase, immunosuppressive cytokines, including IL-10 and TGF-β, are secreted, reducing T helper (Th)1 type responses, MHC II expression, and antigen presentation by APCs [62]. IL-10 also upregulates programmed cell death protein (PD)-1, and TGF-β promotes the expression of cytotoxic T lymphocyte antigen (CTLA)-4, leading to T-cell exhaustion and reduced activation [63]. By downregulating T cells, HIV facilitates its latency and promotes reservoir persistence. Additionally, chemokines such as CXCL9, CXCL10, CCL19, CCL20, and CCL21[64], and cytokines such as IL-2, IL-4, IL-7, and IL-15, enable resting CD4+ T cells to become infected [65]. Interestingly, IL-7 activates the AKT/FOXO3a pathway, reducing the

expression of proapoptotic FasL and Bim, promoting memory T cell survival, and stabilizing the latent reservoir [66]. Thus, the latent reservoir of HIV-infected cells remains constant by the homeostatic and cognate antigen-stimulated proliferation of infected cells.

4. CCR5 – gene, protein, and the Δ32 mutation

At position 21 on the short arm of chromosome 3, the human *CCR5* gene has two or three exons that encode its three transcript variants. The sense strand has two upstream promoters, offering potential binding sites for several interferon-stimulated response elements, κB factors, and cAMP-response elements, providing a binding platform for interferon regulatory elements, NF-κB, and the common activator of transcription CREB-1, respectively [67]. The *CCR5* antisense strand (*CCR5AS*) encodes long non-coding RNA sequences, further regulating *CCR5* expression. Single nucleotide polymorphisms associated with one *CCR5AS* allelomorph increase CCR5 expression. These polymorphisms increase viral load, promote latency, and decrease CD4 T cell counts, accelerating disease progression, and can play a negative role in disease outcomes [68,69].

The *CCR5* gene encodes C-C chemokine receptor 5, a beta chemokine receptor structurally similar to G protein-coupled receptors (GPCRs) [70]. This protein is expressed in many cells, including T cells, monocytes, macrophages, natural killer cells, microglia, astrocytes, and dendritic cells. It is a critical coreceptor for macrophage-tropic HIV strains [71]. Like other GPCRs, CCR5 has one extracellular N-terminal domain (NTD), three extracellular (ECL1–3) loop segments, seven transmembrane hydrophobic α-helical domains, one cytoplasmic C-terminal domain (CTD), and three intracellular (ICL1–3) loop segments [72] (Fig. 2A). The ligand recognition site comprises a tyrosine-rich motif in the NTD and various conserved amino acids in ECL1 and ECL2 [73]. CCR5 ligands are inflammatory chemokines, including RANTES, MIP-1a, MCP-2, and MIP-1b [74]. Site-directed mutagenesis and molecular docking studies demonstrated that core domains of ligands initially interact with ECL1, ECL2, and the NTD, then bind later to the transmembrane helical bundle of CCR5 via the N-terminus of the ligand [75]. Once activated in its two-step binding process, a conformational change in CCR5 desensitizes PKC/GRK-dependent phosphorylation of the CTD and ICL3 and induces CCR5 internalization. This results in the recruitment of cytotoxic lymphocytes and activation of antigen-specific T cells via secondary signaling pathways, including PI3K/Akt and MAPK/ERK, by G-protein subunit release and engagement of other effector molecules [76]. Once the ligand undocks, CCR5 is recycled back to the surface. HIV gp120 hijacks the exact mechanism of ligand binding and activation of CCR5+ cells. The NTD of CCR5 contains four tyrosine residues; sulfation of two residues appears essential for high-affinity binding to gp120. Gp120 has two sites able to interact with sulfated tyrosine residues and two others able to interact with non-sulfated tyrosine residues [77].

In classical molecular dynamics and site-directed mutagenesis models, four gp120s stabilize CCR5 [78]. Simulated structure studies revealed that gp120 molecules stabilize CCR5 in different conformational states, reorienting ECL2 and ECL3 and allowing access to the transmembrane binding cavity. Gp120 also reshapes this cavity giving rise to distinct positions of transmembrane helices 5, 6, and 7 and ICL3 cytoplasmic ends that

may influence G protein binding regions [78]. To facilitate binding and access to the transmembrane cavity, HIV gp120 also changes conformation after its first interaction with the CD4 receptor. This exposes the hypervariable V3 loop, which has a high affinity for ECL2 and NTD of CCR5 (Fig. 2B).

The Δ32 *CCR5* mutation is a 32-bp deletion resulting in a frameshift mutation with a premature stop codon leading to a truncated form of the protein [79]. The mutant protein remains cytosolic, unable to translocate to the cell surface. Moreover, the specific amino acids 298–329 in the hypervariable V3 loop of HIV gp120 that determine cellular tropism bind with the 2D7 epitope in the ECL2 of CCR5 [80]. This truncated form of CCR5 structurally lacks the 2D7 loop since the frameshift occurs before 2D7 in the open reading frame, making the cytosol bound truncated CCR5 protein inaccessible to the free virus.

Predominantly found in Europe and Western Asia, the Δ32 genotypes resulted from positive natural selection of the Δ32/Δ32 alleles [81,82]. The Δ32 mutation evolved approximately 700 years ago and, amid the smallpox endemics of the eighteenth century, which exerted selective pressure on European and Western Asian populations, increasing the penetrance of the allele [83]. Roughly 10% of Europeans retain paired missense mutations C101X and FS299 or C20S and C178R, responsible for the Δ32 mutation [84]. Interestingly, the Δ32 *CCR5* genotype appears protective against several other viruses, including smallpox [85] and SARS-CoV-2 [86], and CCR5 blockade inhibited Dengue and Zika virus infection [87,88]. Similarly, genetic polymorphisms of CCR5 ligands are involved in protection against *Trypanosoma cruzi* infection [89]. Even more surprisingly, individuals with Δ32 mutations show improved neuronal plasticity and faster neuronal recovery following a stroke or traumatic brain injury, indicating a significant role for the immune response in these presumed immune-independent conditions [90]. The Δ32 mutation can also have negative consequences, including increased susceptibility to the West Nile virus [91], increased risk of death from the influenza virus [92], and detrimental bone development due to defective osteoclastogenesis and osteoclast communication [93].

5. Δ32 *CCR5* as a functional HIV cure?

Allogeneic HSCTs carrying the Δ32 mutation have cured five patients to date. Initially, two male HIV+ Caucasians, the ‘Berlin’ and ‘London’ patients, were cured of HIV-1 following allogeneic HSCT from Δ32 *CCR5* donors (Fig. 1) [14,15]. Both patients had been on ART for many years and developed HIV-associated hematologic malignancies. The HSCT replaced recipient immune cells damaged during chemotherapy, HIV-1 infection, and ART with healthy donor immune cells containing the Δ32 homozygous mutations in CCR5, allowing the recipients to reconstitute their immune cells with CCR5-deficient cells. Homozygous Δ32 donors were explicitly selected so that following immune reconstitution, the new immune cells would be highly resistant to HIV-1 infection. Thus, patients could stop ART following immune reconstitution. Findings from distinct HIV-1 reservoir sites in these patients, such as plasma, semen, and cerebrospinal fluid, revealed undetectable levels of HIV-1 RNA, intact proviral DNA, viral packaging signals ψ, or envelope [14,15]. The treatment, however, was rather intense, requiring chemo- or radiotherapy to ablate existing

bone marrow cells and immunosuppressive drug administration to control graft versus host disease (GVHD). Both patients were declared free of HIV-1.

The ‘City of Hope’ patient was the third to be cured. This Caucasian male had lived the longest with the virus before the transplant [16]. He was an ART-compliant patient who presented with acute myeloid leukemia (AML) and received an allogeneic HSCT from a Δ32 *CCR5* donor treating both AML and HIV-1. This patient subsequently stopped ART, and clinical findings at reservoir sites were negative. He has remained in dual HIV-1 and AML remission since 2019.

A 53-year-old Caucasian male, also known as the ‘Düsseldorf patient,’ is the fourth patient to be cured of HIV-1 following allogeneic HSCT from a Δ32 *CCR5* donor [17]. Like the City of Hope patient, this individual also developed AML and underwent HSCT to treat both conditions. However, his AML relapsed after four months, forcing the patient to continue ART. Five years later, in 2018, the patient stopped ART and was assessed for viral load. Residual, non-replicative HIV-RNA and HIV-DNA were detected, yet the patient remains HIV-1-negative.

A fifth case in New York has generated high hopes of developing an HIV cure using a haplo-cord transplant method [18]. This female patient from a mixed genetic background marked a significant step toward an HIV cure.

Haplo-cord transplants involve cord blood SC transfusions followed by adult bone marrow SC transfusions [94]. Cord blood transplants generally contain few SCs and often require serial transplants to achieve a reconstituting dose [95]. Double cord transplants are rarely obtained from individual donors; unfortunately, single cord blood transplants face significant graft failure and a high incidence of infection [95, 96]. Therefore, in haplo-cord transplants, cord blood SCs are accompanied by mismatched (haploidentical) healthy adult marrow SCs, supporting the recipient against diseases [94]. For a patient of African or mixed ancestry like this New York patient, finding a Δ32-matched donor is challenging because, to date, the Δ32 screening registries have only reported Caucasians to be Δ32 homozygous; however, most HIV-infected individuals are of African ancestry. Δ32 pre-screened cord blood banks hold promise for providing hematopoietic Δ32 SCs. In the New York patient’s case, Δ32 cord blood SCs and wildtype adult SCs from a close relative with a partial HLA mismatch were administered. Thus, Δ32 SCs from the cord blood donor could adapt more readily to the new host with the aid of adult SCs. This approach could hold significant promise for allogeneic Δ32 transplants in non-Caucasian populations.

6. R5 and X4 viral tropism

Synthetic CCR5 based cell or gene therapy cannot offer a complete cure for HIV-1, as this therapy does not treat infections caused by CXC chemokine receptor 4 (CXCR4) - tropic (X4) HIV-1 virus. Host cell surface expression of the HIV entry receptor complex composed of CD4 plus CXC chemokine receptor 4 (CXCR4) or CCR5 determines viral tropism [97]. Other chemokine receptors on non-CD4+ target cells, including CCR3 and CCR8, can also aid in viral fusion and expand the pool of infectable cells [98]. R5-tropic viruses bind CCR5

for host cell fusion and are usually present in the early stages of HIV infection. In contrast, X4-tropic strains, which utilize CXCR4 to promote host cell fusion, begin to dominate during later infection stages [99]. Intriguingly, the normal progression of HIV infection begins with R5 tropism and then switches to an X4 tropic virus. Strains that can bind both CCR5 and CXCR4 for host cell fusion characterize intermediate pathogenic stages, in which HIV appears to prefer CXCR4 receptors on naïve T cells and CCR5 receptors on memory T cells [100]. Surprisingly, this R5 to X4 phenotypic switch has not yet occurred in the five clinical Δ32 *CCR5* HSCT cases cured of HIV-1 discussed above, suggesting that CXCR4 tropic viruses were absent or, if present, could not infect donor CCR5-deficient cells [14–18].

It is important to understand the mechanism of phenotypic switching during disease progression. In normal individuals, memory T cells divide nearly ten times as frequently as naïve cells [101]. Therefore, preferential R5 tropism for memory T cells in early infection can augment viral expansion [102]. As the disease progresses, the frequency of naïve and memory T cell division equalizes, allowing viral strains that can exploit both chemokine receptors to dominate. In the latter stages of HIV infection, viral load increases and CD4+ counts drop; consequently, naïve T cell division frequency exceeds that of the remaining memory T cells, and X4 tropic viruses predominate [103].

Notably, genetic ablation of CCR5 is a naturally validated approach to cure HIV-1. However, ablation of CXCR4 did not support a viable treatment option for X4 tropic HIV-1 in a preclinical setting 104]. Nevertheless, X4- (NL4–3) and R5- (YU-2) tropic strains failed to infect *CCR5* and *CXCR4* ablated CD4+ T cells *in vitro,* and this dual ablation was suggested as a prophylactic strategy in developing nascent cell-based therapeutics for curing HIV-1[105].

7. *CCR5* gene editing using CRISPR/Cas9

CRISPR-Cas is an adaptive immune mechanism targeting foreign genetic elements in bacteria and archaea [106]. This intra- or extra-chromosomal genetic system deploys nucleases, such as Cas, followed by a leader sequence and a CRISPR array composed of short, direct repeats stacked with foreign DNA sequences called spacers. CRISPR-Cas systems are highly diverse and divided into many subtypes. The most frequently used gene editing CRISPR system is adapted from *Streptococcus pyogenes*. This type II system uses the Cas9 endonuclease and crRNA (guide), and tracrRNA (scaffold) to perform dsDNA nicks in an RNA-guided manner [107]. Cas9 comprises six domains: Rec I, Rec II, Bridge Helix, HNH, RuvC, and protospacer adjacent nucleotide motif (PAM) interacting. Synthetically designed single guide-RNA (sg-RNA) binds to the Rec I domain, activating Cas9 to interrogate the host DNA for a specific PAM signature. The PAM sequence binds the Cas9-sgRNA complex allowing the sg-RNA to form a heteroduplex with the target DNA. This heteroduplex then activates the endonuclease domains HNH and RuvC, breaking the phosphodiester bonds between the third and fourth nucleotides upstream of the PAM. CRISPR-based targeting exploits two major active DNA repair pathways to facilitate gene editing: HDR (homology-directed repair) for applications such as abnormal gene replacement or wild-type gene insertions and NHEJ (Non-homologous end joining)

for therapeutic deletions [108,109]. However, other DNA repair pathways, such as HITI (homology-independent targeted integration) [110], BER (base excision repair), and PE (prime editing), were artificially developed for more precise CRISPR editing applications (Fig. 3) [111,112].

CRISPR/Cas9 has demonstrated superior outcomes in the last two decades compared to other gene editing approaches, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), small interfering RNAs (shRNA/siRNA), peptide-nucleic acids, and ribozymes, in reproducing the naturally occurring Δ32 *CCR5* mutation [113]. *CCR5* gene editing using CRISPR-Cas9 shows 4.8 times more efficiency than TALENs [114] and is associated with increased biallelic dsDNA breaks at the *CCR5* locus [115]. Indeed, multiple reports show higher efficiency, specificity, and lower cytotoxicity via CRISPR/Cas9-mediated *CCR5* gene editing [114,116].

In vivo, off-target Cas9 activity raises important questions about the utility of applying this approach without further technique modifications [117]. Indeed, wild-type SpCas9-generates dsDNA breaks, leading to unwanted risks by increasing the frequency of insertions and deletions (indels). Consequently, Cas modifications are underway to enhance cleavage, specificity, and PAM recognition [118,119]. AsCpf1, a Cas12a endonuclease recognizing 'TTTN' PAMs, demonstrated enhanced specificity and superior efficiency compared to Cas9, with no off-target events [120]. A high-fidelity Cas9 shows promise for delivering ribonucleoprotein complexes (RNPs) with no off-target events in editing *CCR5* [121]. Engineering gRNAs can increase target specificity and Cas9 activity [122,123]. Assays such as ZeoR and qEva-CRISPR can effectively test for on and off-target activity of Cas9s [124,125]. Nevertheless, preclinical *ex vivo* data suggest that cell editing by CRISPR circumvents many of the recognized cell-associated risks of previous techniques, off-target events, immune-reactivation, and delivery-associated issues [126].

Biological delivery of CRISPR/Cas9 components relies on several methods, including lentiviral, adenoviral, adeno-associated viral (AAV), non-viral vectors (plasmids, transposons), all-RNA CRISPR, and RNPs [127]. However, host exposure to these viruses can lead to immunoreactivity, and integrating vectors such as lentivirus allow permanent expression of Cas9, exposing cells to recurrent dsDNA breaks and increasing vulnerability to neoplastic development [128]. Moreover, since lentivirus integrates, it is also possible that these 'random' viral DNA insertions could occur in oncogenes, albeit this risk is minimal [129]. Adenovirus is non-integrating, but transcription of its components may lead to platelet activation and coagulopathic effects [130]. AAVs are less immunogenic; transfection can be complex, and outcomes could be more efficient. Synthetic vectors, including lipids, polymeric, and inorganic particles, are considerably easier to scale from a pharmaceutical perspective. Lipofection, electroporation, and microinjections effectively deliver CRISPR/Cas9 components to target cells; however, these methods must be thoroughly assessed before clinical use. Although RNP delivery to target cells is routinely accomplished by electroporation, newer approaches using transmembrane internalization assisted by membrane filtration (TRIAMF) [131] and induced transduction by osmocytosis and propane betaine (iTOP) [132] offer promising solutions for *ex vivo* manipulation of target cells, particularly HSCs. TRIAMF is a technique that delivers RNPs into HSPCs

by passing RNPs and cells through a filter membrane. The TRIAMF technique retains the multilineage colony-forming capacity and engraftment potential of HSPCs. iTOP exploits micropinocytosis to uptake RNPs using non-detergent sulfobetaines, achieving efficient CRISPR editing in HSCs. iTOP may also be a viable method to scale transfections for clinical use.

Ex vivo CRISPR studies have failed to achieve comparable *CCR5* disruption as CCR5-ablated lymphocytes (Table 2). In clinical studies, Xu and colleagues successfully created and engrafted CRISPR-mediated HSPCs carrying the Δ32 *CCR5* mutation [133]. Although the cells were well-tolerated in patients, infusing 1 billion HSCs did not produce sufficient CD4+ cells with the *CCR5* mutation—only ~5% [134,135]. Post-infusion HSPC activation is presumed as a significant clinical challenge in *ex vivo* cell therapy. Administration of CXCR4 antagonists in mice, monkeys, and humans [136,137] helps release HSPCs from bone-marrow stroma via blocking stromal cell-derived factors, such as SDF-1α, that interact with CXCR4 [82]. Others have reported that endogenous CD26 protease activity is involved in HSC bone marrow homing, the loss of which resulted in HSC peripheral blood mobilization [82]. Finally, cytokines such as type II IFNs promote differentiation of myeloid-derived HSCs [138], and mesenchymal stem cells can secrete angiopoietin-like 5, supporting HSC expansion in NSG mice [139].

Nevertheless, autologous transplantation of CCR5 edited cells shows limited capacity to clear the HIV latent reservoir, providing strong evidence that an allogeneic immune response is also likely required to clear viral reservoirs in ART-suppressed animals [140]. Indeed, following HSCT, latent virus was cleared which was associated with an increase in donor chimerism leading to a complete remission within 2.5 years [140]. Based on these data, it appears that CCR5-editing in autologous HSCs may not alone be sufficient to eradicate HIV-1, strongly suggesting that allogeneic immunity in the context of CCR5-based cure approaches need further evaluation. Moreover, development of additional preclinical models to further understand the mechanisms of reservoir clearance is highly warranted [141].

A major limiting factor in treating HIV with HSCT is associated toxicity with conditioning protocols [142]. Conventional conditioning procedures employ high doses of radiation and/or chemotherapy resulting in nonspecific tissue injury, potentially inducing or aggravating GVHD. However, antibody-based conditioning methods, including native antibody, radiolabeled antibody conjugates, and antibody drug conjugates result in tissue specific injury [142], suggesting that milder preparative regimens could be critical in developing better CCR5-based HSCT methods.

8. Conclusion

The introduction of the first single-tablet ARV (azidothymidine) to treat HIV infection almost four decades ago, followed by the development of subsequent ARVs, dramatically reduced HIV-associated morbidity and mortality. Today, ART allows restoration of CD4+ T cell counts with the virus becoming undetectable and non-transmissible, yet treatment interruption results in rapid viral rebound. Drug resistance, long-term ART-associated

comorbid conditions, and a stable HIV latent reservoir necessitate further exploration for new curative approaches.

CCR5, in tandem with the CD4 molecule, interacts with gp120 of HIV, promoting host cell-to-virus fusion. Defective Δ32 alleles of *CCR5*, evolutionarily selected during the smallpox pandemic of the eighteenth century, confer significant resistance to HIV-1. Thus, allogeneic HSCTs from Δ32 donors cured several recipients and showed proof-of-concept for a Δ32-based replacement approach; yet, the Berlin patient died from malignant disease, the London patient suffered graft versus host disease complications, and the other three patients are still under observation. Allogeneic transplants were never a real option for generalized HIV treatment. However, an *ex vivo* intervention based on HSCTs carrying Δ32 designs may still be viable.

Several gene editing approaches, including ZFNs and TALENs, were referenced when designing *ex vivo* modified HIV-1-resistant SC infusions. However, the poor generation of HIV-1-resistant lymphocytes derived from CCR5-modified SCs remains challenging. The New York patient was cured of HIV-1 by donor cord blood SCs carrying Δ32 and wildtype donor adult SCs, findings which hold great promise in the pursuit of a cure. Editing *CCR5* with CRISPR/Cas9 shows improved nuclease efficiency compared to previous methods; however, off-target Cas9 activity, *in vivo* SC activation, and required allogeneic immune responses are impeding progress into the clinic. Targeted conditioning and development of animal models to further understand the mechanisms of HIV reservoir elimination are required to develop a better HSC transplantation program.

CCR5 gene-editing using CRISPR/Cas9 to reproduce the Δ32 mutations certainly requires optimization, thorough *in vitro* testing, and preclinical validation. Simple and incremental advances optimizing CRISPR should allow future clinical studies with *ex vivo* generated allogeneic HSCTs.

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Highlights

- Allogeneic hematopoietic stem cell transplant (HSCT) from Δ32 donors has been established as a cure for HIV; however, the procedure involves life-threatening risks limiting its widespread use.
- CRISPR-Cas9 targeting the CCR5 gene generates autogeneic Δ32 hematopoietic stem cell grafts, potentially replacing allogeneic Δ32 HSCTs.
- This review describes difficulties with antiretroviral therapy, the use of CRISPR/Cas9 to replicate the natural Δ32 mutation, and challenges with *ex vivo* edited hematopoietic stem cells in curing HIV.

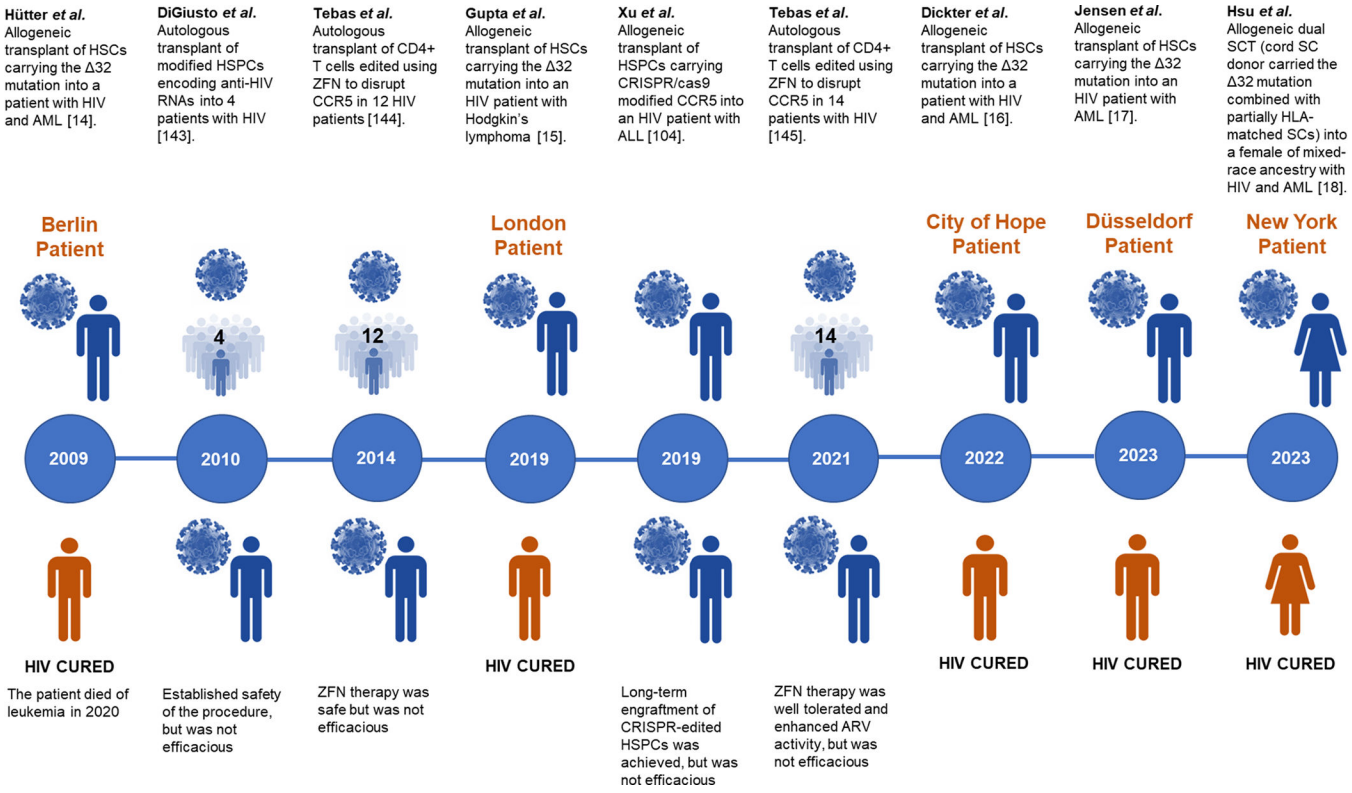
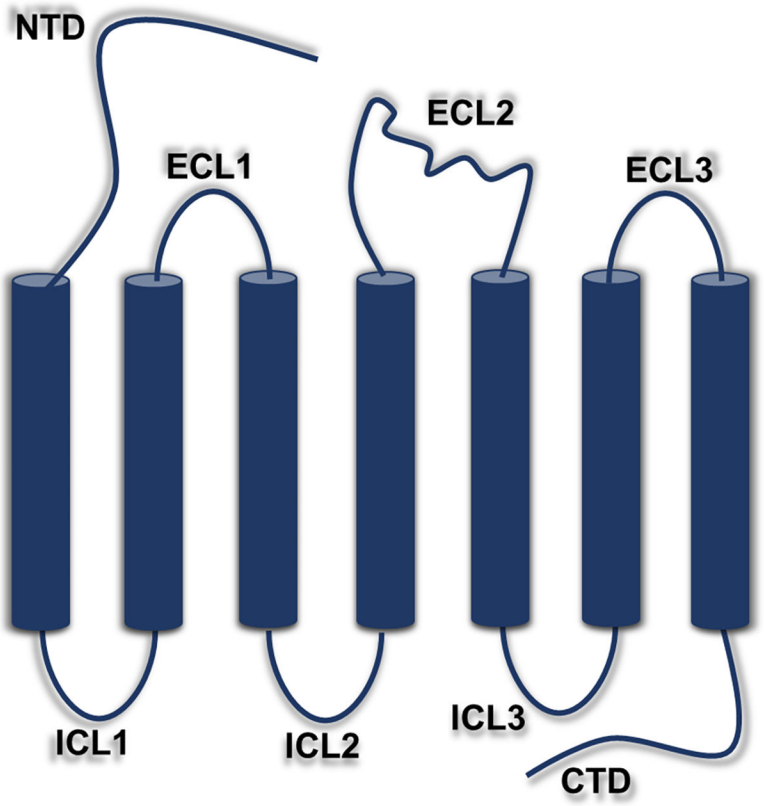
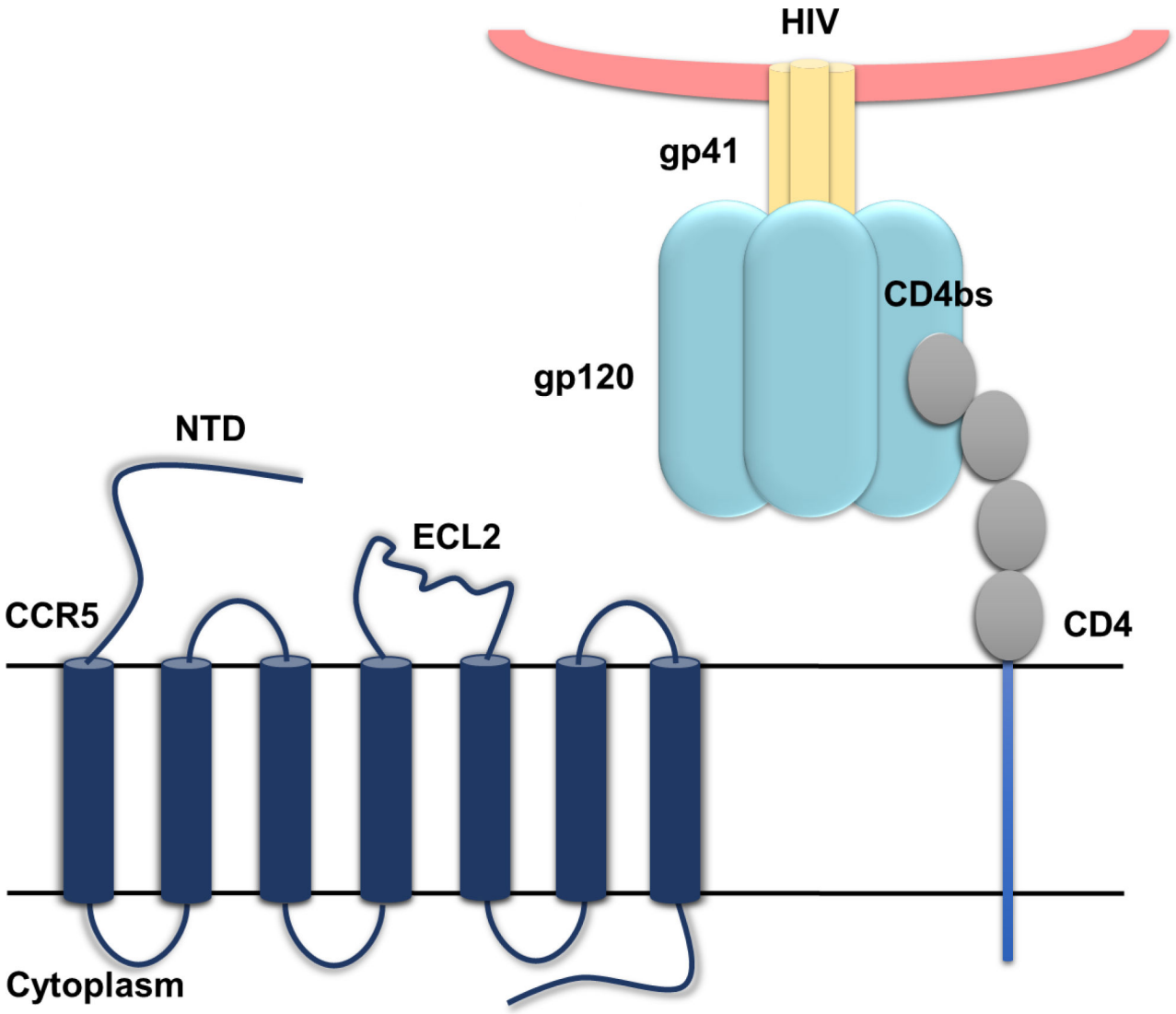


Fig. 1.

Clinical developments in CCR5-based cell replacement therapies.

AML, Acute myeloid leukemia; ALL, Acute lymphocytic leukemia





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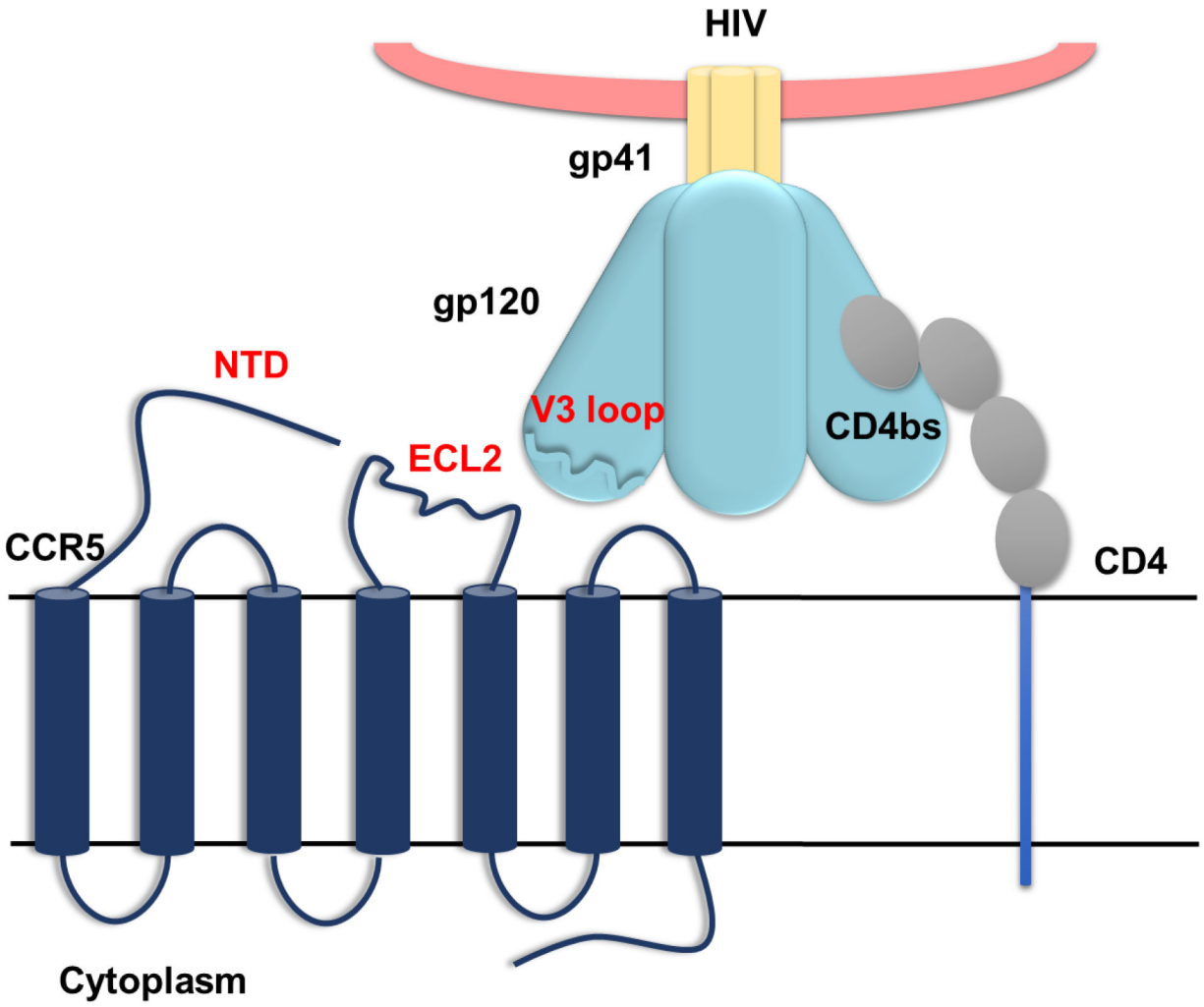

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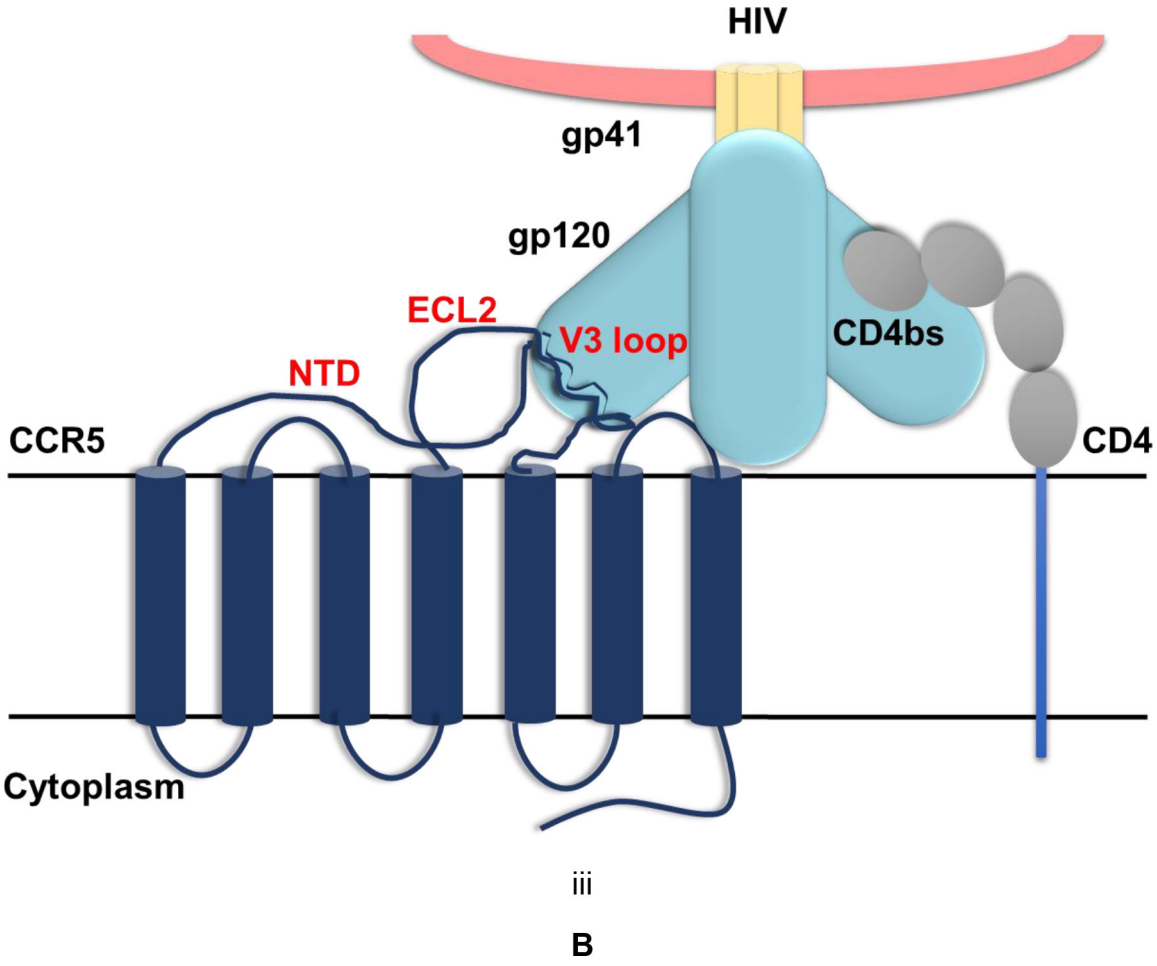
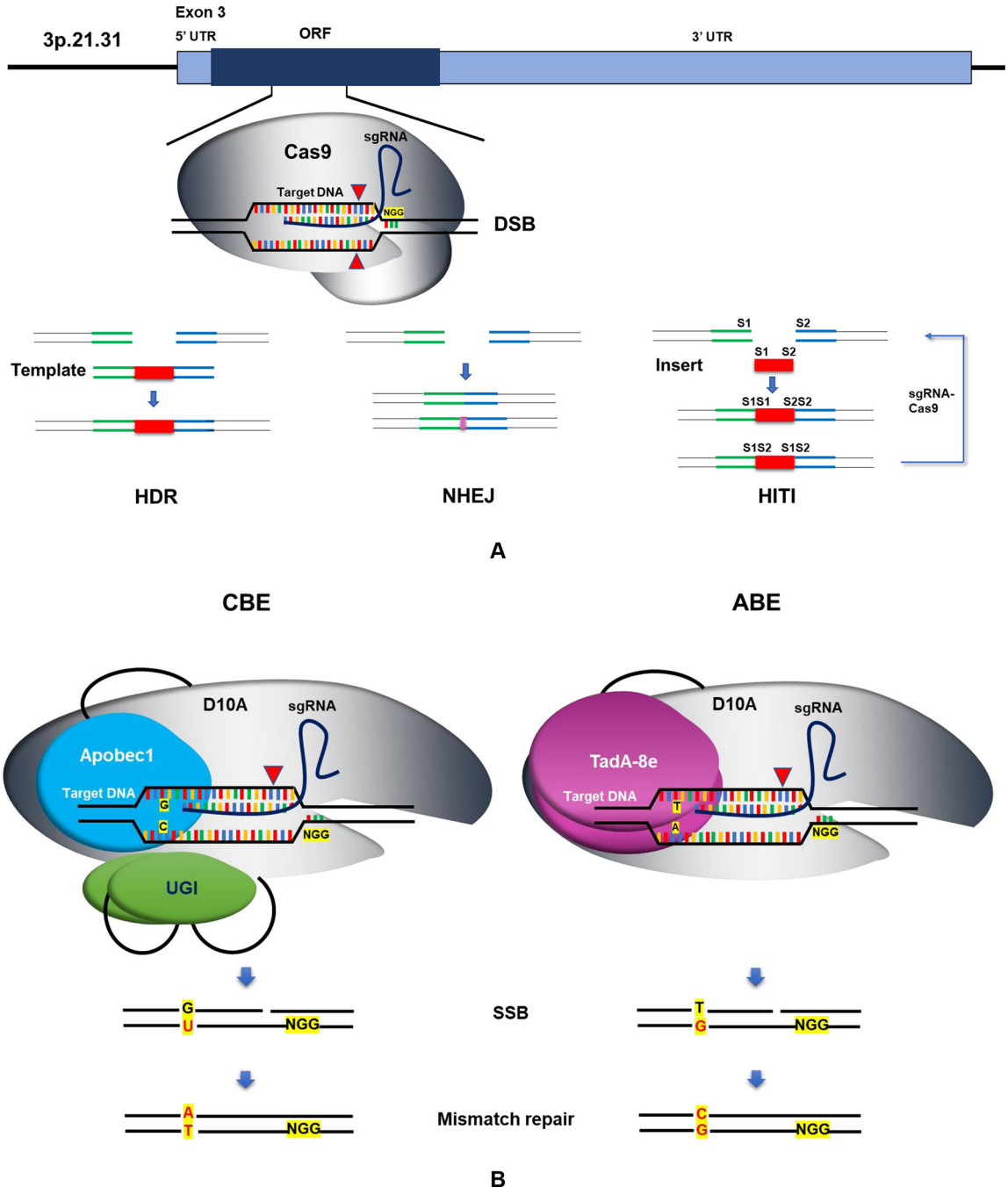


Fig. 2.

CCR5 structure (A); and Stages of CCR5-mediated HIV entry (B).

CD4bs, CD4 binding site.



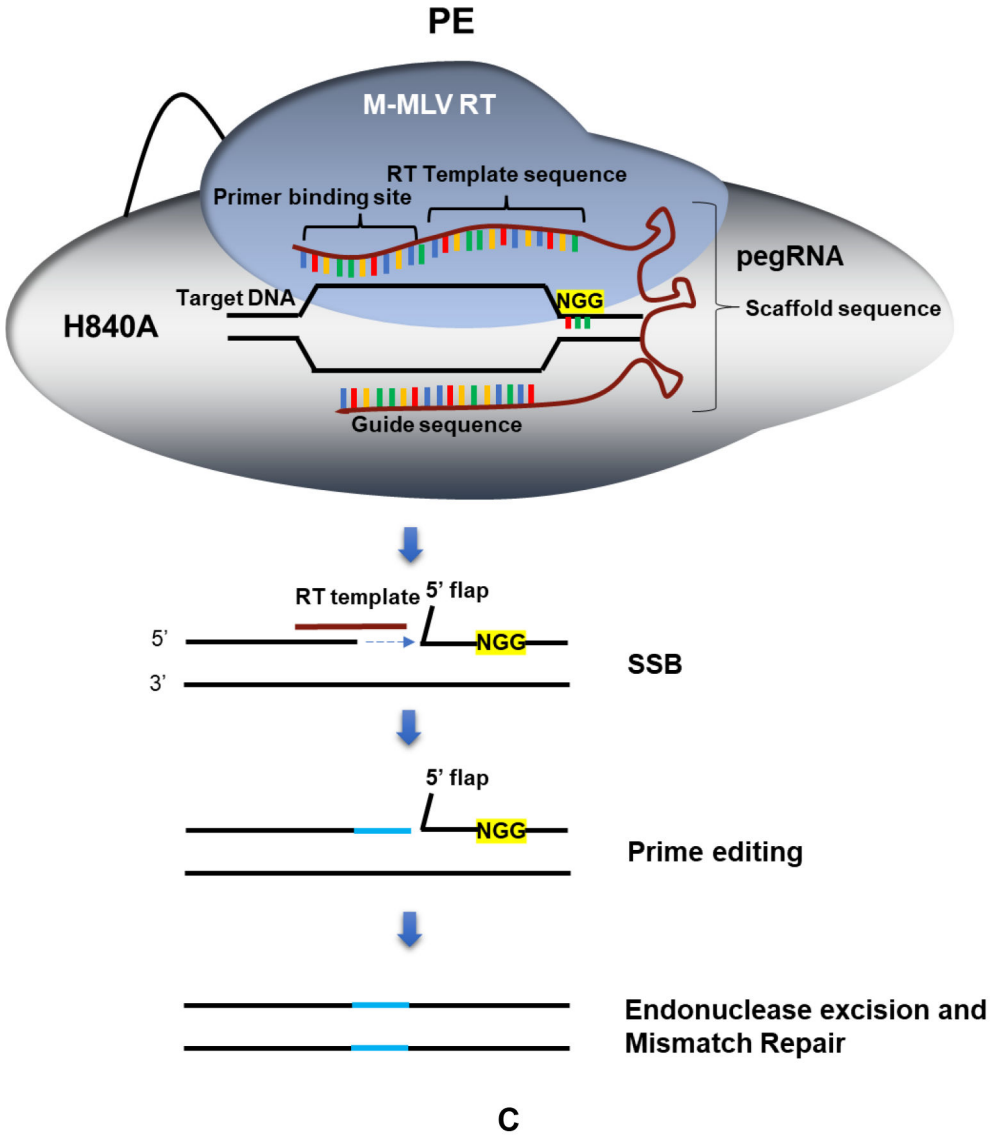


Fig. 3.

The *CCR5* gene is situated at 3p.21.31, and a 32-bp deletion leads to a truncated, non-functional protein. Various CRISPR approaches can artificially reproduce this deletion, including knock-out, knock-in, base-editing, and prime editing.

A. CRISPR-Cas9 editing. Cas9-induced double-stranded DNA breaks (DSBs) initiate intrinsic DNA repair mechanisms, resulting in an edited *CCR5* gene. Primary DSB repair mechanisms include homology-directed repair (HDR) and non-homologous end-joining (NHEJ). HDR, which is accurate and most active in dividing cells, employs an homologous template to mend DSBs. In contrast, NHEJ directly ligates nicked ends in a homology-independent manner, often introducing small insertions and deletions at the joined ends. In non-dividing cells, homology-independent targeted integration (HITI) serves as an assisted endogenous NHEJ method for knock-in applications. The HITI insert cassette contains short target flanking sequences, enabling both the target and insert to share the same sgRNA-Cas9

site. Targeting these sequences produces identical ends (S1 and S2) in genomic DNA and insert cassette. Correct insert orientation eliminates the sgRNA target site, while incorrect orientation recreates the sgRNA target.

B. Base editing. Single-strand DNA breaks (SSBs) yield better results and demonstrate an enhanced insertion and deletion (indel) frequency compared to DSBs. Base editing utilizes single-strand nicking Cas9. The cytosine base editor (CBE) consists of a D10A Cas9 nickase (RuvC mutant) bound to cytidine deaminase (Apobec1) and a uracil glycosylase inhibitor (UGI). Apobec1 converts C to U, while UGI prevents the conversion of U to G. Mismatch repair replaces U with T; subsequently, on the complementary strand, it replaces G with A. Cytidine base editing can transform C-T into T-A and introduce premature stop codons at desired positions within the *CCR5* coding region. The adenine base editor (ABE) converts A to G and comprises D10A bound to adenine deaminase (TadA-8e). TadA-8e converts A to G, and mismatch repair replaces T on the complementary strand with a C. ABE can eliminate the transcription start site by changing the start codon ATG to GTG or ACG.

C. Prime editing. The prime editor (PE) consists of H840A Cas9 nickase (HNH mutant) bound to M-MLV reverse transcriptase (M-MLV RT) and enables rewriting of the target sequence up to 80-bp. PE employs a modified guide RNA called prime editing guide RNA (pegRNA). PegRNA comprises four components: i) guide sequence, ii) scaffold sequence, iii) RT template, and iv) primer binding sequence. H840A nicks the target, creating an open 3’ end for M-MLV RT to initiate DNA synthesis. The newly synthesized sequence replaces the original sequence, resulting in a 5’ flap. Cellular endonucleases remove the 5’ flap, and mismatch repair mechanisms replace the desired sequence on the complementary strand.

Table 1

FDA-approved antiretroviral drugs for HIV.

Drug Class	Mode of action	Drugs
Nucleoside Reverse Transcriptase Inhibitors	Reverse transcriptase blocker	Abacavir *
		Emtricitabine *
		Lamivudine *
		Tenofovir disoproxil fumarate * Tenofovir alafenamide *
		Zidovudine *
Non-Nucleoside Reverse Transcriptase Inhibitors	Reverse transcriptase blocker	Doravirine
		Efavirenz *
		Etravirine
		Nevirapine *
		Rilpivirine
Protease Inhibitors	HIV protease blocker	Atazanavir *
		Darunavir *
		Fosamprenavir
		Ritonavir *
		Lopinavir *
		Tipranavir
HIV capsid inhibitor	Interferes with capsid assembly	Lenacapavir
Fusion Inhibitor	Binds CD4 receptors, blocking fusion	Enfuvirtide
CCR5 Antagonist	Binds CCR5, preventing viral entry	Maraviroc
Integrase Strand Transfer Inhibitors	Binds the integrase-viral DNA complex	Cabotegravir
		Dolutegravir *
		Raltegravir *
		Bictegravir
Attachment Inhibitor	Binds gp120, preventing viral entry	Fostemsavir
Post-Attachment Inhibitor	Binds gp120-CD4 complex, preventing coreceptor engagement	Ibalizumab
Pharmacokinetic Enhancer	Inhibits key liver enzymes that decrease bioavailability of protease inhibitors	Cobicistat
Combination	Multiple targets	Bictegravir Emtricitabine Tenofovir alafenamide

* WHO recommended first and second line regimen drugs.

Table 2

CRISPR-based methods to edit CCR5.

Gene editing tool	Approach	En gra ftm ent type	Target cells	Study outcome
CRISPR-Cas9 knockout	Reproduced Δ32 mutation	*in vitro*	Human CD4+T cells	R5-tropic resistance was conferred to primary cells [146].
CRISPR-Cas9 TALENs	CRISPR and TALENs used to reproduce Δ32 mutation	*in vitro*	iPSCs	CRISPR showed better targeting efficiency than TALENs [115].
CRISPR-Cas9 knockout	Reproduced Δ32 mutation	*in vitro*	iPSCs, human CD4+T cells	Multiple sgRNA pairs generated CCR5 knockouts and resistance to R5-tropic HIV 116,147].
CRISPR-Cas9 knockout	Reproduced Δ32 mutation	*in vitro*	TZM-bl cells	Cas9-sgRNA-edited cells showed few off-target events, and lentiviral delivery was safe [148].
CRISPR-Cas9 RNP technology	Reproduced Δ32 mutation	*in vitro*	Human CD4+T cells	RNP-mediated delivery was safe, disrupted CCR5, and provided R5-tropic resistance [149].
CRISPR-Cas9 knockout	Reproduced Δ32 mutation	*in vitro*	A549, HeLa, primary human skeletal myoblasts	Δ32 mutation induced resistance to HIV [150].
CRISPR-Cas9 HDR	Reproduced Δ32 mutation	humice	HSPCs	Δ32 HSPCs conferred HIV resistance [134].
CRISPR-Cas9 knockout	Flanking regions of the Δ32 locus were targeted to minimize off-target events	*in vitro*	Human CD4+T cells, Jurkat Cells	Knockout conferred resistance to R5-tropic virus without off-target effects [151].
CRISPR-Cas9-tracrRNA structure modification	sgRNA structure was investigated to improve indel outcomes for safer CCR5 ablation.	*in vitro*	Human CD4+T cells	Modified tracrRNA enhanced CCR5 cleavage and improved indel frequency [123].
CRISPR-SaCas9	*S. aureus*(Sa) and *S. pyogenes*(Sp) Cas9s were compared for CCR5 ablating efficiency	humice	CD4+T cells, HSPCs	SaCas9 showed superior CCR5 cleavage efficiency than SpCas9 [152].
CRISPR-Cas9 NHEJ	Series of sgRNAs were assessed for CCR5 ablating efficiency	*in vitro*	HSCs	CCR5 ablation conferred HIV resistance [153].
CRISPR-Cas9 HDR	Reproduced Δ32 mutation	NH P	HSCs	Autologous CCR5-deficient HSCs provided short-term anti-SIV protection [154].
TALEN and CRISPR-Cas9	PiggyBac-mediated CRISPR to reproduce the Δ32 mutation	*in vitro*	iPSCs	Δ32 macrophages showed resistance to HIV [155].
CRISPR-Cas9 knockout	Ablated the CCR5 gene and placed X4 infected cells under ganciclovir-augmented suicidal control	*in vitro*	TZM cells, H7 cells	CCR5 knock-out and Tat-activated conditional X4 inhibition conferred complete resistance to HIV [156].
CRISPR-Cas9 knockout	Deletion of a 198-bp CCR5 fragment from exon 2	*in vitro*	iPSCs from primate T cells and fibroblasts	Knockout cells prevented replication of the CCR5 tropic- SIVmac239 and -SIVmac316 [157].
CRISPR-Cas9 knock-in	Induced a frameshift insertion in the CCR5 gene	*in vitro*	HSPCs, SCs, CD4+ T cells	Knock-ins disrupted CCR5 and conferred HIV resistance [158].
CRISPR-Cas9 knockout	Induced CCR5 and CXCR4 single and dual knockouts	humice	Human CD4+T cells and PBMCs	Single knockouts resulted in HIV resistance, and dual knockouts inhibited CD4+T cells engraftment in BM [159].
Base editing	evoCDA-BE4max converted CAA, CAG, CGA, and TGG to stop codons. ABE, using different PAM-specific nCas9s, showed broader coverage in mutating start codons	*in vitro*	HSPCs, Human CD4+ T cells	CBE and ABE complexes inhibited R5-tropic strains. CBE also inhibited X4 tropic strains [112].

Gene editing tool	Approach	En gra ftm ent type	Target cells	Study outcome
Prime editing	PE3max and uPEn converted C-T, disrupting CCR5	*in vitro*	HEK293T cells	uPEn significantly reduced indel frequencies and ablated CCR5 [120].

TALENs, Transcription activator-like effector nucleases; HDR, Homology-directed repair; hu, humanized; NHP, non-human primate; NHEJ, Nonhomologous end joining; CBE, Cytosine base editor; ABE, Adenine base editor