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# CCR5 and Its Mutation in HIV Infection, and the Therapeutic Approach with CRISPR-Cas9

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ABSTRACT: HIV/AIDS is a major global public health issue. While antiretroviral therapy (ART) is effective for suppressing the viral load, the presence of HIV latent reservoirs causes unavoidable viral rebound and relapse of disease after discontinuation of ART. This paper aims to visualize and analyze the transmembrane structure of the C-C Motif Chemokine Receptor 5 (CCR5), an essential HIV-1 coreceptor, and its mutant protein, CCR5 Δ32, to investigate how structural alterations may influence its function as a coreceptor and provide natural resistance against HIV infection. Additionally, this paper compares the level of conservation of CCR5 amino acid sequences across organisms to understand the significance of the Δ32 mutation. Finally, the paper simulates a CRISPR-Cas9 gene editing process *in silico* and further proposes its hypothetical application to mimic the CCR5 Δ32-like mutation in a patient's hematopoietic stem and progenitor cells (HSPCs), as a potential long-term treatment option to reduce susceptibility to HIV and prevent relapse after discontinuation of treatment. However, despite the potential of CCR5 Δ32 as a treatment for HIV/AIDS, one must also address the challenges faced by the application of gene therapy, regarding safety, ethics, cost, and accessibility.

KEYWORDS: Biomedical Science, Genetics, HIV/AIDS, CCR5, CRISPR-Cas9.

## Introduction

***Introduction to HIV/AIDS:***

Human Immunodeficiency Virus (HIV) is a retrovirus that attacks the immune system, and if left untreated, can lead to Acquired Immunodeficiency Syndrome (AIDS), a condition in which the immune system becomes severely weakened and unable to fight off infections or certain cancers.[1,2] As a retrovirus, HIV primarily infects CD4+ T lymphocytes, or helper T cells, which play a crucial role in immune response.[3] 2 major variants of HIV are: HIV-1 and HIV-2, with HIV-1 being considerably more infectious and responsible for most HIV cases in humans.[4]

HIV is believed to have originated in the 1920s, being passed on from chimpanzees after feeding on them. The first recognized cases of HIV infection were reported in the Democratic Republic of Congo,[5] and since then, the virus has become a global health issue. According to the World Health Organization (WHO), by 2024, HIV will have claimed around 42.3 million lives worldwide, with around 630,000 in 2023 alone. HIV infection consists of 3 different phases: acute infection, chronic asymptomatic infection (clinical latency), and AIDS. During the acute infection phase, flu-like symptoms, such as fever and headache, are often seen. During the chronic asymptomatic phase, individuals may not develop any symptoms of the disease for years;[6] without treatment such as Antiretroviral therapy (ART), the rate of progression to AIDS can vary from a few months to more than 20 years, depending on the individual.[7] A person is said to have AIDS when they are diagnosed with opportunistic infections, or AIDS defining diseases,[6] such as tuberculosis, pneumonia, and oral candidiasis, which often appear with a CD4+ T cell count of less than 200 cells/mm$^3$.[8] If untreated, the weakened immune system cannot fight off opportunistic infections, leading to death.[9]

***Virus Biology:***

HIV-1 infection is initiated when the viral surface glycoprotein gp120 binds to the CD4 receptor on the host cell surface, causing a conformational change that exposes the coreceptor binding site.[10,11] gp120 then engages with a coreceptor; C-C Motif Chemokine Receptor 5 (CCR5) is used by R5 tropic HIV, C-X-C Chemokine Receptor Type 4 (CXCR4) is used by X4 tropic HIV, and dual tropic (R5X4) viruses can use either.[12,13] This interaction triggers further conformational change in the gp120 and transmembrane glycoprotein gp41 complex, allowing gp41 to penetrate the cell surface membrane. After fusion of HIV and the host cell, the viral capsid is released into the cytoplasm[10,11] and transported towards the nucleus. Here, reverse transcription of viral RNA to DNA occurs: The enzyme reverse transcriptase produces a single-stranded DNA from the viral RNA template, then synthesizes the complementary strand to form double-stranded DNA.[9] The viral DNA is integrated into the host cell's genome by integrase, allowing synthesis of more viral RNAs to be used as genetic material for new HIV or as mRNA to produce viral proteins.[14]

Coreceptors used by HIV differ throughout the course of infection. R5 tropic HIV, which uses CCR5, is typically seen during early infection,[15] likely due to the high abundance of CCR5+ cells in this stage.[16] In contrast, X4 tropic viruses are rare during initial infection, and appear later in around half of the patients,[15] possibly due to the accumulating damage on the immune system.[16] Surprisingly, studies have shown that the switch from R5 to X4 tropic virus is not influenced by the abundance of coreceptors. Instead, it has been associated with high



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viral load and low CD4+ counts early in infection.[15] In later stages, some patients develop dual tropic (R5X4) or X4 tropic viruses, which are often associated with faster progression to AIDS and a severe decline of immune response. However, the presence of X4 viruses alone does not necessarily guarantee rapid disease progression, suggesting it could also be due to other virological factors.[16] Overall, these indicate that CCR5 targeted treatment may be most effective in earlier stages of HIV infection, when R5 tropic viruses are more common.

***CCR5:***

The C-C Motif Chemokine Receptor 5 (CCR5) gene is found on chromosome 3, at the cytogenetic location 3p21.3120.[17] CCR5 protein is part of the G-protein-coupled receptor (GPCR) family, and has an overall similar structure to other GPCRs: It has 7 transmembrane regions (TMRs), 3 intracellular loops, and 3 extracellular loops.[18] CCR5 is expressed on the surface of CD4+ T lymphocytes, and individuals with chronic HIV have the highest percentage of CCR5+ CD4+ cells.[19] A naturally occurring mutation, CCR5 Δ32, is a result of the deletion of 32 base pairs, beyond nucleotide 522,[20] in the CCR5 gene. This causes a frameshift, leading to the translation of 7 new amino acids and a premature stop codon. Therefore, the mutant CCR5 protein contains 215 amino acids compared to 352 amino acids in a healthy protein. In this paper, a visualization of the transmembrane structure of the CCR5 protein and its mutant, Δ32, will be used to explore how structural alterations in CCR5 influence its function as a coreceptor.

The CCR5 Δ32 allele is most frequently found in Caucasian populations, less frequently in other North American populations, and is mostly absent in African and Asian populations.[21,22] For heterozygous individuals, the reduced production of wild-type (WT) CCR5 delays their progression to AIDS, especially in the earlier years of infection.[23–25] In addition, the mutant protein exerts a transdominant negative (TDN) effect by forming heterodimers with WT CCR5 and CXCR4, reducing their expression on the cell surface and further reducing susceptibility.[23,26] For homozygous individuals, inhibited expression of WT CCR5 on the cell surface, combined with the TDN effect reducing the expression of CXCR4, leads to high resistance to R5-tropic HIV-1 and partial resistance to X4 and R5X4 tropic HIV-1. However, some homozygous individuals do not always express the Δ32 protein, thus lacking the TDN effect against CXCR4,[23] and are still susceptible to infection by dual tropic or X4 tropic HIV-1 through CXCR4.[27]

***Latent Reservoirs:***

Stable viral reservoirs are established early on in HIV infection within latently infected, resting CD4+ T cells.[28] These latent reservoirs decay very slowly and lie dormant in host cells until triggered.[29,30] After stopping treatment, patients experience viral rebound,[31] due to the virus in latent reservoirs replicating, and replenishing other reservoirs, even after achieving undetectable levels of plasma viremia following ART or Highly Active Antiretroviral Therapy (HAART).[32] This guarantees lifelong persistence of HIV-1, and requires individuals to take medication throughout their lifetime. Therefore, a true cure to HIV will only be achieved after complete eradication of these latent reservoirs.[33]

***Treatment & CRISPR-Cas9 Gene Editing:***

ART or HAART is the primary treatment for HIV infections, effectively suppressing viral load through combinations of drugs that target different stages of the virus's life cycle, such as entry inhibitors, reverse transcriptase inhibitors, integrase inhibitors, and protease inhibitors.[34] However, the long-term effectiveness of CCR5 antagonists can be limited for some patients due to the natural shift from R5 tropic to X4 tropic HIV-1 throughout the course of infection, which increases the proportion of X4 viruses even under treatment.[35] Targeting CXCR4 presents even more difficulties, since this receptor is involved in many essential body processes such as immune cell and organ development, making CXCR4 blocking agents harder to develop and implement. Nonetheless, some studies suggest that strategies to downregulate CXCR4 expression may be beneficial for HIV positive patients.[2] Given this information, CCR5 remains the most promising target for therapy. Its inhibition does not interfere with essential cellular processes, and the natural resistance against HIV-1 presented by the Δ32 mutation provides substantial evidence that CCR5 disruption can confer resistance to HIV-1. This possibility has been explored using gene editing technology such as Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated Protein 9 (CRISPR-Cas9), to induce a disruption in the CCR5 gene of an infected individual.

CRISPR and CRISPR-associated proteins are originally part of the defense mechanism in bacteria and archaea. The CRISPR locus consists of short, repeat sequences, interspaced by variable sequences (spacers) derived from previously encountered extrachromosomal elements, such as viruses and bacteria.[36–38] This system acts like a genetic memory; In response to reinfection by an invader, CRISPR RNAs (crRNAs) guide the Cas proteins to recognize and cleave complementary sequences in the invader's DNA, interfering with their replication,[38–41] and thus providing sequence-specific immunity.[42] Inspired by this naturally occurring process, the CRISPR-Cas9 system was developed in 2012, which uses a designed, single guide RNA (sgRNA) to direct the Cas9 nuclease to cleave specific target DNA in virtually any organism, providing an efficient and versatile genome editing tool.[43]

***Stem Cells:***

Stem cells are undifferentiated cells with the ability to reproduce through mitosis and differentiate into various specialized cells. In clinical settings, hematopoietic stem cell transplantation (HSCT) can be carried out using either allogeneic or autologous stem cells, and each has its own advantages and risks. Allogeneic HSCT involves using hematopoietic stem cells from a healthy donor, which can help eliminate infected cells and reduce the risk of relapse. However, it also carries significant risks, including graft vs host disease (GVHD), graft failure, and opportunistic infections. Treatment-related mortality is significantly higher than in autologous transplantation,

and this risk increases with donation from HLA-mismatched or unrelated donors. In contrast, autologous transplantation uses the patient's own hematopoietic stem cells, which means there is no need for HLA-matched donors and eliminates risks associated with GVHD. Furthermore, it allows for faster immune recovery and lower treatment-related mortality, making it more suitable for a wider range of patients. The main risk of this approach is the potential for disease relapse due to contamination of the harvested cells.[44] Given these considerations, autologous transplantation offers a more practical approach for introducing gene-edited hematopoietic stem and progenitor cells (HSPCs). Used in combination with the CRISPR-Cas9 technology to modify the CCR5 gene, this approach may be able to provide an effective long-term solution for HIV-1 infection. This paper explores this approach through *in silico* modelling and a hypothetical experimental design of the proposed strategy.

***Previous Cases:***

Timothy Brown, known as the 'Berlin patient', was the first person to be considered cured of HIV. He was diagnosed with HIV during his studies at university and initially managed his symptoms with low doses of zidovudine and protease inhibitors. 10 years later, he developed acute myeloid leukemia (AML) and required chemotherapy, followed by an allogeneic stem cell transplant. He received the transplant from an HLA-matched, unrelated donor, homozygous for the CCR5 Δ32 mutation, and stopped ART.[45,46] Typically, viral load in HIV patients rebounds after stopping treatment,[31] however, in Brown's case, active HIV-1 was not found for nearly a year. Unfortunately, his pneumonia and leukemia relapsed, and he received a second transplantation from the same donor in 2008.[46] This second transplant resulted in complete remission of AML and full donor chimerism. Although a small proportion of X4 tropic HIV-1 was detected before the transplant, the virus was not detected in peripheral blood, bone marrow, or rectal mucosa for over 20 months after discontinuation of HAART after the transplant, no longer requiring him to continue ART.[45]

Inspired by this case, Xu *et al.* carried out allogeneic stem transplantation using hematopoietic stem and progenitor cells (HSPCs) with CRISPR-Cas9 induced CCR5 Δ32 mutation into a patient infected with HIV-1 and acute lymphoblastic leukemia (ALL). Cas9 was delivered via non-viral transfection methods to prevent entry of foreign DNA and avoid long-term presence of Cas9 in target cells, reducing the risk of unexpected off-target effects.[47,48] The transplantation and long-term engraftment of CRISPR-edited HSPCs were successful; ALL was in complete remission with full donor chimerism, and donor cells carrying the mutated CCR5 were present for over 19 months. Although no adverse off-target effects related to CCR5 gene editing or CRISPR modification were observed, the low percentage of CCR5 disruption in lymphocytes highlighted the need to improve the gene editing efficiency of the CRISPR-Cas9 system and transplantation methods.[49]

Together, these cases highlight the therapeutic potential of the CCR5 Δ32 mutation to inhibit CCR5 function as an HIV-1 coreceptor through transplantation or gene editing, as a possible treatment option for HIV-1.

## ■ Aim of This Study

Based on the role of the CCR5 gene and Δ32 mutation in HIV resistance, this paper aims to analyze the structural differences between the CCR5 wild type and mutant protein, compare the level of conservation of CCR5 across organisms, simulate the CRISPR-Cas9 gene editing process via *in silico* designing tools, and propose a hypothetical CRISPR-Cas9-mediated experimental design for a potential autologous stem cell transplant. We hypothesize that mimicking the CCR5 Δ32-like mutation in the hematopoietic stem cells of a patient infected with HIV-1 could help better understand the disease mechanism and reduce susceptibility to the disease. Ultimately, this approach may help prevent relapse after discontinuation of HAART in the future.

## ■ Methods

***2.1: Amino acid sequences of CCR5 protein and CCR5 Δ32 mutant protein were retrieved from the NCBI database:***

The amino acid sequences of the human CCR5 protein (GenBank accession number: AAB57793.1) and CCR5 Δ32 mutant protein (GenBank accession number: AAB09551.1) were obtained from the National Center for Biotechnology Information (NCBI) protein database in FASTA format.

NCBI > Gene > FASTA

***2.2: Hydrophobicity of amino acids was analyzed via Deep TMHMM for the prediction of transmembrane regions:***

The FASTA format amino acid sequences obtained from NCBI were submitted to the Deep TMHMM website to generate a prediction for their transmembrane regions.

NCBI > Gene > FASTA > Deep TMHMM

***2.3: Transmembrane structure of CCR5 and CCR5 Δ32 were generated using Protter:***

The FASTA format amino acid sequences obtained from NCBI were submitted to the Protter website by entering the corresponding amino acid accession numbers to visualize their transmembrane structure.

NCBI > Gene > FASTA > Protter

***2.4: Clustal Omega was used to compare amino acid sequences between organisms:***

Human, chicken, mouse, and chimpanzee CCR5 amino acid sequences were obtained from NCBI. Then, it was submitted to the Clustal Omega website under "Protein" sequence type for comparison between organisms.

NCBI > Gene > FASTA > Clustal Omega

***2.5: CRISPR/Cas9 gene editing on the CCR5 gene was simulated via SnapGene with guideRNAs designed by CRISPR Finder:***

The CCR5 gene sequence was obtained from the NCBI Gene section. Then, on a new SnapGene file, the gene sequence was annotated for exons and introns, with the exonic


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and intronic information gathered from ENSEMBL. A region was picked for CRISPR/Cas9 targeting, and the guideRNA was chosen using CRISPRFinder. To minimize the probabilities of off-target effects, chosen gRNAs were shown to have 0 predicted off-targets with up to 2 nucleotide mismatches. For the Cas9 nuclease, a designed humanized Cas9 (hCas9) should be used for higher efficiency. Primers were generated on SnapGene to simulate PCR for fragment amplification and genotyping, and create a visual representation of hypothetical agarose DNA gel electrophoresis. An experimental design of CRISPR-Cas9-mediated autologous stem cell transplantation was proposed based on the simulated gene editing and chosen gRNAs. Insight into the procedures of autologous stem cell transplantation was gathered from the Leukemia and Lymphoma Society. The illustrations used were downloaded from Servier Medical Art.

NCBI > Gene > ENSEMBL > SnapGene file > CRISPRFinder > SnapGene

## Results and Discussion

### *3.1: CCR5 mutations causes loss in the number of predicted transmembrane domains:*

Amino acid sequence of wild type (WT) CCR5 and CCR5 Δ32 obtained from NCBI (Figure 1A, B) shows the difference in length of WT CCR5 (352 amino acids) and CCR5 Δ32 (215 amino acids), due to the 32 base pair deletion causing a frameshift and coding for a premature stop codon.[20] Transmembrane region (TMR) is the region of a protein that is integrated in the cell membrane. TMR prediction with DeepTMHMM shows 7 predicted TMRs for WT CCR5 (Figure 1C, D). In contrast, the mutated protein only shows 4 predicted TMRs (Figure 1E, F), showing that this mutation resulted in a significant change in predicted topology. The Δ32 mutation prevents correct folding of the protein and thus prevents CCR5 from being expressed on the cell surface, inhibiting HIV penetration into the cell and giving immunity against R5 tropic HIV-1.[23]

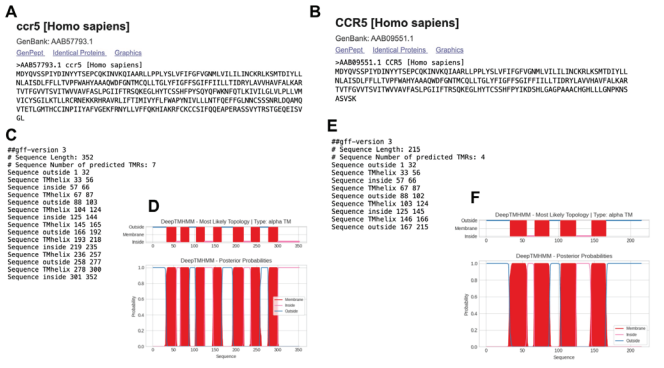


**Figure 1 :** Transmembrane region prediction of CCR5 and CCR5 Δ32. The Δ32 mutation results in the loss of multiple TMRs and amino acids, indicating significant disruption of CCR5 topology. 1A. CCR5 protein sequence retrieved from NCBI in FASTA format. 1B. CCR5 Δ32 protein sequence retrieved from NCBI in FASTA format. 1C. ~ D. DeepTMHMM prediction of WT CCR5 shows 7 predicted TMRs. 1E. ~ F. DeepTMHMM prediction of CCR5 Δ32 shows 4 predicted TMRs results.

### *3.2: Structural differences between WT CCR5 and CCR5 Δ32 #2:*

The topology diagram of CCR5 (Figure 2A) shows its 7 predicted TMRs, 3 intracellular and 3 extracellular loops. The apparent 4th intracellular loop is not part of its structure, but a result of palmitoylation, which is a form of post-translational modification of proteins, where a palmitic acid molecule is bound to the cysteine side chains via a reversible thioester bond. CCR5 has a cluster of 3 cysteines in the carboxyl-terminal tail, which is palmitoylated. This provides a membrane anchor to the c-terminus domain, creating a fourth intracellular loop.[50] In contrast, the topology diagram of CCR5 Δ32 shows its 4 predicted TMRs, 1 extracellular loop, and 2 intracellular loops. The Δ32 mutation results in a premature stop codon,[24] deleting the cysteine cluster required for palmitoylation. The misfolded protein is not palmitoylated and is not expressed at the cell membrane.[23]

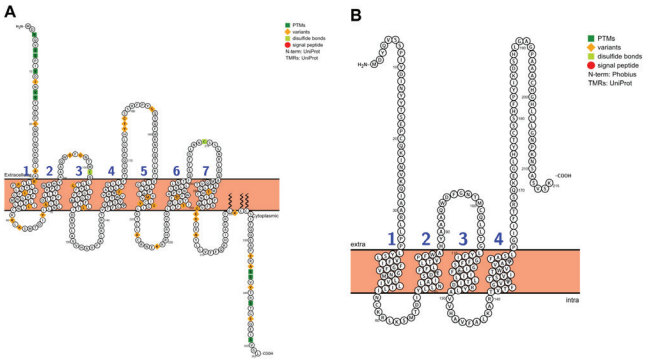


**Figure 2:** Comparison of predicted 3D structure for CCR5 and CCR5 Δ32. The Δ32 mutation results in an altered 3D topology with reduced TMRs compared to WT CCR5. 2A. Predicted transmembrane structure of CCR5, showing 7 TMRs with 3 intracellular loops and 3 extracellular loops.[18] 2B. Predicted transmembrane structure of CCR5 Δ32, showing 1 extracellular loop and 2 intracellular loops.

### *3.3: Assessing the level of conservation of CCR5 amino acids across different species:*

Comparison of CCR5 protein sequences of chicken, mouse, chimpanzee, and human revealed that CCR5 is a mostly conserved protein among the 4 organisms. In total, there are 210 asterisks, meaning 210 amino acids are fully conserved throughout all 4 organisms, or 59.7%. This suggests that CCR5 structure and function are also generally conserved across these organisms. Proteins that perform essential functions tend to have their amino acid sequences conserved across species by purifying selection, which eliminates harmful mutations.[51] In addition, natural selection strongly resists changes in regions of the amino acid sequence that contribute to several functions at the same time, since any alteration could significantly disrupt structure and function.[52] Therefore, since the amino acids are generally conserved among the 4 organisms, it can be inferred that these evolutionarily conserved regions are crucial for maintaining CCR5 structure and receptor function. Hence, mutations that disrupt these conserved regions, such as the CCR5 Δ32, may significantly affect receptor function, potentially explaining the HIV resistance associated with this mutation.

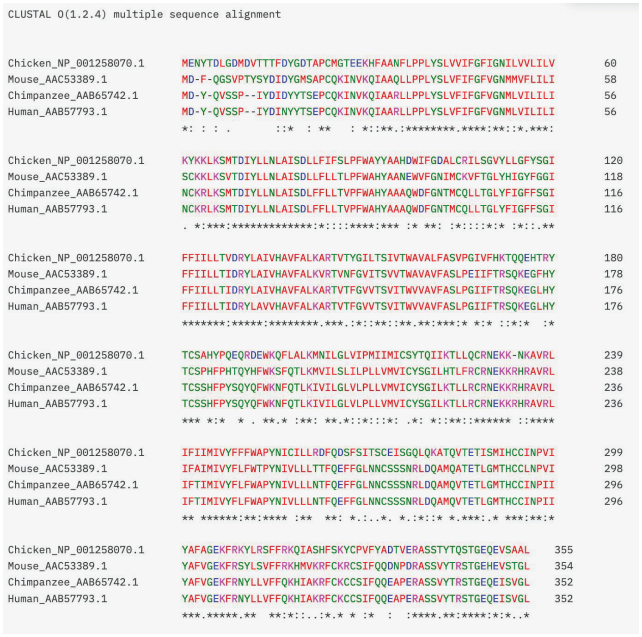


**Figure 3:** Multiple protein alignment of CCR5. CCR5 amino acid sequences of chicken, mouse, chimpanzee, and human obtained from NCBI are compared using Clustal Omega. Approximately 59.7% of amino acids are fully conserved across species, suggesting strong evolutionary conservation of CCR5 structure and function. Asterisks (*) indicate positions with fully conserved amino acids. Colons (:) indicate strong similarity, periods (.) indicate weak similarity, and a blank indicates no conservation. The colors represent: Red: Positively charged (Basic), Magenta: Negatively charged (Acidic), Blue: Hydrophobic, and Green: polar.

### *3.4: Hypothetical CRISPR-Cas9 gene editing strategy for CCR5:*

A hypothetical gene editing strategy targeting the CCR5 gene (Figure 4) was designed to generate potential mutants for investigating how CCR5 mutations may influence relapse after HAART treatment. This strategy could be tested in a wet lab setting. Here, mutant outcomes were simulated (Figure 5) for training purposes. Gene editing provides a potential tool to understand why relapses happen and improve treatment outcomes. Potential guide RNAs were predicted using the CRISPRFinder (Sanger) tool (Figure 4A), and 3 gRNAs with the lowest predicted off-target scores were selected to increase the precision and the diversity of potential mutants. Primers were also designed (Figure 4B) for the simulation of PCR amplification of mutated sequences.

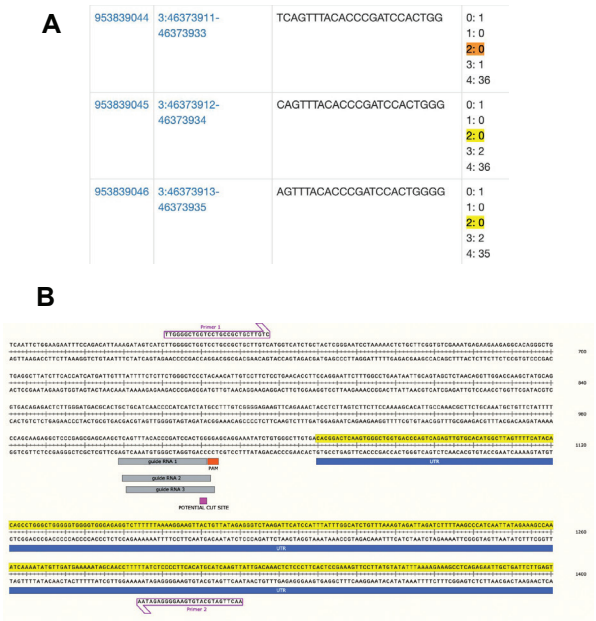


**Figure 4:** CRISPR-Cas9 gene editing strategy on the CCR5 gene designed by using SnapGene. This figure shows the positioning of the selected primers and CRISPR-Cas9 target region within the CCR5 gene. 4A. Predicted guide RNAs from CRISPRFinder (Sanger). Ideal gRNAs should have minimal off-target effects, so 3 gRNAs were selected for testing based on these scores, showing 0 predicted off-targets with up to 2 nucleotide mismatches. 4B. SnapGene view of the CCR5 gene sequence with annotated UTR region. The CCR5 gene editing is aimed to occur before the beginning of UTR. 2 primers (Primer1 and Primer2) were designed for PCR and simulated DNA gel electrophoresis. The length that primer 1 and primer 2 will amplify is 720bp.

Potential mutations were predicted and simulated for this study. The single-nucleotide alterations cannot be completely confirmed by DNA agarose gel electrophoresis, due to the very small size differences. Therefore, to confirm these mutations, a further analysis by DNA sequencing is required.

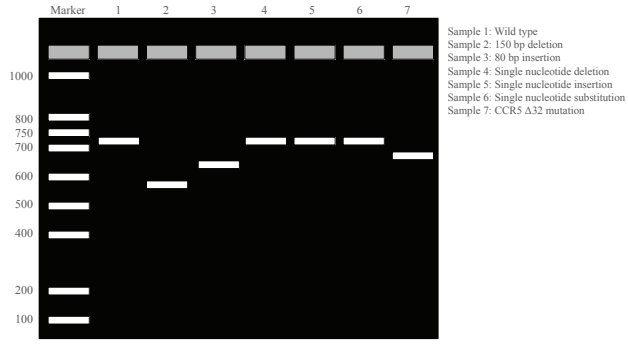


**Figure 5 :** Hypothetical band patterns from DNA gel electrophoresis showing potential outcomes of the gene editing approach. The first lane contains a DNA size marker (100-1000bp). Sample 1 shows the wild-type fragment with a size of 720bp. Samples 2 - 7 show the potential mutant fragments generated by the gene editing process, including deletions and insertions. This visualizes how band patterns of successful gene editing would appear relative to the 720 bp WT fragment.

### *3.5: Hypothetical experimental design for application of autologous transplantation of CRISPR-Cas9 edited HSPCs:*

This design outlines a potential therapeutic strategy using autologous transplantation of CRISPR-Cas9 edited HSPCs.


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By collecting and editing the patient's own stem cells *ex vivo*, issues such as finding a matching donor and graft versus host disease (GVHD) are avoided. Quality control ensures that only viable cells with the least off-target effects are reinfused. Conditioning with chemotherapy or radiation is necessary to create space in the bone marrow for the edited stem cells to engraft. After reinfusion of these edited cells, they are expected to replace the immune system with CD4+ T cells lacking CCR5, providing long-term resistance against HIV-1 infection.

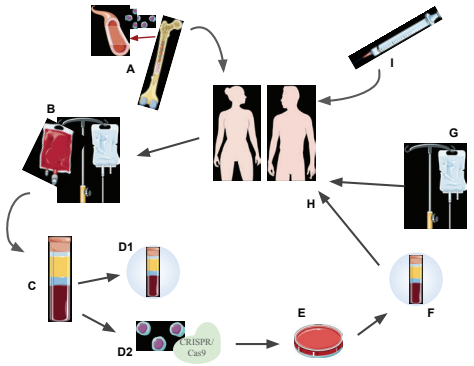


**Figure 6 :** A flowchart illustrating the hypothetical experimental design for the application of autologous transplantation of CRISPR-Cas9 edited HSPCs.[53,54] 6A. Release stem cells from the bone marrow into the bloodstream. 6B. Collect blood from the patient. 6C. Stem cells are separated and removed. 6D1. Some unedited stem cells are cryopreserved to keep as a backup. 6D2. Edit stem cells using CRISPR-Cas9. 6E. Quality control: Check for percentage off-target effects, cell viability, and the karyotype. 6F. Cryopreserve edited cells. 6G. Conditioning: Patient receives high-dose chemotherapy and/or radiation therapy to make 'space' in the bone marrow. 6H. Thawed stem cells are reinfused back into the patient. 6I. The patient receives treatment to help the bone marrow regrow. *Image adapted from Servier Medical Art (https://smart.servier.com/), licensed under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/)*

***Overall Discussion:***

This paper discusses the significance of the CCR5 coreceptor and the naturally occurring CCR5 Δ32 mutation in HIV infection and proposes the application of CRISPR-Cas9 technology to mimic the mutation in hematopoietic stem and progenitor cells (HSPCs) as a potential long-term treatment approach. While HAART is effective at suppressing viral load and preventing progression to AIDS, it does not eradicate latent viral reservoirs, meaning patients are required to be on medication throughout their lives. Prolonged treatment may lead to accumulating toxicities and comorbidities, especially in aging patients who may already face chronic diseases associated with aging. Furthermore, the health complications and economic burden of long-term HAART are not fully understood.[55] These challenges highlight the importance of long-term solutions such as CCR5 targeted therapies. As shown in Figure 1 and 2, the CCR5 Δ32 mutation results in major structural differences between the mutated protein and the wild type (WT) protein. The loss of transmembrane regions (TMRs) 5, 6, and 7 prevents correct folding of the mutant, causing it to be retained in the endoplasmic reticulum and absent on the CD4+ cell surface. This disruption in structure leads to strong immunity against R5 tropic HIV-1, especially in individuals homozygous for the mutation. The therapeutic potential of this mutation was demonstrated by the Berlin patient, who achieved a complete cure after undergoing successful allogeneic transplantation from a donor homozygous for CCR5 Δ32.[45] However, considering the scarcity of the population homozygous for Δ32, an allogeneic transplantation from a homozygous donor is not a realistic approach. To address such limitations, this paper proposes the use of CRISPR-Cas9 to recreate the CCR5 Δ32 mutation in HSPCs for autologous transplantation. Improving the accuracy of CRISPR-Cas9 is crucial for its therapeutic application. Therefore, a humanized Cas9 (hCas9) and carefully selected gRNAs (Figure 4) should be used for higher efficiency and minimal off-target effects. Akcakaya *et al.* showed that a well-designed sgRNA can carry out precise *in vivo* gene editing with no detectable off-target effects, suggesting CRISPR-Cas9 is a very specific genome editing tool.

As outlined in the proposed hypothetical experimental design (Figure 6), this strategy involves autologous stem cell transplantation using CRISPR-Cas9 edited HSPCs. Autologous transplantation provides several advantages. There is no need to find a donor homozygous for CCR5 Δ32, no need for HLA matching, and no risk of graft versus host disease (GVHD). After transplantation, the edited stem cells should produce immune cells lacking CCR5, leading to strong resistance against R5-tropic HIV-1, and potentially some reduced susceptibility to dual-tropic HIV-1. Crucially, while *in vivo* delivery of CRISPR-Cas9 faces challenges such as a higher risk of off-target effects, selection of delivery methods, and low efficiency,[56] *ex vivo* editing allows gene editing to be performed under controlled conditions, avoiding many of these issues. Furthermore, generating HIV-1-resistant cells by disruption of the *CCR5* gene has shown immediate loss of the receptor after transfection, with minimal off-target effects due to the protein complex being quickly degraded within the cell.[57,58]

While these advantages highlight the potential of *ex vivo* CRISPR-Cas9 gene editing as a therapeutic approach, several major challenges remain before it can be applied clinically. Although tools such as CRISPRFinder and Cas-OFFinder[59] can be used to predict off-targets, and studies suggest that overall, CRISPR-Cas9 is a very specific gene editing tool,[60–62] the possibility of unintended edits remains a major issue. In addition to the technical aspects, challenges regarding patients remain. HIV infected patients are significantly more susceptible to opportunistic infections and certain cancers due to their deteriorating immune system. In these situations, allogeneic stem cell transplantation is often applied, since it is more effective than autologous transplants. Therefore, genetically modified autologous transplants may not be a priority for patients battling cancer or other opportunistic infections. Furthermore, for patients who were able to suppress viral load with HAART and are without opportunistic infections, the risks of this approach and the unfamiliarity of CRISPR-based gene editing may outweigh the potential benefits.

Aside from technical and clinical considerations, ethical and social issues must also be addressed. Some may argue that enhancement of the immune system through gene editing, such as proposed in this paper, is considered genetic enhancement.[63] Therefore, this urges for further discussion on where we draw

the line between gene therapy and genetic enhancement.[64] Last but not least, challenges remain in managing cost, scalability, and accessibility. Gene editing is a fairly new and expensive technology, and more work must be done to allow treatment to be available to patients in low and middle-income countries, where HIV infection is more common and presents a greater challenge.

## Conclusion

HIV/AIDS remains an incurable disease, mostly due to the persistence of the latent viral reservoirs requiring lifelong antiretroviral therapy. While ART can suppress viral load and has been effective at increasing the life expectancy of patients, it cannot completely eradicate the virus, and it also poses long-term risks. The CCR5 Δ32 mutation has provided groundbreaking insight into the possibility of providing patients with HIV resistance, especially with cases of patients cured after transplantation from CCR5 Δ32 homozygous donors. The application of CRISPR-Cas9 technology to induce the Δ32 mutation directly to hematopoietic stem cells of a patient offers a possible strategy towards finding a cure for HIV/AIDS. Although challenges remain regarding safety, off-target effects, accessibility, and ethical concerns with gene editing, the ongoing research in the gene editing field continues to bring its application closer to a therapeutic option.

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## Author

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## Supplementary data

>Chimpanzee_AAB65742.1 CCR5 receptor, partial [Pan troglodytes]

MDYQVSSPIYDIDYYTSEPCQKINVKQIAARLLP-
PLYSLVFIFGFVGNMLVILILINCKRLKSMTDIYLLN-
LAISDLFFLLTVPFWAHYAAAQWDFGNTMCQLLT-
GLYFIGFFSGIFFIILLTIDRYLAIVHAVFALKART-
VTFGVVTSVITWVVAVFASLPGIIFTRSQKEGL-
HYTCSSHFPYSQYQFWKNFQTLKIVILGLVLPLL-
VMVICYSGILKTLLRCRNEKKRHRAVRLIFTIMI-
VYFLFWAPYNIVLLLNTFQEFFGLNNCSSSN-
RLDQAMQVTETLGMTHCCINPIIYAFVGEK-
FRNYLLVFFQKHIAKRFCKCCSIFQQEAPERASSVY-
TRSTGEQEISVGL

>Chicken_NP_001258070.1 C-C chemokine receptor type 5 [Gallus gallus]

MENYTDLGDMDVTTTFDYGDTAPCMGTEEKH-
FAANFLPPLYSLVVIFGFIGNILVVLILVKYKKLKSMT-
DIYLLNLAISDLLFIFSLPFWAYYAAHDWIFGDA-
LCRILSGVYLLGFYSGIFFIILLTVDRYLAIVHAV-
FALKARTVTYGILTSIVTWAVALFASVPGIVFHK-
TQQEHTRYTCSAHYPQEQRDEWKQFLALKM-
NILGLVIPMIIMICSYTQIIKTLLQCRNEKKNKA-
VRLIFIIMIVYFFFWAPYNICILLRDFQDSFSITSCE-
ISGQLQKATQVTETISMIHCCINPVIYAFAGEK-
FRKYLRSFFRKQIASHFSKYCPVFYADTVERASS-
TYTQSTGEQEVSAAL

>Mouse_AAC53389.1 CC chemokine receptor-5 [Mus musculus]

MDFQGSVPTYSYDIDYGMSAPCQKIN-
VKQIAAQLLPPLYSLVFIFGFVGNMMVFLILISCK-
KLKSVTDIYLLNLAISDLLFLLTLPFWAHYAAN-
EWVFGNIMCKVFTGLYHIGYFGGIFFIILLTIDRY-
LAIVHAVFALKVRTVNFGVITSVVTWAVAVFASL-
PEIIFTRSQKEGFHYTCSPHFPHTQYHFWKS-
FQTLKMVILSLILPLLVMVICYSGILHTLFRCRNEK-
KRHRAVRLIFAIMIVYFLFWTPYNIVLLLTTFQEF-
FGLNNCSSSNRLDQAMQATETLGMTHCCLNPVI-


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YAFVGEKFRSYLSVFFRKHMVKRFCKRCSIFQQDN-
PDRASSVYTRSTGEHEVSTGL

>AAB57793.1 ccr5 [Homo sapiens]
MDYQVSSPIYDINYYTSEPCQKINVKQIAARLLP-
PLYSLVFIFGFVGNMLVILILINCKRLKSMTDIYLLN-
LAISDLFFLLTVPFWAHYAAAQWDFGNTMCQLLT-
GLYFIGFFSGIFFIILLTIDRYLAVVHAVFALKART-
VTFGVVTSVITWVVAVFASLPGIIFTRSQKEGL-
HYTCSSHFPYSQYQFWKNFQTLKIVILGLVLPLL-
VMVICYSGILKTLLRCRNEKKRHRAVRLIFTIMIVY-
FLFWAPYNIVLLLNTFQEFFGLNNCSSSNRLDQA-
MQVTET