# HIV-1: tackling the obstacles that limit the effectiveness of CRISPR-Cas9 gene editing of the T cell co-receptor CCR5

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## ABSTRACT

HIV-1 is a complicated and perplexing virus. It infects T cells, reverse transcribes its RNA into DNA, utilizes its host DNA machinery to replicate its HIV-DNA, translates the HIV-DNA into proteins, assembles itself for a budding escape from the T cell, and rapidly mutates its conformation. Partially, due to its complexity, there remains no cure for HIV or AIDs. However, recently with the discovery of TALENs, the use of zinc fingers, and most of all the applications of CRISPR-Cas9 technology, has given researchers new hope in finding alternative gene therapies and treatments for diseases. With more focus on CRISPR-Cas9, this new and novel technology uses a guiding RNA, sgRNA, to lead a Cas9 nuclease to its target for deletion or to change that DNA site. CRISPR-Cas9 can delete point mutations and multiple DNA sites. Because CRISPR can alter DNA sequences, several scientists have conducted research into CRISPR, possibly treating more diseases such as cancer, diabetes, and even HIV. HIV-1 drew the focus of a researcher named Dr. Ebina in 2013 when he was the first to design and apply CRISPR-Cas9 to genes found in the binding sites of HIV-1, inhibiting HIV-1 gene expression. Since 2013, several other researchers have blocked HIV replication and infection through CRISPR-Cas9 targeting the receptors of T cells called the CC chemokine receptor 5 or CCR5. HIV-1 binds to the CD4 receptor of T cells, which consists of co-receptors CCR5 and CXCR4. If CCR5 expression can be removed, the HIV virus cannot bind to T-cells, blocking the initial attachment stage, and discontinuing the infection. However, there remain obstacles and issues for the CRISPR deletion of CCR5 for treating HIV-1. The issues include: 1) finding new and safe methods of CRISPR-Cas9 delivery, 2) clearing the latent HIV reservoirs, 3) improving the sgRNA design to avoid off-target mutations or deletions, and 4) effectively analyze the viral escape of HIV from CRISPR-Cas9 modifications. Therefore, the purpose of this review is to discuss possible techniques for removing the obstacles that can lessen the potential of CRISPR to delete CCR5, repressing HIV-1 into long-term remission or a functional cure.

Keywords: HIV-1; CRISPR-Cas9; T-cells; lipid nanoparticles; gut-associated-lymphoid tissue; Co-receptors; Probiotics; GI Tract; Gene Editing

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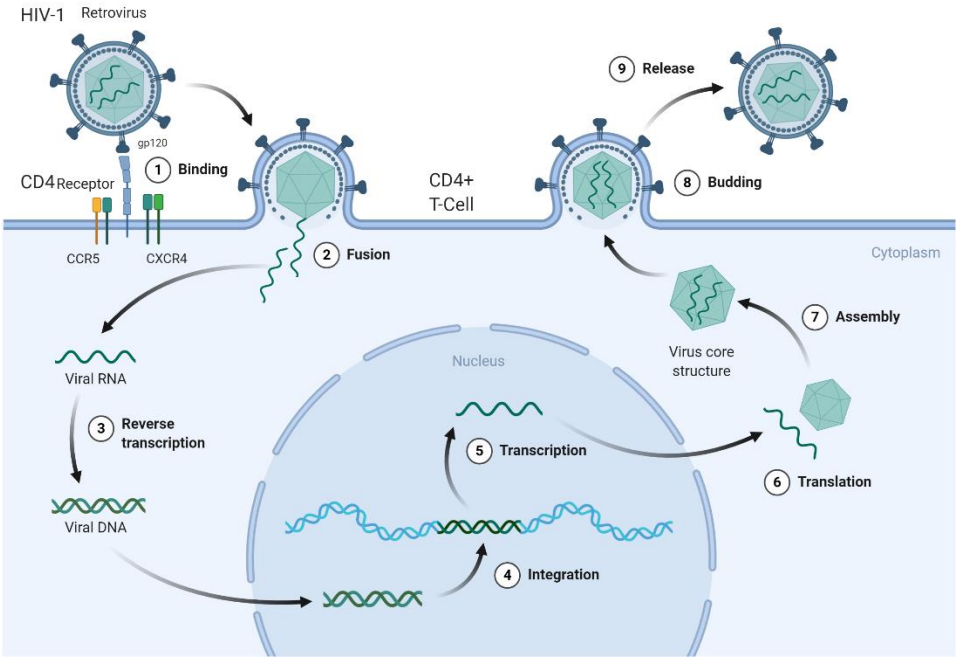
## INTRODUCTION

The World Health Organization or WHO reports 36.9 million people were living with HIV in the year 2017. The 1.8 million newly infected persons were provided with highly active antiretroviral therapy or HAART. HAART medicines are the most effective for lessening the symptoms and death rates of HIV and AIDS. HIV-1 causes AIDS. HIV-1 infects CD4+ T cells and, in the long term, eradicates most infected T cells. T cells are white blood cells, a part of the immune system's adaptive immune response. The adaptive immune response includes cytotoxic CD8+ T cells, B cells, and helper CD4+ T cells. The helper CD4+ T cells monitor and regulate the activity of CD8+ T cells and antibody-producing B cells. The structure of the HIV virus includes a capsid core containing RNA and enzymes. It has an outer surface called the envelope. From the envelope, protrudes glycoproteins as glycoprotein 120 and 41. RNA is viral genetic material. The life cycle of HIV begins when the glycoproteins attach to the $CD4^{+}$ receptors of T cells with assistance from the CCR5 and CXCR4 co-receptors shown in Figure 1. After binding, the HIV envelope fuses with the cell membrane of the T cell, entering the cell. RNA and enzymes are released. Reverse transcriptase converts its RNA into DNA, which the DNA then enters through the CD4+ cell nucleus.

**Figure 1 The Life Cycle of HIV** The HIV-1 virus binds to the CD4 receptor of a T cell. It binds and fuses with the white blood cell and releases its viral RNA into the cytoplasm of the T cell. The virus reverse transcribes its RNA into DNA: the DNA integrates into the genome of the T cell. HIV-DNA enters transcription and translation. After translation, the viral core is assembled containing the newly synthesized HIV genetic material. The assembled HIV provirus buds, releasing it from the T cell for further maturation.




The HIV-DNA binds to CD4+ T cell DNA and is inserted into the cell DNA with the integrase enzyme. The HIV virus then uses the polymerases of the cell to begin replicating and

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and RNA were transported to the surface of the cell membrane. The new HIV proviruses bud and exit the T cell. The new HIV viruses secrete a protease, dividing the long chains of proteins within the capsid core, forming a mature infectious HIV virus. In addition, the capsid core controls the uncoating process. Mutations in the integrase enzyme stored inside the capsid core produce weakened capsid cores. The less stable capsid core after an integrase mutation shows that the integrase fortifies the capsid core (Campbell and Hope, 2016). Reverse transcription is involved in stabilizing the capsid core. When mutating capsid proteins or enzymes, the core is highly degraded. Therefore, a faulty integrase and reverse transcriptase can delay the uncoating process.

In addition, the degradation of the GI tract's epithelial layers and fewer T cells present in the GIT can help trigger and activate HIV-1 viruses in patients who are HIV positive. By adding probiotics plus interleukin-IL-21 can heal the intestinal walls of the GIT, and can restore and increase T-cells in SIV-infected monkeys, where SIV is a virus alike HIV-1. By studying animal models. Probiotics have been shown to benefit persons with HIV, but not many studies have closely examined the gut microflora and structure of the GIT. Therefore, more research is needed to link probiotics to improve health of HIV-1 positive individuals. After treatment with probiotics, researchers found less T-cell activation, an increase in T-cells, and probiotics restored the gut epithelial walls, and the structure of the mitochondria was repaired. The placement of a chaperonin, called HPS60, specific for the mitochondria, rebuilt the mitochondrial structure (d'Ettore et al., 2017). Additionally, there were fewer immune responses to and less activation of CD4+
and CD8+ T-cells.

The CCR5 delta 32 mutation disables the CCR5 receptor, which is expressed on the surface of white blood cells in the immune system. HIV utilizes CCR5 as a key by latching onto it for entrance into the CD4+ T cell. A malfunctioning CCR5 receptor blocks HIV from entering the white blood cells (Fig 2). There is no cure for HIV-1. However, a person was cured, known as the Berlin Patient. The Berlin patient received a transplantation of stem cells from a donor. The stem cells from the donor possessed a mutation in the HIV coreceptors of CCR5. The mutation was homozygous or a (CCR5Δ32/Δ32) that deleted the presence of the CCR5 co-receptor in T cells (Fig 2). Transplantation was applied to treat his leukemia. Another positive HIV-1 patient, known as the London patient, was transplanted with stem cells to treat his lymphoma. Again, the cells had mutations in the (CCR5Δ32/Δ32). After 16 months, he no longer needed antiretroviral medicine. HIV-1 was in remission for an additional 18 months. HIV-1 RNA in the plasma was not traceable at <1 copy/mL and HIV-1 DNA could not be detected surrounding CD4 T cells (Gupta et al., 2019). The antibodies against HIV-1 also decreased. Targeting CCR5 with gene therapies may result in remission of HIV-1.

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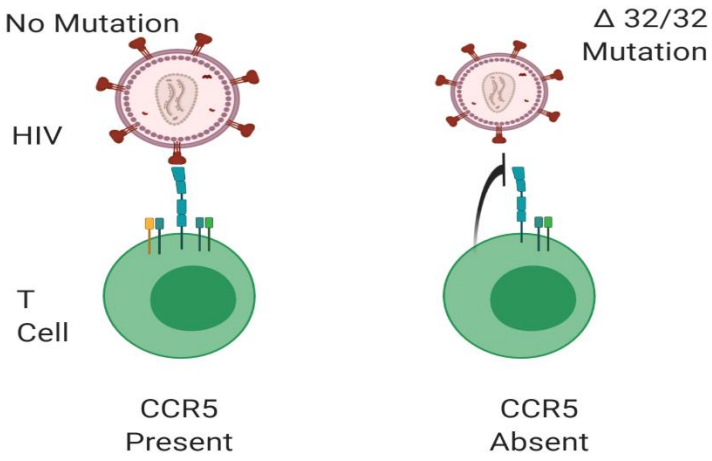
**Figure 2 The Absence of CCR5 and the Inhibition of HIV Attachment** A mutation of CCR5 causes the receptor to malfunction and become inactive. The CCR5 is present on the exterior of white blood cells. By disabling CCR5 HIV cannot attach to the CD4 receptor of T cells. HIV glycoproteins cannot use CCR5 as a key to bind and unlock white blood cells.




Currently, there are many new and promising treatments for HIV-1 as gene therapy, transcription activator-like effector nuclease or TALENS, clustered regularly interspaced short palindromic repeats, or CRISPR-Cas9sgRNA. Nevertheless, this review centers on CRISPR-Cas9 gene editing of CCR5. The gene for CCR5 can be deleted or changed through CRISPR-Cas9 technology. However, there remain present obstacles that can decrease the effectiveness of targeting and modifying the CCR5 receptors of CD4+ T cells. In this review, possible suggestions to address these restrictive issues are: 1) formulating a safer delivery method, 2) improving sgRNA design, 3) decreasing HIV-1 reservoir size, and 4) avoiding HIV-1 viral escape. The purpose of this study was to describe the possible alternative processes for developing an effective and safe delivery of CRISPR-Cas9, for modifying CCR5 receptors, into a T cell. The GI tract linked to the GALT may provide an alternative route for delivering CRISPR-Cas9 into T cells.

## 1. SURVEY METHODOLOGY

The literature was examined to discuss and outline possible solutions for CRISPR-Cas9 targeting CCR5. One-hundred fifteen total articles were screened from NIH and PubMed Central combined. Forty-seven articles were retrieved after 68 articles were excluded if the title or abstract of these articles did not describe HIV-1, CCR5, methods of delivery, gut microbes, or CRISPR as key terms. After a full-text screen, 22 articles were excluded. The data of the 8 articles excluded from this study included data points from clinical trials and from diseases other than HIV-1, as cancer. The 17 articles, chosen and included, displayed data results, and

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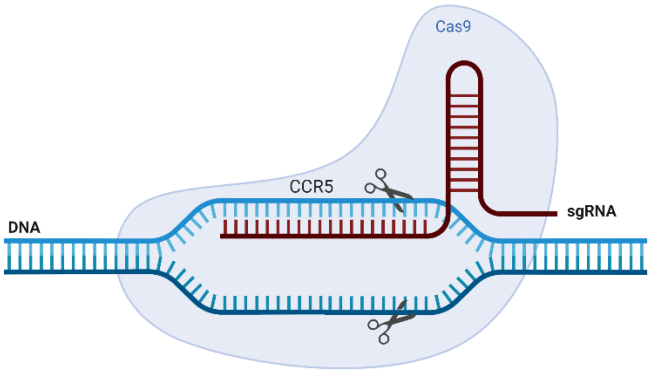
CCR5 of HIV-1, which are described in this review.

## 2. CRISPR-CAS9 MODIFICATION OF CCR5

Cas9 is an RNA-guided endonuclease. Cas9 is a part of the prokaryotic immune system. The clustered regularly interspaced short palindromic repeats or CRISPR can bind to a gene locus with the assistance of short RNA guides or sgRNA. The CRISPR Cas9 nuclease uses sgRNA to target specific DNA sequences through base-pairing. The RNAs are similar to bacteriophages, and the RNAs produce an immune response to combat foreign viral genetic material. The CRISPR Cas9 can identify specific DNA sequences and then cleave it, acting as a nuclease (Fig 3). The development of CRISPR occurred by studying bacterial and archaeal immunology and diversity.

CRISPR gene editing has been successfully implemented for treating HIV-1 virus in human cells and in small laboratory animals (Xiao et al., 2019). Ebina et al. in 2013 was the first researcher to design CRISPR-Cas9 to limit the progression of HIV-1 gene expression. Ebina et al. (2013) targeted the binding sites of NK-kB in the area of the LTR called U3 and deleted TAR sites in the R surface region. Cho et al., (2013) used CRISPR-Cas9 and deleted the CCR5 gene in human embryonic kidney cells by transfecting kidney cells with Cas9-sgRNAs. Ye et al. in 2014 conjoined CRISPR/Cas9 with TALENS to produce pluripotent stem cells, which induced the homozygous CCR532/32 mutation. The iPSCs derived into white blood cells that cannot become infected with HIV.

**Figure 3 CRISPR Cas9-sgRNA Excision of CCR5** The Cas9 nuclease uses sgRNA as a guide to target the specific CCR5 DNA sequence. The sgRNA is sequenced as a complement to the CCR5 nucleic acid sequence. The sgRNA binds to the CCR5 sequence, and then the Cas9 regconizes CCR5. The Cas9 nuclease begins to dissever CCR5 DNA.




Li et al. (2015) designed CRISPR-Cas9-sgRNAs to target exon number four of the CCR5 gene, which was delivered by the adenovirus. Xu et al. (2017) deleted CCR5 for longer

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term, ceasing the infection of T-cells with HIV-1. Xu et al. (2017) established a foundation for the engrafting of CCR5-modified hematopoietic stem cells, an HIV-1/AIDS treatment, for clinical implementation. However, the issues that remain for CRISPR modification of CCR5 include: 1) finding new and safe methods of CRISPR-Cas9 delivery, 2) clearing the latent HIV reservoirs, 3) improving the sgRNA design to avoid off-target mutations or deletions, and 4) effectively analyze the viral escape of HIV from CRISPR-Cas9 modifications.

## 3. POSSIBLE METHODS TO ADDRESS ISSUES OF CRISPR-CAS9 DESIGN AND DELIVERY FOR THE CO-RECEPTOR CCR5 TARGET

**ISSUE 1. DESIGN DELIVERY AND SAFETY** Lipid nanoparticles are ideal for the delivery of CRISPR-Cas9 because they can passively diffuse across the lipid bilayer of cells. Using lipidnanoparticles, we can avoid the unfavorable immune responses induced by adenoviral vectors, which are viral vectors used to transport Cas9 nucleases. Lipidnanoparticles also have a propensity to aggregate near places of disease, for example, usually within the proximity of tumors. Near and adjacent to the endothelial layers of tumors, the tumors maintain specificity for lipid nanoparticles with smaller diameters. Lipid nanoparticles can preserve their cargo when traversing through the circulatory system, avoid interaction with non-infected tissue, and only gather at locations of disease (Bolhassani et al., 2011). Lipid-oligonucleotides can be used as vehicles to deliver oligonucleotides into the cell for gene delivery or gene inhibition, developing new and novel delivery technology of medicines, and identifying RNA in live cells. To design lipid-oligonucleotides, the outer membrane fasteners are oriented at the posterior edges of the lipid-oligonucleotide duplex, which ties the DNA between the liposomes after it is chained, secured, and hybridized to the outer membrane lipid bilayer (Ries et al., 2015). Because of the diverse activities of lipid membrane fasteners, a strand of DNA can help assemble or fuse together lipid nanoparticles into a liposome. However, liposome assembly is contingent upon the location of the site for the lipid-DNA alterations.

**ISSUE 2. TARGETING THE HIV-1 RESERVOIRS** The reservoirs of HIV-1 found in the gut-associated lymphoid tissue or in the GALT contribute to the continued replication of the HIV virus. HIV is dormant in the GALT even when it is absent from the blood. The replication of HIV-1 takes place within the lymph nodes, and T-cells are first bound to the virus within the GALT. Antiretroviral drugs have limited access to reservoirs in the GALT. However, nanoparticles such as drug polymers, micelles, and liposomes may offer improved delivery of antiretroviral drugs to the GALT reservoir. M cells more specifically absorb nanoparticles. As a result, Ogunwuyi et al. (2016) proposed a method called site-specific targeting of ARV drug-loaded nanoparticles to those M cells. They added an M-cell-targeting ligand to the outer region of the nanoparticle. The addition of the ligand to the nanoparticle increased the M cell endocytosis of the antiretroviral drug.

They used in situ polymerization of monomers for the nanoparticle core, crosslinks that stabilized the core, a redox system, and a water-soluble macromonomer called polyethylene glycol or PEG. PEG allows the nanoparticles to directly target the protein receptors on the outer surface of M cells. This macromonomer with ligand specific for M cell receptors called

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GRGDS peptide had a minimally low molecular weight of methacrylate at MAv=400 (n=7) and 2000 for the PEG region (n=4) (Ogunwuyi et al., 2016). The structure of GRGDS has a non-polar hydrophobic core with a polar hydrophilic outer covering. The ARV drugs called zidovudine, lamivudine, nevirapine, and raltegravir all become compacted into and formed into nanoparticles and dispersed in vitro. The loaded nanoparticles effectively blocked HIV from infecting T-cells. PEG on the antiretroviral nanoparticles allowed for a tighter fit of the ligands bound to the M cell receptors.

**ISSUE 3. DESIGNING AND PREVENTING CRISPR CAS9-SGRNA OFFTARGET MODIFICATIONS** Hu et al., in 2014 engineered CRISPR-Cas9 to delete sites in the HIV-1 LTR U3 region, ceasing gene expression and replication within HIV-1 viruses. No off-target mutations were observed. Also, Hillman (2019) described a study, which designed an sgRNA to target and delete the Crygc gene mutation, which results in the development of cataracts in the eyes. The sgRNA called sgRNA4 was manufactured to target one base-pair downstream of the initial base pair deletion. An sgRNA 4 successfully modified the Crygc gene, correcting the mutation with less phenotypic expression of the cataracts in the eyes in mouse models. Also, many computational tools and software packages are now available to assist with designing the sgRNA. However, researchers detected data with algorithmic overfitting and recommended using data from the same guide RNA expression system (Lino et al., 2018).

**ISSUE 4. POSSIBLE WAYS FOR AVOIDING HIV-1 VIRAL ESCAPE** HIV-1 virus quickly and vigorously mutates. HIV changes its speed, velocity, and conformation to avoid or escape immune cell responses. For the CRISPR-Cas9 modification of CCR5, the HIV virus rapidly mutates and shifts from attaching to the CD4 receptor of T cells via CCR5 to bind through the co-receptor called CXCR4, infecting more T-cells. After suppressing the viral load, HIV-1 can still shift into binding to T cells, but only with the assistance of the co-receptor, CXCR4. The change in HIV viral binding from CCR5 to CXCR4 is characterized by high viral load, low CD4+ T cell count, and AIDs.

An improved understanding of the switch from CCR5 to CXCR4 is greatly needed to avoid HIV-1 viral escape. Hutter et al. (2015) proposed decreasing the HIV reservoir, using gene therapy that targets CCR5, and tested the application of CCR5-independent inhibitors. A gene therapy called C46 has been approved for clinical use. The C46 consists of 46 amino acids synthesized from the second repeat of the HIV-1 envelope glycoprotein 41 or gp41. C46 results from repeat experimentation revealed no trace or detection of the viral escape mutants after 9 weeks of PCR analysis on the HR1, HR2, and V3 loop domains of gp41 (Hutter et al., 2015). The drugs Plerixafor and a newer less toxic drug called Burixafor are CXCR4 inhibitors, which can prevent the proliferation of HIV quasi-species. Plerixafor is injected through subcutaneous skin; however, the drug, Burixafor, is a small organic molecule that can be orally ingested and is currently being studied in clinical trials.

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## CONCLUSION

The ultimate purpose of this review was to present possible alternative ways and means to overcome the obstacles for targeting HIV-1 with CRISPR-Cas9 editing of CCR5 of T-cells. HIV-1 infects T cells, which are cells that largely populate the GI tract and more specifically reside in the Peyer's patches of the small intestines, approximately 70% of the immune system is within the GI tract (Hillman, 2018). The degradation of the GI tract can create more space for developing larger HIV reservoirs. The use of probiotics was shown in this review to restore the epithelial lining of the intestines, which improves the outcome, prognosis, and the symptoms of HIV. However, further research is needed to find novel methods to treat HIV-1 through gut-associated-lymphoid tissue or the GALT. Evidence from literature was presented confirming the successful editing and modification of the T cell co-receptor CCR5 by CRISPR-Cas9 technology.

Issues that may limit the efficacy of CRISPR-Cas9 for editing CCR5, which can result in long-term remission of HIV, were provided. The present issues are sgRNA design, new methods of delivery, hard to reach HIV reservoirs, and HIV-1 viral escape. However, possible solutions were also given, which included the use of lipid nanoparticles for safer delivery of CRISPR-Cas9, enhancing antiretroviral drug design to effectively lessen the HIV reservoir size, to modify sgRNA design to prevent off-target DNA changes, and administering CCR5 and CXCR4 inhibitors to combat viral escape of HIV-1. The review also suggests increasing the effectiveness of delivering CRISPR-Cas9 for CCR5, using lipid nanoparticles as carriers and transporters to T-cells. Further studies are needed to confirm the safe delivery of CRISPR-Cas9 within lipid nanoparticles. The delivery technology for CRISPR-Cas9 needs to be safe, therefore, more research is necessary to find solutions to formulate lipid nanoparticles with less toxicity and more safety. Also, the final location in the body where each component of the nanoparticle remains needs further research and studies to confirm the precise mechanism for excretion of the nanoparticles.

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