

Original Article

Inhibition of CCR5 and CXCR4 prevents HIV infection

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ABSTRACT: CCR5 and CXCR4 are chemokine receptors recognize by HIV to enter into the host cell. In this review, we focus on their biology, function and pivotal role in HIV-1 infection, and also, how HIV quasi-species change tropism depending on their expression on the cell surface. We also discuss about the state-of-the-art strategies for targeting CCR5 and CXCR4, with emphasis on novel gene therapies that mimic a natural mutation called CCR5-delta32, enabling innate protection against HIV R5 strains. Trials with gene therapies can knockout both co-receptors and confer protection in vitro without mutants. These techniques include zinc finger nucleases (ZFN), clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9 nuclease (CRSIRP/Cas9), transcription activator-like effectors nuclease (TALEN), short hairpin RNA (shRNA), and ribozymes.

KEYWORDS: Chemokine receptors, HIV infection, inhibition, gene therapy, gene editing tools

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INTRODUCTION

The Human Immunodeficiency Virus (HIV) is a lentivirus that attacks and weakens the human immune system, primarily targeting lymphocytes and macrophages, which leads to the acquired immunodeficiency syndrome (AIDS). HIV-1 is the most common type of HIV virus and it is the main cause of AIDS, and the responsible of a pandemic that affects around 35 million people world-wide (UNAIDS, 2014).¹ AIDS is characterized by a persistent virus replication and a progressive loss of T helper cells (CD4+T cells), and is in close association with progressive increasing susceptibility to opportunistic infections.

More than thirty years after the identification of HIV,^{2,3} a cure for HIV infection is still to be achieved. The combination of multiple antiretroviral therapy (cART) has reduced AIDS-related morbidity and mortality and transformed HIV-infection into a chronic disease. Unfortunately, in spite of the favorable outcomes in enabling HIV-infected individuals to live a longer and healthy life, cART is not curative and only partially mitigates the malicious symptoms of HIV-infection. In addition, patients under cART

are at risk of accelerated aging and developing age related diseases such as cardiovascular disease, metabolic syndrome, solid organ malignancies, neurocognitive, functional decline and osteoporosis.^{3,4,5,6} Moreover, also financial obstacles restrict the universal access to antiretroviral treatment. Undoubtedly, the search for an HIV treatment is needed to bypass the limitations of the current therapy and restore health.⁵

During cART, the plasma virus levels decrease to below detection (50 copies of viral/HIV-1 RNA per ml of plasma) during three phases. The first phase represents the decrease of infected CD4+T cells (half-life of ~1 day). The second phase, which has a half-life of ~14 days, reflects virus production by another population of infected cells. These cells are not identified (possibly partially activated CD4+T cells or other cells such as macrophages or dendritic cells).

During the third phase, viremia reaches levels below the limit of detection of clinical assays. There is residual viremia derived from the activation of latently infected resting CD4+T cells

and from another unknown cell source, for that reason HIV isn't cured.^{3,4,6} If cART therapy is stopped, a rapid rebound of viremia is observed and AIDS might be present again, for that reason is urgent to research other ways to find a sterilizing cure. (Figure 1).^{3,7}

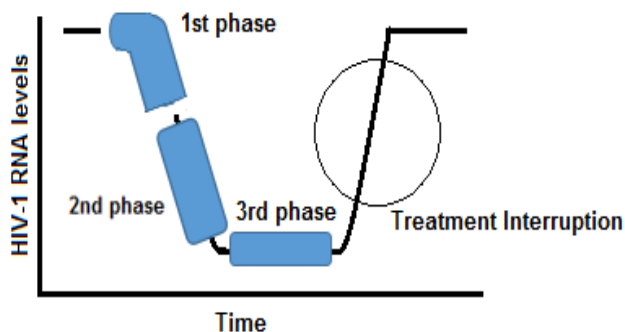


Figure 1. Dynamics of plasma virus levels in a cART-treated HIV⁺ individuals.

Virus replication is attenuated by continuous cART (phase 2 and phase 3). Once the treatment is stopped, high level of virus replication is recovered.

One of the main obstacles in the treatment of HIV is its genetic variability.^{3, 5, 7} HIV does not have any enzymes to check that its RNA sequence is being copied properly, and its copying machinery is not as accurate as ours¹

The lack of quality and repair checkpoints entails a high-mutagenesis rate, which poses a big challenge for vaccination and drug-treatment, since some copies of the virus might mutate to become resistant to chemical and vaccination.³

In order to infect vital cells of the human immune system, HIV-1 carries on a glycoprotein, called gp120, which recognizes and binds tightly to CD4, a cell-surface glycoprotein found on immune cells such as CD4⁺T cells, monocytes, macrophages and dendritic cells. The binding to CD4 allows the attachment and merging of the virus to the host-cell surface (**BOX 1 and Figure 2**).

The binding/fusion is a vital stage of the HIV life cycle, which occurs unless a co-receptor, such as CXCR5, CXCR4 or CCR5,^{3, 8} is also present on the cell surface in addition to CD4. The importance of chemokine co-receptors in HIV-1 pathogenesis is underlined by the observation that individuals deficient in CCR5 are resistant to infection by HIV-1.¹⁰

These observations confirm that co-receptors are promising targets in the prevention or therapy of HIV.^{10, 14} A number of small molecules have been designed to potentially interfere with the CCR5 and CXCR4 viral interaction and inhibit HIV entry into human cells. 41

In this review we will discuss about HIV replication (BOX 1 - HIV life cycle), CCR5 and CXCR4 biology, function and pivotal role in HIV-1 infection and state-of-the-art strategies for targeting CCR5 and CXCR4, with emphasis on novel gene therapies that mimic a natural deletion CCR5- delta32 (CCR5 Δ 32), enabling innate protection against HIV-infection.

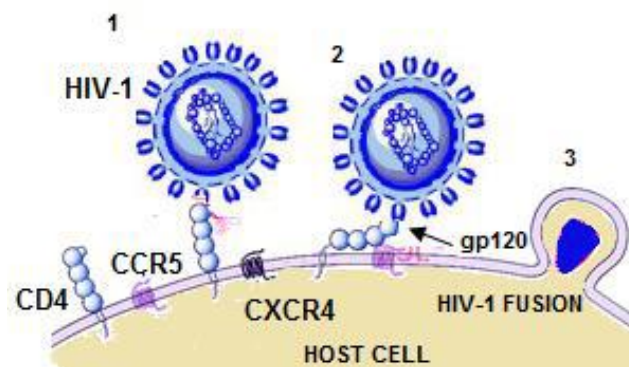


Figure 2. HIV Attachment to a CD4⁺T cell. 1)

The viral protein gp120 attaches to CD4. 2) The gp120 variable loop binds to a co-receptor either CCR5 or CXCR4. 3) HIV merges and enters the host-cell. The viral core is release inside the cell. Viral core contains viral RNA, reverse transcriptase, viral integrase and accessory and regulatory proteins (e.g. Vpr).

HIV GENOME AND STRUCTURE ORGANITZATION

HIV-1 encodes for nine open reading frames. Three of them are common to all retroviruses: **gag**-the antigen which encodes the precursor (Gag) of the major internal structural proteins of the virus (matrix [MA], capsid [CA], nucleocapsid [NC] domains and protein p6 in HIV), **pol**-which encodes the catalytic enzymes of the virus (**protease**, a cleavage of precursor proteins for the maturation of viral particles, reverse transcriptase, **integrase**, which lets the integration of viral RNA into host cell genome and lead to recombinant RNA and **reverse transcriptase**, that is used for transcription of viral RNA) and are also

encapsulated within the particle, and the **env** frame, which encodes gp160, the precursor of the viral envelope proteins (gp41 and gp120).^{11,12}

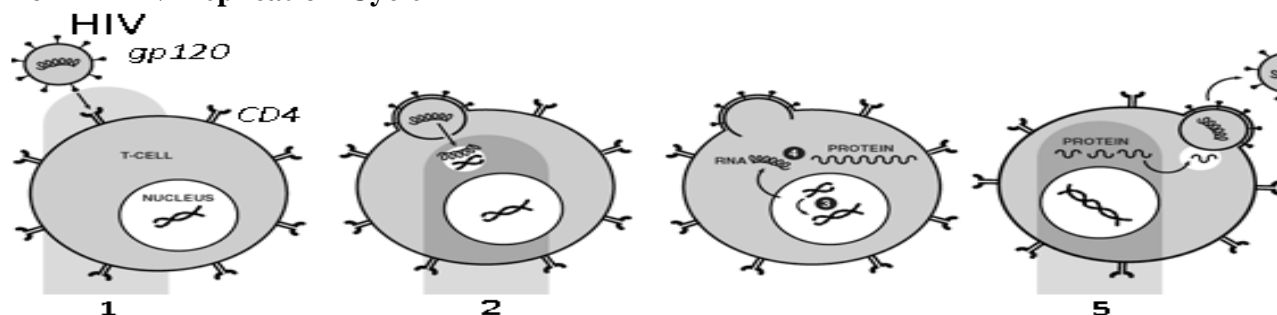
CCR5 AND CXCR4

CCR5 and CXCR4 are chemokine receptors belonging to the superfamily of the seven-transmembrane G-protein receptors.^{39, 10} Chemokines, small molecular proteins, are the ligands that activate CCR5 and CXCR4 to mediate some cellular functions including development, leukocyte trafficking, angiogenesis and immune response. Those proteins are highly hydrophobic, an only stable when are embedded in the lipid bilayer, traits that explain why there is a lack of crystallography on them.¹⁰

muscle and fibroblasts; and microglia, neurons, and astrocytes in the central nervous system (14). CCR5 has shown to be the major coreceptors for HIV-1 entry into cells. A CCR5 deficient mouse model (CCR5 $-/-$) developed normally in pathogen-free environment, but it showed a partial defect in macrophage.¹⁴

In addition, CCR5-deficient mice had an enhanced Delayed-type hypersensitivity (DTH) reaction and increased humoral responses to T-cell dependent antigenic challenge (15). CCL3, CCL4, CCL5 and CCL8 are the CC chemokines that have shown the most suppressive activities in HIV-1 infection.¹⁶ The lack of CCR5 expression is presented in 2-3% of Caucasian.¹⁷

Box 1 - HIV Replication-Cycle



Step 1. Binding – A protein in the HIV surface (gp120) binds to the immune cell via the receptor CD4 using CXCR4 and CCR5, cell's chemokine receptors to complete of fusion with the host cell membrane. HIV fuses with the membrane to allow the viral core to enter into the cell.

Step 2. Reverse Transcription – HIV gets uncoated and the viral RNA is released into the immune cell, transported to the nucleus where it is converted into DNA by reverse transcriptase released by the viral core.

Step 3. Viral genome integration – Viral DNA enters the cell nucleus where, with the help of HIV integrase, it is integrated to the cell's DNA.

Step 4. Transcription of viral genes – The cell produces viral RNA (viral transcripts). Some of the RNA is translated into protein and enzymes including protease. Viral transcripts are expressed under the control of Tat protein.

Step 5. Virus Assembly and shedding – Production of viral particles from the host chromosome. HIV protease cuts newly produced elements of virus into essential functional viral proteins assisting virus assembly and maturation... Viral particle is assembled with the viral mRNA inside. Newly produced virus "buds" from the cell to infect other cells. Host cell is eventually destroyed in the process.

CCR5: BIOLOGY AND STRUCTURE

Biology of CCR5

The chemokine receptor CCR5 is expressed on various cell populations including macrophages, dendritic cells and memory T cells in the immune system; endothelium, epithelium, vascular smooth

muscle and fibroblasts; and microglia, neurons, and astrocytes in the central nervous system (14). CCR5 has shown to be the major coreceptor for HIV-1 entry into cells. A CCR5 deficient mouse model (CCR5 $-/-$) developed normally in pathogen-free environment, but it showed a partial defect in macrophage.¹⁴

It's caused by a 32 base pair deletion in the CCR5 gene. This naturally genetic mutation, known as CCR5- Δ 32 is the responsible for innate HIV resistance. This mutation causes the CCR5 coreceptor to develop smaller than usual and no longer sits outside the cell.^{17, 18} This mutation locks "the door" which prevents HIV to enter the host cell.

Patients with heterozygous CCR5 $\Delta 32$ reduce carrier's chances of infection and delay the progress of AIDS, but patients with homozygous CCR5- $\Delta 32/\Delta 32$ are the ones which present this resistance.¹⁸

Unfortunately, only the 1% of the population present it and they are majority Northern Europeans, particularly Swedes.¹⁹ Some scientists suggest the mutation previously protected from another deadly viral disease, the Black Death, a continuing series of a lethal, viral, haemorrhagic fever that used CCR5 as an entry into the immune system.^{18, 19}

CCR5 Gene Structure

The CCR5 gene is localized to chromosome 3p21. It's composed of three exons, two introns and two promoters. The promoter consists of a 1.9 kb region preceding exon 1. Exon 1, is the start of the encoding region. Exon 2 is intronless. The second promoter encompasses the intron 1 and exon 2 regions. Exon 3 is intronless and contains the entire open reading frame (ORF) of the CCR5 gene.^{20,21}

CCR5 protein structure

The CCR5 protein consists of 352 aminoacids. The protein is composed of conserved residues, specific motifs and hydrophobic regions.²² These regions are important for chemokine ligand binding, functional response of the receptor, and HIV co-receptor activity. Eliminating of the palmitoylation between fatty acids provided a reduced surface expression by intracellular trapping of the receptor in organelles and its degradation (**Figure 3**).^{22, 23}

CXCR4 is a used along with CD4 by HIV-1 to infect T cells. The binding of gp120 to CD4 induces to conformational changes on gp120, enabling it to interact with CXCR4's N-terminal,

ECL2 and ECL3 domains and the ligand binding cavity through the V3 loop of gp120. This interactions induce a conformation on gp41 which leads to the fusion stage of the viral cycle (Figure 4).^{26, 27, 28} CXCL12y, an isoform of CXCL12, is 332 very weak agonist for CXCR4, but it provides effectiveness in HIV-blocking assays²⁵

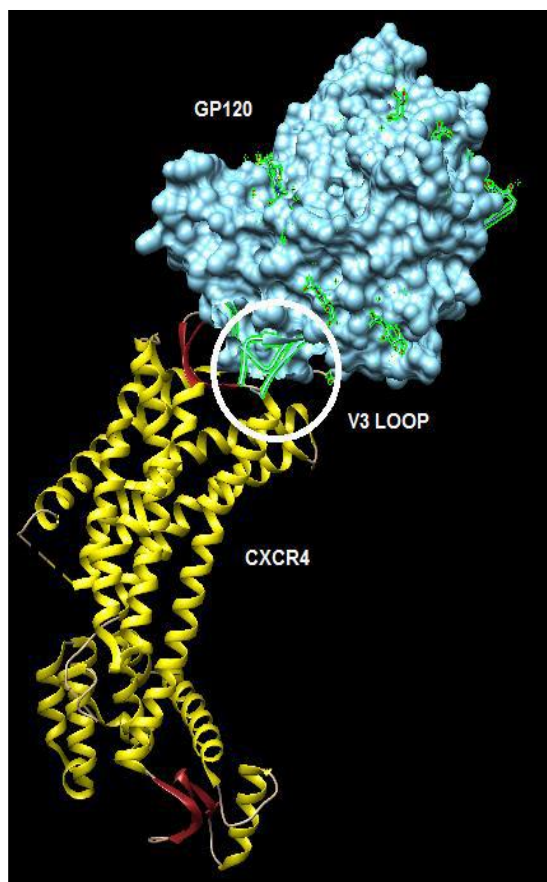


Figure 4. Model of gp120 bound to CXCR4 during HIV-1 entry process

The N-terminus of CXCR4 binds first with the loop of the envelope protein inducing conformational changes in gp120 which will enable V3 to bind with the extracellular side of CXCR4.

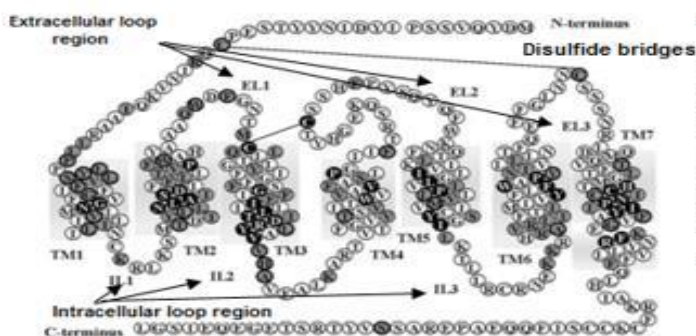


Figure 3. Schematic for CCR5 protein structural topology El and IL denote extra and intracellular loops regions. Disulfide bridges are shown as lines connecting cysteines. Gray circles denote conservation of strong groups and the ones with outlines denote similarities between CCR1 and CCR5. Black circles denote highly conserved residues in the rhodopsin family of GPCR.

Role of CCR5 and CXCR4 in HIV-1 infection

The HIV fusion to the cell is initiated by binding gp120, first to CD4 and then to a specific chemokine receptor, either CCR5 or CXCR4.²⁹ Gp 120 interaction with coreceptors triggers gp41 to promote the fusion reaction to the cell via a series of complex conformational changes. These receptor interaction trigger gp41 to promote membrane fusion; this reaction involves a gp41 subunit to allow insertion of its N- terminal 'fusion peptide' into the T cell membrane, followed by refolding the pre-fusion into an energetically favourable six- helix bundle which brings the two membrane together so the fusion can take place.³⁰
³¹ It's accepted that R5-strains of HIV appear early in the infection, whereas X4 strains appear late and are associated with a faster decline of CD4+T cells. The depletion of this cells switches in viral co-receptor usage from CCR5 to CXCR4 in approximately 40-50% of infected patients³²

HIV tropism change

The co-receptor usage of HIV virus has got preferences to CCR5, but it changes to a CXCR4 in approximately 50% of infected individuals. This is the result HIV interaction with various cell populations of the immune system (**Figure 5**).³³ Cell tropism is determined by CCR5 and CXCR4 expression on the cell surface.³⁴ The reduction of CCR5 expression via inhibitors lead to functional changes in R5 envelope, which decline the entry of R5-strain viruses and this increases the usage of CXCR4 co-receptor.^{35, 36} HIV quasi-species are more sensitive to both CCR5 and CXCR4 inhibition than the parental R5 strain and the X4 strain.³⁵ CCR5-mediated virus entry is thought to be determined by a few charged residues in the V3 loop env gene sequence (residues 296 to 331 of gp120).^{35,36} Another study reported that the entire gp41/gp120 glycoprotein sequence is the ones which determines which the co- receptor to use. This is useful to understand the sequence and predict it.³⁷ Nowadays, predictors for HIV quasi-species identification range from 71 to 84% for nonsubtype B virus and as high as 91% for subtype B HIV-1 virus.³⁶

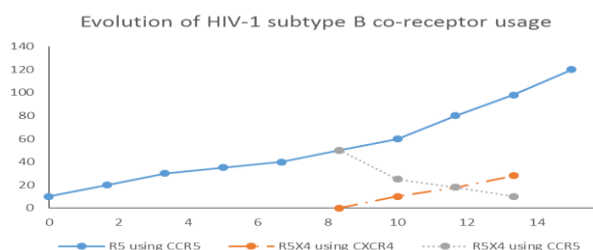


Figure 5. Evolution of co-receptor use by HIV-1 subtype B

Schematic representation of CCR5 and CXCR4 use during the course of HIV-1 subtype B infection. Approximately a 50% viral infections change CCR5 to CXCR4 due to CCR5 inhibition, as virus changes tropism depending on the co-receptor concentration on the cell surface, leading to HIV-1 quasi-species.

CCR5/CD4/CXCR4 oligomerization blocks HIV from binding to the cell surface

CCR5 expression in CD4+T cells reduce X4 strains cell entry and infection because it alters CD4/CXCR4 heterodimer conformation, blocking gp120IIIB to bind to CD4/CXCR4/CCR5 complexes. CCR5 co-expression impaired the cell-cell fusion that allow HIV-1 to enter into the T-cells.³⁸ Heterologomerization also increases cell plasticity and the engineering of compounds which could mimic the CCR5- triggered changes in CXCR4 homodimers or CD/CXCR4 heterodimers could reduce virus- induced damage to the immune system, making them suitable to block X4 HIV-1 infection.³⁸

INHIBITION OF CXCR4

One of the problems with the *Essen patient*, which rebounded viremia after hematopoietic stem-cell transplant (HSCT) was the presence of HIV quasi-species which could use both CCR5 and CXCR4 co-receptors (39). Plerixafor, a CXCR4 inhibitor was synthesized in the late 1980s. The effect on HIV was disappointing, but the ability to block HSC homing mechanism was considerable. Plerixafor has been approved by the FDA as a CXCR4 antagonist for use in combination with granulocyte-colony stimulating factor (G-CSF) to mobilize hematopoietic stem cells to the bloodstream for collection and subsequent autologous transplantation in patients with non-Hodgkin's lymphoma and multiple myeloma.^{39, 40, 41}

Currently, the most promising CXCR4 antagonists so far are the bicyclam agents, in which two cyclam rings are tethered by an aromatic bridge, such as AMD070, which was generally well tolerated and after the treatment the patients had elevated white blood cell counts but later on about a quarter of the participants developed tachycardia.

^{39,41} Another candidate of the same type, AMD3451 has demonstrated both CXCR4 and CCR5 antiviral activity with no secondary effects.⁴¹

INHIBITION OF CCR5

Maraviroc

Maraviroc is a CCR5 antagonist with potential *in vitro* and *in vivo* approved by the FDA (Food and Drug Administration) for treatment of patients with R5-tropic HIV virus.^{46, 57} Maraviroc doesn't affect CCR5 cell surface levels or intracellular signaling, it only blocks binding of gp120 to the cell. It has no detectable cytotoxicity (46). In a 10-day monotherapy trial, administration of maraviroc at doses up to 600 mg resulted in a big decrease of viral plasma.⁵⁷ Two of those patients developed a dual- or mixed- tropic virus by 11th day.⁴⁷ After 48 weeks, 60% of participants receiving the drug reduced the viral load to less than 400 copies/mL compared with the 26% of those with the placebo, proving effectiveness against HIV-1.⁴⁷

There are other CCR5 antagonists such as Viriviroc or Aplaviroc, but currently Maraviroc is the most used as it's approved by the FDA and it has no dangerous secondary effects. Resistance to CCR5 antagonist emerges as viruses, which have the ability to use the inhibitor-bound form of CCR5 to enter into the cell.⁵⁵ These mutants, such as Maraviroc-resistant HIV-1 have been generated by mutations in the V3 loop.⁵⁵

DUAL INHIBITION OF CCR5/CXCR4 HIV ENTRY

A part from the single CCR5/CXCR4 inhibitors, there are novel compounds with combined dual host-pathogen pharmacology against both co-receptors at the same time. A compound composed of a pyrazole-piperidine core exhibits three mechanisms of action: CCR5 and CXCR4 viral entry inhibition and non-nucleoside reverse transcriptase inhibition, proving viral new prototypes to block HIV-1 entry.⁵⁶ Clinical tests suggest that dual entry inhibition develops an advantage over HIV as it isn't able to switch tropisms, leading to non HIV-quasi species and a possible functional cure of HIV (Figure 6).^{39, 56}

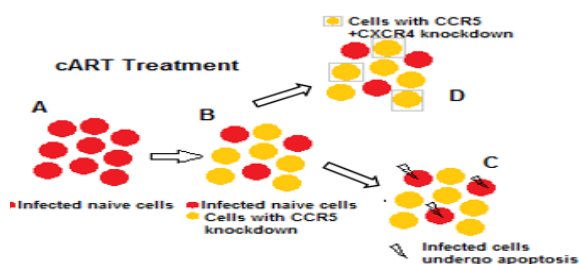


Figure 6. Selective advantage of dual entry inhibition through clinical tests with an ART plus gene therapy treatment A) Modified autologous CCR5 cells will be transplanted into a patient with ART treatment. B) ART is discontinued. Based on the cytopathic effect of HIV, there will be an enrichment of CCR5 cells; C) Infected cells apoptosis decreases the possibility of CCR5 as a target for cell entry. HIV may switch the tropism and use CXCR4, an alternative co-receptor; D) Dual entry inhibition (CCR5 modified cells and CXCR4 inhibited cells) could potentially be a sterilizing cure of HIV

GENOME EDITING TECHNIQUES AND EXPERIMENTAL TRIALS AGAINST CCR5 SYNTHESIS

1. Zinc-finger nucleases (ZFNs)

ZFNs can bind specific genomic sites and conduct gene editing breaking DNA double- strands (57, 39). With this technique, ZFN made by fusions of the non-specific DNA cleavage domain from the *FokI* restriction endonuclease with zinc-finger proteins is used to target the sequence in the genome of T-cells which produce CCR5 and bind it or lead to mutations which won't produce the protein. Locations of DNA breakage can undergo either non homologous end joining (NHEJ) or homologous-directed repair (HDR) by insertion of donor DNA.^{57, 39}

Clinical trials⁵⁸ in 2014 enrolled 12 patients in an open-label, the patients had aviremic HIV infection while they were receiving highly antiretroviral therapy. A transfusion was made to them with autologous CD4 T cells in which the CCR5 gene was rendered permanently by a ZFN.^{39, 58} One week later, the median CD4 T-cell count was 1517 per cubic millimeter, a significant increase from the 448 per cubic millimeter from the preinfusion. HIV RNA became undetectable in four patients. HIV DNA level in the blood decreased in most patients.⁵⁸ A group of patients underwent a 12-week interruption in treatment that began 4 weeks after SB-728-T, which is a type of autologous CCR5 modified T-cell product infusion. All of them rebounded rapidly, so the manipulated cells did not protect from viral replication (Figure 7).^{39, 58}

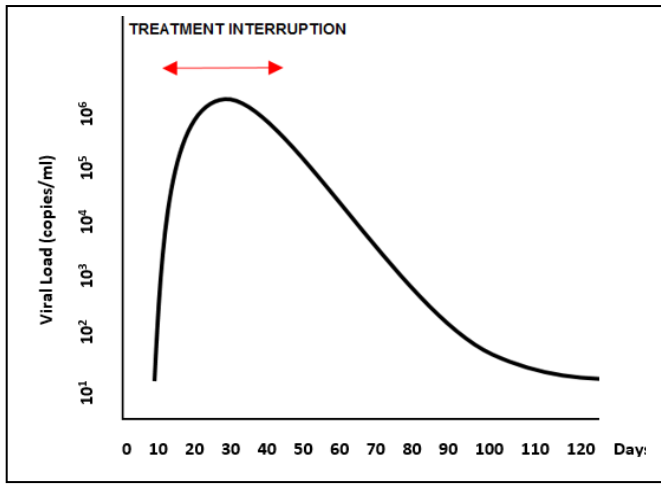


Figure 7. Changes in Viremia during Treatment Interruption

Treatment interruption leads to a rebound of viremia and, as a result a reduction of uninfected cells, proving that manipulated cells did not protect from viral replication.

2. Transcription activator-like effectors nucleases (TALEN)

TALEs are proteins found in phytopathogenic bacteria of the genus *Xanthomonas*, whose function is to induce the expression of specific host plant genes. TALE nucleases (TALENs) operate similarly to ZFN in that a pair is assembled at a given DNA sequence of two half-targets separated by a spacer sequence which induces the dimerization of the *FokI* portions resulting in site-specific DNA cleavage. There are no indications of an individual TALE repeat to its cognate base pair is altered by neighboring sequences.^{39, 60} TALENs can bind a wider range of DNA sequences, they can be programmed in an easier and more predictable manner and with less cytotoxicity.^{39, 59, 60} But they can only recognize one nucleotide instead of three.⁶¹

A clinical assay used TALEN to knockout CCR5 from T-cells. First of all, a codon-optimized TALEN targeting the functionally relevant first intracellular loop of the CCR5 receptor (CCR5-Uco-TALEN) was used to increase the efficiency. Then, CCR5-Uco-TALEN was introduced into T-cells from different donors by mRNA electroporation observing gene-editing rates of app. 50%. However, this clinical assay only reports one long term (12 days) exposure to HIV, which showed incomplete suppression of HIV replications (**Figure 8**).⁶²

	% CCR5 POSITIVE
ISOTYPE	0.59
■ UNTREATED	17.0
■ 2x10ug L+R	6.3
■ 1x10 ug R	19.2
% CCR5-KNOCKOUT (FC)	63.1%

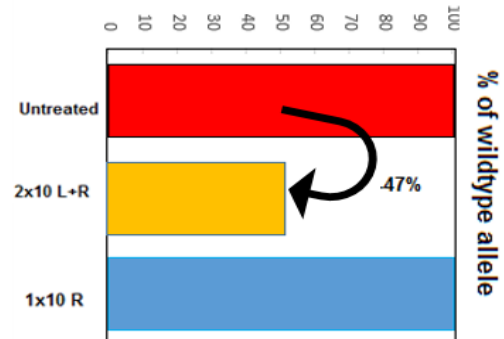


Figure 8. CCR5-Uco-TALEN enable high-level gene knockout leading to efficient protection against R5- tropic HIV independent of initial CCR5 expression

mRNA electroporation shows rates 46,9% of CCR5-knockout. Almost all of those cells showed resistance towards transduction with HIV-1, whereas the other ones changed HIV-tropism, proving that TALEN is an effective gene editing method.

3. The clustered regularly interspaced short palindromic repeats/Cas9 System (CRISPR/Cas9)

The CRISPR/Cas9 system is a novel gene modification method in which guide RNAs (gRNA) direct Cas9 to determined sequences of DNA, and Cas9 cuts both strands at a precise location. The genomic DNA is repaired by NHEJ or HDR, leading in mutations, which can cause gene inactivation. To keep its cleavage efficacy, the dual-crRNA: tracrRNA complex was designed as a single transcript (sgRNA) that is required for Cas9's binding and cutting DNA targets.⁶³

Recent experimental trials transduced a CXCR4-gRNA/Cas9 to Jurkat T-cells with the aim of disrupting the CXCR4 gene. To test whether this alteration conferred protection to HIV-1, cells were infected with CXCR4 viral strains and later on they performed p24 antigen ELISA assays. Results show that the p24 level of HIV-1 is much lower in genome-edited Jurkat T cells compared to the control one (**Figure 9**).^{63, 64}

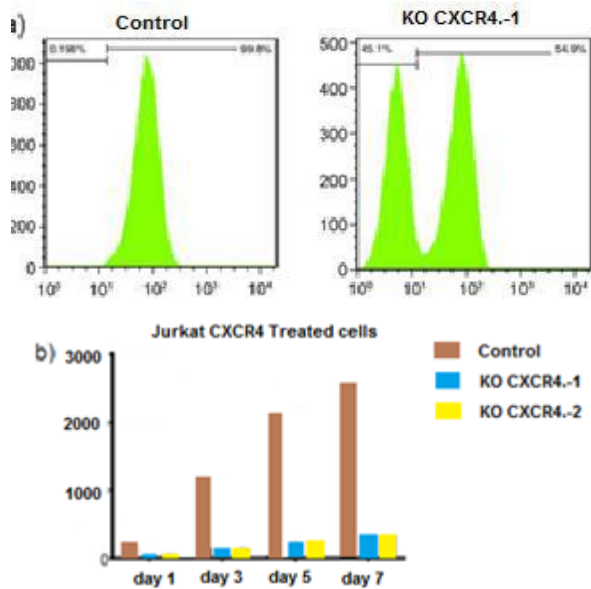


Figure 9. Jurkat T-cells treated with CRISPR/Cas9 confer resistance against HIV-1 X4 strains A)

CXCR4 gRNA/Cas9 was transduced to Jurkat T-cells. FACS analysis show the lack of CXCR4 expression in treated cells (KO CXCR4.-1) in front of the control ones. **B)** Jurkat treated T-cells are highly resistant to HIV-1 infection, as shown in the p24 antigen expression, proving that CXCR4 knockout avoids viral entry to the cell.

4. Small interfering RNA (SiRNA)

SiRNAs are small pieces of synthetically derived RNA of 20-25 base pairs. SiRNA can target specific regions of the genome and it is used to promote the degradation of messenger RNA (mRNA) after the transcription, resulting in no gene expression.

Clinical tests have used FUGW, an HIV-1 based lentivirus vector as the backbone of the siRNA. The short hairpin was transcribed from a human 6-RNA polymerase III (Pol III) promoter.^{39, 65, 66} The anti-CCR5 siRNA construct directed to the 186–204 region [CCR5-siRNA (186)] resulted in >90% reduction of CCR5 expression. SiRNA directed to CXCR4 did not suppress CCR5 expression or vice versa.^{65, 66} Transfection with siRNA containing mismatches to the target sequence in the middle of the siRNA molecule reduced the gene silencing efficiency, so the inhibition of HIV is incomplete.^{66, 67} Even though siRNAs are target specific, some viral mutants have been documented, so different regions of the viral genome with shRNA would be need to be targeted

to reduce the probability of generating escape mutants.^{14, 67, 68}

5. Small hairpin RNA (shRNA)

ShRNA is an artificial RNA molecule which differs from siRNAs by a more stable secondary structure (hairpin loop) that can be used to inhibit gene expression by a sequence-specific RNA degradation mechanism termed RNA interference (RNAi)^{69, 70}

In some in vitro assays, LVsh5/C46 has been used as a lentiviral vector expressing two viral-entry inhibitors to block HIV-1 cycle: a short hairpin RNA (shRNA) to inhibit CCR5 and C46, the HIV-1 fusion inhibitor peptide. 69CCR5-targeted shRNA (sh5) and C46 peptide were able to protect gene-modified cells against CCR5- and CXCR4-tropic strains of HIV-1.^{69, 70} Over 40% transduction of LVsh5/C46 into various hematopoietic cells, including T cells, was effective. The treatment was nontoxic as assessed by cell growth and viability, it was noninflammatory, there was no apoptosis, had no adverse effects and resulted active viral particles with very low mutagenic potential and absence of replication-competent lentivirus^{69, 70, 71}. Co-expression of the two anti-HIV-1 genes has no influence on their stability. Recent studies have shown detection of gene modified T lymphocytes 11 years after the infusion, so it suggests the high viability and that they may persist for decades with continued expression and activity.⁷² There is currently an open clinical trial that employs the use of lentiviral vectors to express shRNA with C46 peptide to CC55 (NIH clinical trial NCT01734850).⁷³

6. Recombinant adenovirus containing antisense CCR5

Antisense RNA can be introduced into a cell through an adenovirus and inhibit translation of a complementary mRNA by base pairing and obstruct the translation machinery.^{39, 74}

Experimental trials have used recombinant adenovirus containing antisense CCR5 cDNA (Ad-antiR5) which was obtained by homologous recombination of the plasmid with the adenoviral backbone plasmid in *E.coli* and then packed in AD-203 cells.⁷⁴ In the antisense RNA method, the appropriate size of the foreign gene for effective suppression of the target is around 700 pb, whereas this study used a fragment of 653 bp in order to

have a higher efficiency of inhibition.⁷⁴ Positive CCR5 on U937 (human white histiocytic lymphoma cells) surface decreased from 89,35% to 1,88% after 24 hours. Treated cells also produced less p24 antigen when challenged against HIV-1 virus. The adenovirus can protect cells from HIV-1 without effects on their chemotaxis activity and proliferation function.⁷⁴

7. Ribozymes cell-delivered gene therapy

Ribozymes are small catalytic RNA molecules that can act like protein enzymes and can target specific RNA sequences.^{39, 75}

To test its effectiveness to inhibit CCR5, a CCR5-specific single-chain antibody was expressed intracellularly. This CCR5-intrabody blocked surface expression and prevented cellular interactions with nonhuman HIV-1 because the ST6 antibody reacts with a conserved primate epitope on CCR5, so this strategy can be used in SIV and chimeric simian-human immunodeficiency virus (SHIV).^{39, 75, 76} In another study with gene therapy,⁷⁴ HIV-1 infected adults received OZ1, which is a retroviral vector that contains a gene encoding a ribozyme that targets the *vpr* and *tat* reading frames of HIV-1, or placebo delivered in autologous CD34+ hematopoietic progenitor cells. There was no difference in viral load between them until weeks 40-48 and 40-100, which time weighted areas were lower in the OZ1 group.^{76, 77, 78} During the treatment interruption, control patients needed a re-initiation of cART after 29 weeks, whereas for the OZ1 group it was greater than 60 weeks. Throughout the 100 weeks, CD4+ lymphocytes were also higher in the OZ1 group. There was no notice of resistance mutations in the region of HIV-1.^{76, 77}

This gene therapy is a combination of Tat/Rev shRNA, and Tat activation-response region (TAR) decoy.^{77, 78} Reports revealed that CCR5 ribozyme maintained the stability for up to 24 months and that there was no evidence of viral resistance through the 100 weeks trial, but an improvement in the transduction process is required.⁷⁸

KNOCKOUT OF CCR5 AND CXCR4

Knockout of CCR5 and CXCR4 through gene editing tools has proved to be effective against viral infection, relying on the fact that HIV-1

requires them to entry into the cell and that without them viral infection is not possible. Still, now we're going to discuss about CCR5 and CXCR4 known-roles and the necessity of inhibiting it instead of knocking them out. First of all, CCR5 doesn't seem to play a vital role in the cell, as deficient CCR5 mouse have develop healthy without any diseases. Humans which suffer from the $\Delta 32$ have also developed healthy lifestyles with no problems related with the lack of the co-receptor.^{22, 23} Whereas CCR5 doesn't seem to be vital, CXCR4 plays a key role in immune system cells development as it's up-regulated by T cell receptor activation, so it's a vital protein to maintain the homeostasis of immune system cells (13). Its knockout will decrease our immune barrier and, in fact, we'll suffer from more bacterial and viral infections. CXCR4 deficient mouse died in utero and were defective in vascular development, showing that they are necessary for cell's survival.^{24, 25}

We suggest a partial inhibition of CCR5 and CXCR4 instead of knocking them out using either antagonists or epigenetical changes on their expression. A study reports the use of Zebularine to inhibit DNA methylation in order to up-regulate CCR5 expression in Jurkat T cells, but it hasn't been tested to downregulate its expression as a possible novel HIV-1 therapy.¹³

INHIBITION OF CCR5 AND CXCR4 FROM CD4+T CELLS WITH ZFN

In an experimental trial *in vitro*, CD4+T cells were treated with 2 ZFN pairs targeting the HIV-co-receptors CCR5 and CXCR4 to stop their expression. To determinate which ZFN would be used, human SupT1-R5 T-cell line were co-transduced with vectors encoding the R5-ZFN and X4-ZFN.^{82, 83} The administration of both ZFN caused a reduction of the expression of both co-receptors with 9% of cells no longer expressing either co-receptor. After 25 days, 96% to 99 of the treated cells did not express either CCR5 or CXCR4 and the most part of them were highly resistant to either R5 or X4-HIV strains. Low levels of residual infection were observed in the R5/X4-ZFN group, and the majority of this residual infection occurred in the 1% to 4% of cells expressing CCR5 and CXCR4.^{39, 82, 83} By 32 days postinfection of the CD4+T cells with a mix of HIV R5-strains and X4- strains, there were no live

cells in the groups that receive ZFN or R5-ZFN alone, whereas cells from the R5/X4 ZFN group continued to expand.⁸²

Then, this was tested *in vivo* with a group of 18 mice treating them with CD4+T cells with no ZFN, the R5-ZFN alone and both ZFNs simultaneously. The results showed that animals that received both ZFNs were better able to maintain the CD4+T-cells counts following challenge with R5- and X4-HIV, and the counts were higher than the other groups.^{39,83} The next step is to test it in humans to see possible effects and the rating of effectiveness that it has into T-cells *in vivo* (Figure 10).⁸³ This data suggest that the use of two ZFN pairs might be a viable strategy to disrupt CCR5 and CXCR4, resulting in cells resistant to HIV.

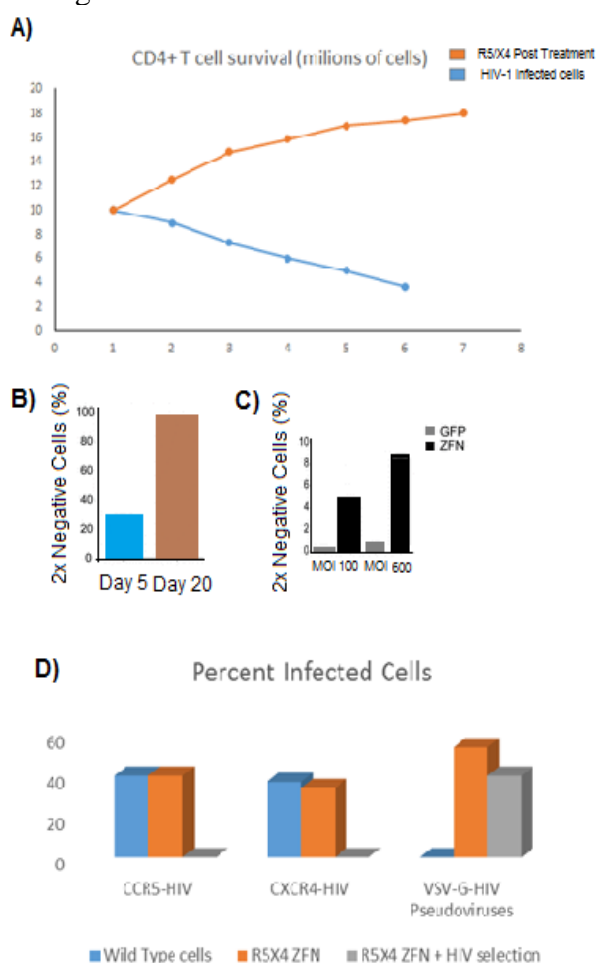


Figure 10. Simultaneous ZFN inhibition of CCR5 and CXCR4 protects SupT1R5 T cells from infection with viruses that use one of those co-receptors.

A) Treatment with ZFN provides a higher cell survival rate than HIV-1 infected cells. B) Measurement of double negative cells 25 days postinfection. As previously seen, treated cells survive whereas infected cells levels decrease in

time as well as HIV-1 viremia levels. C) Proportion of cells lacking of surface co-receptors expression (2x negative cells). D) Dual ZFN treated cells rechallenged with HIV proves effective protection against the virus, as shown in the graphic.

DISCUSSION

Today, HIV has entered in a new era. cART treatment helps controlling infection and increasing people's survival. Lack of an effective vaccine to beat HIV makes it the first obstacle into HIV prevention. The case of the *Berlin Patient* opened a window on this kind of therapies. Currently, we know that protection against HIV can be achieved by suppressing the chemokine receptor CCR5 to low levels. We know that a small percentage of population, less than 1% have got a mutation called *delta32*, which gives an innate protection against R5-HIV strains, and that is what new potential therapeutic strategies are mimicking artificially with gene therapy. The first patient who was successfully cured from HIV is the *Berlin Patient*. He received a HSC with CCR5-Δ 32 homozygous modified cells and, after a few weeks, the number of viremia decreased and the modified T-cells reproduced while infected cells died. After this, the patient controlled HIV without the use of ART treatment and after 7 years, he is now the first man to be cured from HIV.

With the achievement obtained with the *Berlin Patient*, the same clinical trial was done again with another man, the *Essen Patient*. He also received a HSC with autologous CCR5- d32 homozygous modified T-cells, but this time the trial failed because the patient rebound viremia with HIV quasi-species using an alternative chemokine receptor, CXCR4. This let us know that we need to inhibit both receptors and don't stop ART during HSC treatment.

In this review, we highlighted some of the different gene therapy strategies used to inhibit the expression of both receptors. Among these therapies, the more promising seem to be the ZFNs and CRISPR-Cas9 because they proved very high efficiency, low toxicity, complete suppression of CCR5 or CXCR4 in the threatened cells and a long-time live period of this cells. Other strategies showed some disadvantages: TALEN did an incomplete suppression of HIV replication in

clinical trials and could only recognize one nucleotide instead of three; siRNA treatment produced HIV-mutants; Ribozymes cell-delivered therapy proved effectiveness with the SIV and chimeric simian-human immunodeficiency virus, but not with HIV. Recombinant adenovirus and shRNA need further investigation, but the clinical assays have showed us effectiveness in CCR5 expression. The first thing we have to do is to reduce HIV infections. Recently, a new biomedical tool, called *Truvada*, has been able to reduce HIV infections because it has been approved as a prophylactic against HIV.

In our opinion, autologous HSCT with modified cells without HIV is a very useful technique, but we also need to think for a cure which everyone would have access to. People with homozygous $\Delta 32$ mutation have no effects without expressing CCR5 on their cells, but the knock-down of CXCR4 *in vivo* could produce disadvantages because this receptor is essential in immune responses and also serves as a vital factor for humans. Even therapies targeted against CCR5 and CXCR4 are very promising, long-lived reservoirs still remain infected with HIV, making this a functional cure instead of a sterilizing cure because the viral genome remains. Even if there is no further infection of new cells, this does not ablate any affects caused by viral RNAs and proteins that may be synthesized and secreted by persistently infected cells, so gene therapies targeting co-receptors would be functional cure instead of a sterilizing one. A sterilizing cure achieves a complete eradication of all replication-competent forms of HIV. The reservoir is gone whereas in a functional cure the reservoir remains there but there is a permanent control of viral replication without the need of cART. Recently, CRISPR/Cas9 has been to target the viral genome but it has escape due to the development of some mutations in the place where CRISPR's Cas0 enzyme had been programmed to cut.

The main study suggests to target multiple sequences at the same time using different gene therapies like the ones reported above or to combine them with HIV-attacking drugs.

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