 doi:10.20944/preprints201907.0211.v1

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

Tatiana Hillman[1] https://orcid.org/0000-0002-7539-0512

[1] Biological Sciences, TheLAB Inc., Los Angeles, California, United States

Corresponding Author:
First Author[1]
Email address: thillman@usc.edu

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 application of CRISPR-Cas9 technology, has given researchers a 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. The CRISPR-Cas9 can delete point mutations and multiple DNA sites. Because CRISPR can alter DNA sequences, several scientists have conducted research into CRISPR possibly curing more diseases 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 the HIV-1, inhibiting HIV-1 gene expression. Since 2013 several other researchers have blocked HIV replication and infection through the 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 that consists of co-receptors CCR5 and CXCR4. If CCR5 expression can be removed, the HIV virus can not bind to T-cells, blocking the initial attachment stage, 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) improve 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.

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

 doi:10.20944/preprints201907.0211.v1

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 the death rate of HIV and AIDS. HIV-1 causes AIDS. HIV-1 infects CD4+ T cells and in the long term eradicates most infected T cells. The structure of the HIV virus includes a capsid core containing the RNA and enzymes. It has an outer surface called the envelope. From the envelope protrudes glycoproteins as glycoprotein 120. RNA is the viral genetic material. The glycoproteins attach to the $CD4^+$ receptors of T cells with assistance from the CCR5 and CXCR4 co-receptors. After binding, the HIV envelope fuses with the cell membrane of the T cell, entering the cell. The RNA and enzymes are released. The reverse transcriptase converts its RNA into DNA, which the DNA then enters through the CD4+ cell nucleus.

The HIV-DNA binds to CD4 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 then translating the HIV-DNA for making more viruses. The newly synthesized HIV proteins and RNA are transported to the surface of the cell membrane. The new HIV proviruses bud and exit from the T cell. The new HIV viruses secrete a protease, breaking a part 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 of the capsid core produce weakened capsid cores. The less stable capsid core after an integrase mutation show that integrase fortifies the capsid core (Campbell and Hope, 2016). Reverse transcription is involved with stabilizing the capsid core. When mutating the 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 less 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, can restore and increase T-cells in SIV- infected monkeys, which 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 the improved health of HIV-1 positive individuals. After treatment with probiotics, researchers found less T-cell activation, an increase of T-cells, 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 mitochondria structure (d'Ettore et al., 2017). Also, there were fewer immune responses to and less activation of CD4 and CD8 T-cells.

There is no cure for HIV-1. The only cured person known within the past 10-year span was the Berlin Patient. The Berlin Patient was transplanted with stem cells from a donor. The stem cells possessed a mutation in the HIV coreceptors of CCR5. The mutation was homozygous or a (CCR5Δ32/Δ32). The transplantation was applied to treat his leukemia. Another positive

 doi:10.20944/preprints201907.0211.v1

HIV-1 patient was transplanted with stem cells to treat his lymphoma. Again, the cells had mutations in the (CCR5Δ32/Δ32). After 16 months of the stem cell treatment, he no longer needed antiretroviral medicine. The 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 the CD4 T-cells (Gupta et al., 2019). The antibodies of the HIV-1 decreased also. Targeting CCR5 with gene therapies may result in the remission of HIV-1.

Currently, there are many new and promising treatments of HIV-1 as gene therapy, Transcription activator-like effector nuclease or TALENS, and CRISPR-Cas9sgRNA. Nevertheless, the central idea of this review focuses on the 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 the 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

Literature was examined to discuss and outline possible solutions for CRISPR-Cas9 targeting CCR5. One-hundred fifteen total articles were screened from google scholar and NIH 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 deliver, 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 conclusions that confirmed the effectiveness of the techniques for CRISPR-Cas9 targeting the CCR5 of HIV-1, which are described in this review.

2 THE CRISPR-CAS9 MODIFICATION OF CCR5

CRISPR gene editing has successfully been implemented for treating the 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 for limiting 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 through a transfection of Cas9 plus the sgRNAs. Ye et al. in 2014 conjoined CRISPR/Cas9 with TALENS, producing pluripotent stem cells, which induced the homozygous CCR532/32 mutation. The iPSCs derived into white blood cells that cannot become infected with HIV.

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 the CCR5 for a longer

 doi:10.20944/preprints201907.0211.v1

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 cure, 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) improve 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, 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 proximity of tumors. Near and adjacent to the endothelial layers of tumors, the tumors maintain a 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 to identify 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, the assembly of the liposome is contingent upon the location of the site of the lipid 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 the reservoirs in the GALT. However, nanoparticles as drug polymers, micelles, and liposomes may offer an 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 a M-cell-targeting ligand to the outer region of the nanoparticle. The addition of the ligand to the nanoparticle would increase 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. The PEG allows the nanoparticles to directly target the protein receptors on the outer surface of M cells. This macromonomer with the ligand specific for M cell receptors called

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 18 July 2019 doi:10.20944/preprints201907.0211.v1

the GRGDS peptide had a minimally low molecular weight of methacrylate at a MAv=400 (n=7) and 2000 for the PEGs region (n=4) (Ogunwuyi et al., 2016). The structure of the 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. The 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 a 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. The 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 available now 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 The 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 binding 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 of 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 for avoiding HIV-1 viral escape. Hutter et al. (2015) proposed decreasing the HIV reservoir, using gene therapy that targets CCR5, and testing 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. The 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, which can be orally ingested and is currently being studied in clinical trials.

 doi:10.20944/preprints201907.0211.v1

CONCLUSION

The ultimate purpose for this review was to present possible alternative ways and means to overcome the obstacles for targeting HIV-1 with the 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 the gut-associated-lymphoid tissue or the GALT. Evidence was presented confirming the successful editing and modification of the T cell co-receptor, CCR5, by CRISPR-Cas9 technology.

The issues that may limit the efficacy of CRISPR-Cas9 for editing CCR5, which can result in a 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: use of lipid nanoparticles for safer delivery of CRISPR-Cas9, enhancing antiretroviral drug design to effectively lessen the HIV reservoir size, modifying 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 study is 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 for finding solutions to formulate the lipid nanoparticles with less toxicity and more safety. Also, the final place in the body where each component of the nanoparticle remain needs more research and studies to confirm the precise mechanism for excretion of the nanoparticles.

ACKNOWLEDGEMENTS

Special thanks are given to TheLAB Inc., and their staff. The weekly genetic engineering lab workshops provided and the meetings for the discussion of our projects helped to increase my understanding of the fundamentals of homologous recombination and the role microbes play in the field of gene editing.

Author Contributions: T.H., wrote, reviewed, and edited the manuscript.

Funding: The author received no specific funding for this work.

Conflicts of interest: The author declares no conflict of interest.

REFERENCES

1. Bolhassani, A., Safaiyan, S. and Rafati, S., 2011. Improvement of different vaccine delivery

systems for cancer therapy. *Molecular cancer*, *10*(1), p.3.

2 Campbell, E.M. and Hope, T.J., 2015. HIV-1 capsid: the multifaceted key player in HIV-1 infection. *Nature Reviews Microbiology*, *13*(8), p.471.

3 Cho, S.W., Kim, S., Kim, J.M., and Kim, J. S. (2013). Targeted genome engineering in human cells with the Cas9 RNA-guided endonuclease. Nat. Biotechnol. 31, 230–232. doi: 10.1038/nbt.2507

4 d'Ettorre, G., Rossi, G., Scagnolari, C., Andreotti, M., Giustini, N., Serafino, S., Schietroma, I., Scheri, G.C., Fard, S.N., Trinchieri, V. and Mastromarino, P., 2017. Probiotic supplementation promotes a reduction in T-cell activation, an increase in Th17 frequencies, and a recovery of intestinal epithelium integrity and mitochondrial morphology in ART-treated HIV-1-positive patients. *Immunity, inflammation and disease*, *5*(3), pp.244-260.

5 Ebina, H., Misawa, N., Kanemura, Y., and Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci. Rep. 3:2510.doi: 10.1038/srep02510

6 Gupta, R.K., Abdul-Jawad, S., McCoy, L.E., Mok, H.P., Peppa, D., Salgado, M., Martinez-Picado, J., Nijhuis, M., Wensing, A.M., Lee, H. and Grant, P., 2019. HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature*, p.1.

7 Hillman T. 2018. Integrating the extracellular, intracellular, and intercellular pathogenic processes of the microbiome through glucose saturation, inhibition of the acetyl-CoA carboxylase subunit accA with asRNA, and through quantifying cell-to-cell quorum sensing. PeerJ Preprints 6:e27459v1 https://doi.org/10.7287/peerj.preprints.27459v1

8 Hillman T. 2019. A review: Possible optimization of Cas9-sgRNA nuclease delivery via ingested lipid nanoparticles bioencapsulated within plant cell-based enfolding. PeerJ Preprints 7:e27709v2 https://doi.org/10.7287/peerj.preprints.27709v2

9 Hütter, G., Bodor, J., Ledger, S., Boyd, M., Millington, M., Tsie, M., & Symonds, G. (2015). CCR5 Targeted Cell Therapy for HIV and Prevention of Viral Escape. *Viruses*, *7*(8), 4186–4203. doi:10.3390/v7082816

10 Lino, C.A., Harper, J.C., Carney, J.P. and Timlin, J.A., 2018. Delivering CRISPR: a review of the challenges and approaches. *Drug Delivery*, *25*(1), pp.1234-1257.

11 Li, C., Guan, X., Du, T., Jin, W., Wu, B., Liu, Y., et al. (2015). Inhibition of HIV-1 infection of primary CD4+ T-cells by gene editing of CCR5 using adenovirusdelivered CRISPR/Cas9. J. Gen. Virol. 96, 2381–2393. doi: 10.1099/vir.0.000139

12 Ogunwuyi, O., Kumari, N., Smith, K.A., Bolshakov, O., Adesina, S., Gugssa, A., Anderson, W.A., Nekhai, S. and Akala, E.O., 2016. Antiretroviral drugs-loaded nanoparticles fabricated by dispersion polymerization with potential for HIV/AIDS treatment. *Infectious Diseases: Research and Treatment*, *9*, pp. IDRT-S38108.

13 Ries, O., Löffler, P.M. and Vogel, S., 2015. Convenient synthesis and application of versatile nucleic acid lipid membrane anchors in the assembly and fusion of liposomes. *Organic & biomolecular chemistry*, *13*(37), pp.9673-9680.

15 Xiao Q, Guo D and Chen S (2019) Application of CRISPR/Cas9-Based Gene Editing in HIV-1/AIDS Therapy.Front. Cell. Infect. Microbiol. 9:69.doi: 10.3389/fcimb.2019.00069

16 Xu, L., Yang, H., Gao, Y., Chen, Z., Xie, L., Liu, Y., et al. (2017). CRISPR/Cas9-Mediated CCR5 ablation in human hematopoietic stem/progenitor cells confer HIV-1 resistance in vivo. Mol. Ther. 25, 1782–1789.doi: 10.1016/j.ymthe.2017.04.027

17 Ye, L., Wang, J., Beyer, A. I., Teque, F., Cradick, T. J., Qi, Z., et al. (2014). Seamless modification of wild type induced pluripotent stemcells to the natural CCR5Delta32 mutation confers resistance to HIV infection. Proc. Natl. Acad.Sci. U.S.A. 111, 9591–9596. doi: 10.1073/pnas.1407473111