CRISPR BASED GENE EDITING CONFERS RESISTANCE TO HUMAN IMMUNODEFICIENCY VIRUS (HIV)

Goals

1. **Creation of a novel CRISPR lentiviral vector and lentiviral particles**
2. **Gene disruption of CCR5 to eliminate phenotypic expression of the CCR5 co-receptor**
3. **Contribution of a new set of immune cells that confer resistance to HIV infection**

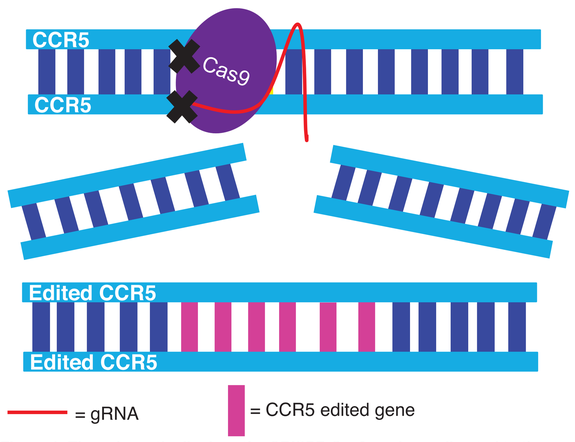


Figure 1: The schematic displays the CRISPR Cas9 nuclease directed to the site of cleavage by the specific gRNA. Cas9 induces a double stranded break in the DNA, leading to Non-Homologous End Joining/inaccurate DNA repair. The result is an edited CCR5 gene.

Introduction

Nearly 50,000 people are infected by HIV in the US each year and 2.2 million people were newly infected globally with HIV in 2014. Current HIV therapies lead to drug resistance and fail to address the latent viral reservoir which reactivates and inevitably leads to AIDS and mortality. Therefore, HIV infection is an unmet medical need.

Chemokine Receptor Type 5 (CCR5)

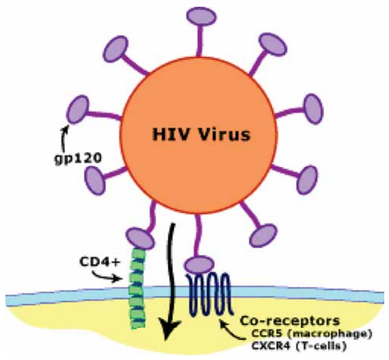


Figure 2: Graphic adapted from HIV/AIDS Newsroom, showing coreceptor mediated HIV infection into T-cells

HIV infects human T-cells via the cluster of differentiation glycoprotein (CD4) receptor and one of two coreceptors, chemokine receptor 5 (CCR5) or chemokine receptor type 4 (CXCR-4) (figure 2). Research has demonstrated that most infecting HIV virus strains have a strong preference for CCR5 (Shaw & Hunter, 2012), making them CCR5 or R5 tropic. Consequently, individuals who are naturally homozygous defective for CCR5 expression (CCR5Δ32) are protected from HIV-1 infection (Swan, 2006). This mutation does not appear to be detrimental. In fact, the "Berlin patient" was a HIV patient treated for acute myeloid leukemia by a restorative transplantation with bone marrow cells from a HLA-matched donor who also happened to be CCR5Δ32. This resulted in repopulation of these HIV resistant CCR5Δ32 cells in his body which rendered him aviremic and cured him of HIV.

CRISPR

The type II CRISPR (clustered regularly interspaced palindromic repeats) system is a versatile tool for gene editing and can be employed for introducing changes in DNA gene sequences. CRISPR-based systems employ two key components; a guide RNA (gRNA) and a non-specific nuclease (Cas9). The chimeric gRNA directs Cas9 to the target sequence. It is designed to cleave the bound DNA at that site. Cas9 causes a double strand break (DSB), which can be repaired through either Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR) repair systems in the cell. NHEJ leads to insertions and deletions at the target site, which disrupt the target (Figure 1). HDR introduces specific nucleotide modifications. Therefore, one can change the target sequence by careful gRNA design. Accordingly, mutating the CCR5 gene using the CRISPR-Cas9 system should lead to a gene therapeutic strategy for HIV.

Lentiviral Delivery

A major challenge of gene therapy for HIV to date has been effective delivery of gene editing systems into primary T cells, the major target for HIV. As HIV, a lentivirus effectively infects and gets stably integrated into T cells. Therefore, a lentivirus based, non-proliferative viral vector allows efficient transfer of the gene editing system into T-cells (Dampier, Nonnemacher, Sullivan, Jacobson, & Wigdahl, 2014). **The overall goal of this project is to disrupt the CCR5 gene using the CRISPR-Cas9 system delivered via lentiviral vectors to generate a pool of HIV resistant human T cells that can be used in clinical gene therapy to confer resistance to HIV-1**.

Results

- CCR5 targeting gRNA was successfully inserted into the pL.CRISPR.SFFV.GFP vector: colony PCR revealed that 100% of the colonies contained the insert (Figure 8). Furthermore, the cloned lentiviral vector (Figure 7) was sequenced to confirm that the nucleotide sequence of the insert matched the original sequence of the designed gRNA.
- T7 endonuclease assay of genomic DNA isolated from human T cells treated with lentivirus generated from 293T cells (see Methodology) revealed nearly 90% gene editing of CCR5 as determined by fragment analysis of the average molar concentrations of the uncut and cut products (Figure 9).
- Flow cytometric analysis of gene edited T cells revealed that surface CCR5 expression was significantly reduced. 31% of cells did not express the CCR5 co-receptor while surface expression of other T cell markers like CD3 and CD4 remained unchanged demonstrating the specificity of the approach to CCR5 (Figures 10, 11). Therefore, HIV will no longer be able to infect these cells via CCR5.

Molecular Cloning

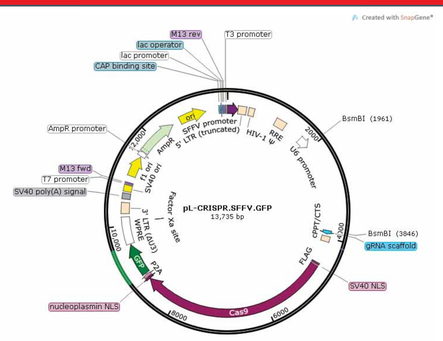


Figure 6: Plasmid map of lentiviral vector plasmid pL-CRISPR.SFFV.GFP acquired from AddGene Only the used restriction enzyme is displayed (BsmBI). Noteworthy in the plasmid is the presence of the Cas9 endonuclease and of a gene for expression of the reporter green fluorescent protein (GFP). The map was created using a software, SnapGene.

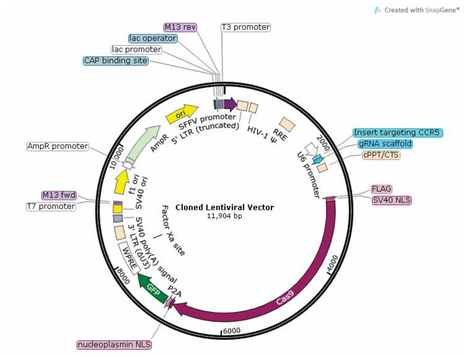


Figure 7: Plasmid map of the cloned lentiviral vector plasmid pL-CRISPR.CCR5.SFFV.GFP now containing the gRNA targeting CCR5. Note: green fluorescent protein (GFP) is in green, the insert targeting CCR5 in light blue, the gRNA scaffold in blue, and the Cas9 nuclease in dark purple. The map was created using a software, SnapGene.

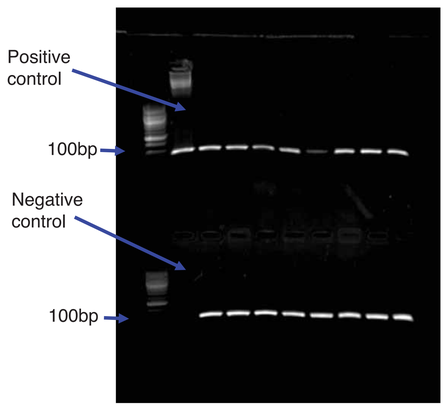
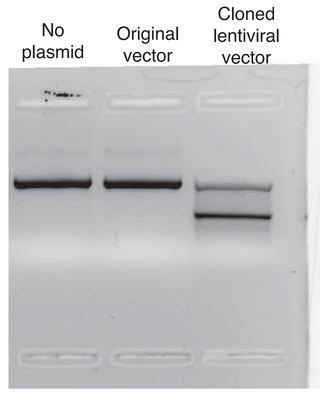


Figure 8: A 0.8% agarose gel with PCR products from DNA in bacterial colonies detailed in Methodology. 100% of the checked colonies contained the amplified insert. The clones were further sequenced and confirmed to have the CCR5 targeting gRNA.

CCR5 Gene Editing

Figure 9: A 0.8% agarose gel with products from T7 endonuclease assays indicates gene editing of CCR5 in 293 T-cells. The presence of multiple bands indicates gene editing with the bands at ~6000bp and 4000bp denoting wild type and edited gene forms respectively. The first lane corresponds to genomic DNA isolated from cells which were not transfected with any plasmid. The second lane corresponds to genomic DNA isolated from cells which were transfected with L.CRISPR.SFFV.GFP (the lentiviral vector not containing the CCR5 targeting gRNA). The third lane corresponds to genomic DNA isolated from cells which were transfected with L.CRISPR CCR5.SFFV.GFP (the lentiviral vector containing the CCR5 targeting gRNA). The last lane depicts nearly 90% gene editing computed by fragment analysis of the average molar concentrations of the uncut and cut products.



Results: CCR5 Expression

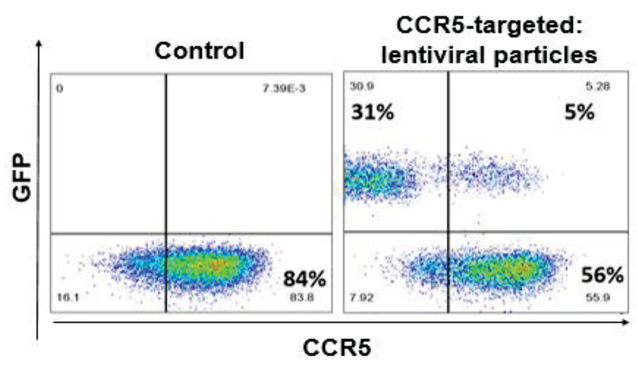


Figure 10: Flow cytometric dot plots of CD3+ CD4+ T cells depicting GFP expression (gene marked cells) and CCR5 expression. The plot on the left shows untransduced cells (negative for GFP expressions) have a high level of CCR5 expression (84%). After transduction with CCR5-targeting lentiviral particles, a majority of the cells that are gene marked (positive for GFP expression, so in the upper two quadrants) are negative for CCR5 expression (31% of the total cells or 86% of the gene marked cells). However, among the GFP negative cells (lower quadrant), all the cells continued to express CCR5.

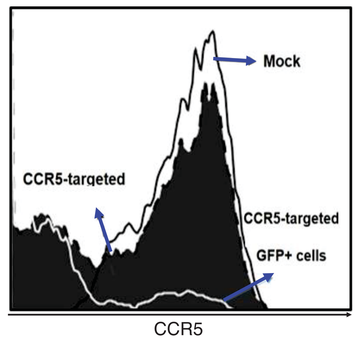


Figure 11: Flow cytometric histograms for CCR5 cell surface expression showing a reduction in CCR5 cell surface expression in total T cells exposed to lentivirus (solid gray histogram) in comparison to cells that were not exposed to lentivirus (open gray histogram). The white histogram depicts CCR5 expression in GFP positive cells alone showing a marked reduction in marked cells.

Discussion

Without CCR5, R5-tropic HIV cannot infect cells. Therefore editing CCR5 expression presents a potent therapeutic option for HIV patients, as opposed to the option of lifelong antiretroviral drug therapy. This project demonstrates a method to reach a significantly high level of CCR5 gene editing (nearly 90%) with a single transduction with lentivirus. This high level of gene editing was due to efficient delivery by the lentiviral vector. One caveat of the protocol is that the lentiviral vector and its components get integrated into the genome. The effects of integration over time are not currently known, however, current clinical trials using lentiviral vectors for gene therapy of HIV and other diseases have not shown untoward effects for greater than a 10 year period. In the future, it would be beneficial to devise a strategy that can limit the expression of Cas9 and the gRNA in the host cell to a small period of time.

In vitro results indicated that functional CCR5 editing can occur in 56% of T cells exposed to the lentivirus even though genomic analysis demonstrated 90% editing at the genomic level. This discrepancy is because the T7ENI assay does not distinguish between single and double allelic gene editing events and cells that were edited at a single allele could continue to express CCR5 albeit at lower levels than the wild type counterparts. Nevertheless, there is ample evidence to indicate that even these hemizygous cells have a greater resistance to HIV infection. It is important to note that upon HIV infection, even if there is a mixed population of resistant and sensitive cells, the sensitive cells will ultimately die due to the infection while the resistant gene edited cells will continue to expand due to a survival advantage and eventually reconstitute the previously depleted immune system. The entire immune system is then resistant to HIV.

There are some limitations to the current research. A primary concern with gene editing therapy is the potential of other edits within the genome, i.e., off-target effects. Although these off-target effects are yet to be fully assessed here, significant off-target effects are unlikely because of the specificity of the CCR5 gRNA and lentiviral vector. The CRISPR lentiviral vector system solely targets the T-cells and the CCR5 gene and the high percentage of gene editing demonstrated that.

Conclusions

- The primary goals were successfully met: (1) creation of a novel CRISPR lentiviral vector with a gRNA sequence that can bind to and disrupt the CCR5 gene, (2) generation of lentiviral particles, and (3) testing of these lentiviral particles in human T- cells.
- The results indicated successful disruption of the CCR5 gene that will confer resistance to HIV specifically in the HIV target cells (about 90% editing at the genomic level and 31% CCR5 knockout in T-cells). These gene edited T-cells will enable the immune system to reconstitute as the newly contributed immune cells are resistant to HIV infection via CCR5.
- HIV resistance, via HIV infection assays, is underway and will be translated to an *in vivo* setting.
- **This genetic editing project presents a potential for HIV treatment with future applications of single round gene therapy to confer complete resistance to HIV as opposed to the current lifelong HIV therapies.**
- **In summation, a novel CRISPR based lentiviral vector system was developed to disrupt the CCR5 gene and confer resistance to HIV.**
- The gene therapy method successfully designed and implemented here can be applied to countless other diseases including leukemia in the future. It also enables easier access to personalized medicine, where therapy is specific to the patient's genetics.

Methodology

gRNA Design for CRISPR targeting of CCR5

Potential gRNA sequences were aligned with the CCR5 gene sequence and a target sequence was identified within the CCR5 gene. The gRNA was selected based on predicted off-target and on-target activity. The designed gRNA sequences were synthesized as forward and reverse oligonucleotides/primers containing the overlapping 'sticky' sequences for the restriction sites BsmB1present in the lentiviral plasmid detailed below.

Molecular Cloning

A plasmid encoding a lentiviral vector, pL-CRISPR.SFFV.GFP (figure 6) (AddGene #57827) was chosen based on (i) the presence of the Cas 9 nuclease and (ii) the presence of a reporter, green fluorescent protein (GFP) under the control of a SFFV promoter. This plasmid has a multiple cloning site with restriction sites for the enzymes BsmB1 that were used for cloning. The plasmid was supplied in E.coli cells. These cells were cultured and amplified in LB-Ampicillin medium and the plasmid DNA was isolated using a DNA Purification kit. Next, the forward and reverse gRNA oligonucleotides were phosphorylated for cloning. The lentiviral vector DNA was digested with BsmB1 to create the proper ends for ligating. This ligated DNA was transformed into Stbl. 3 E. coli and cultured again in LB-Amp. The presence of the insert was confirmed by a colony PCR screening of 32 Stbl. 3 colonies. The colonies were used as the template DNA in a PCR reaction. The amplified region was the insert and the colony PCR is able to confirm whether the insert is present in the transformed bacteria. Lastly, the recombinant lentiviral vector DNA containing the gRNA sequences was isolated (Figure 3).

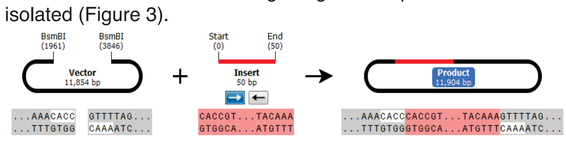


Figure 3: Schematic of cloning process: The vector is digested with BsmBI, the overhangs are ligated to the annealed oligonucleotides that encode gRNA.

Cell Culture

T-cells were cultured as following:
1. Cells were seeded at the appropriate density.
2. Cells were washed before fresh culture medium (DMEM) was added as required. DMEM was removed and cells were transferred from a previous culture to a fresh growth medium as appropriate. Returned the cells to the 37 °C incubator, 5% CO2.

Lentivirus Generation

1. Recombinant plasmids encoding lentiviral helper proteins (called helper plasmid), a viral envelope (called packaging plasmid) and the gRNA/Cas9 (called vector plasmid) described above were incubated at room temperature for 5 minutes with a lipid transfection reagent- Lipofectamine 2000.
2. The mixture was added to 293 cells seeded to be 70-90% confluent.
   - Cells were observed for GFP expression using a fluorescent microscope, which is in the plasmid.
3. After 2 days, the supernatant was collected.
4. Then, the supernatant was ultracentrifuged and the concentrated pellet of lentiviral particles re-suspended in a small volume.

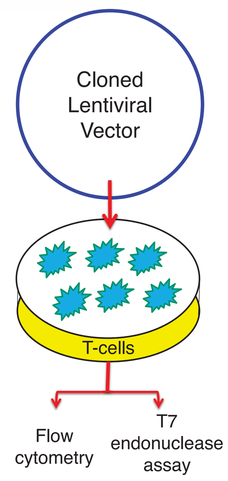


Figure 4: Schematic for testing CCR5-targeting lentiviral vector on human T cells

CCR5 Gene Editing

1) Peripheral mononuclear cells were isolated.
2) These cells were activated with phyto-hemagglutinin and the purified lentivirus was added to them.
3) These cells were monitored for GFP expression.
4) The cells were then stained with fluorescently labeled antibodies that detect human T cells as well as CCR5 after 7 days in culture.
5) The cells were then analyzed by flow cytometry.

T7 Endonuclease Assay (T7EI)

The genomic DNA was isolated and the edited gene was amplified in a PCR reaction. Then, the amplified products were digested by T7 endonuclease which cleaves if the DNA is not perfectly matched and, thereby, indicates the gene editing. The subsequent, fragment analysis of the uncut and cut products indicated a percentage of gene editing. All procedure specifics are found in the report.

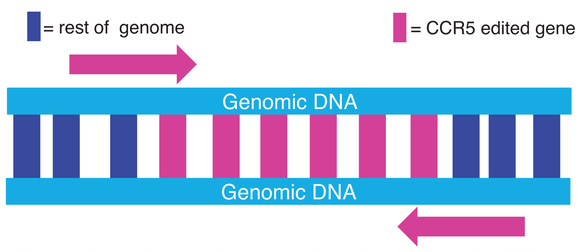


Figure 5: Schematic shows the amplification of the modified edited CCR5 gene in isolated genomic DNA from transfected T-cells. The pink arrows are the primers amplifying only the edited CCR5 gene out of the genomic DNA in order to assess the CCR5 gene editing. The amplified DNA was then digested with T7EI to indicate gene editing.

All graphics were created by the student researcher unless cited otherwise