EFFICACY OF CRISPR-Cas9 IN TREATMENT OF HIV-AIDS

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Abstract/Executive Summary

AIDS is a lethal viral infection that kills more than thousands of individuals every year as it spreads the virus via various modes of transmission. The modern era of biological research is exploring genome editing as a potentially effective way to prevent, treat and cure myriads of diseases with a novel genome engineering tool called CRISPR-Cas system which is now being studied worldwide as an attempt to treat and cure HIV-AIDS. Investigations have been carried from multiple research perspectives- from attempts to inhibit viral replication directly, preventing viral integration into host genome and disrupting specific cell-receptor coding genes in host to excision of provirus in latently infected cells in-vivo. Data collected from these investigations suggest potential of successful treatment of the disease. The limitations faced in the experimental protocols and results along with the gaps can be utilized as new focus of research investigation in this area.

Keywords: HIV-AIDS; CRISPR-Cas; gRNA; genome editing

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List of Acronyms

HIV	Human Immunodeficiency Virus
AIDS	Acquired Immunodeficiency Syndrome
CRISPR-Cas	Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR-associated
gRNA	Guide-ribonucleic acid
AAV-vector	Adeno associated virus-vector

Chapter 1

INTRODUCTION:

HIV-AIDS is a viral infection caused by Human Immunodeficiency Virus, whose primary symptoms range from flu-like mild infections and turns fatal due to extreme immunodeficiency, resulting in opportunistic infections and cancers in the host as the infection progresses.

According to WHO, HIV-AIDS is defined as a severe epidemic throughout the globe, infecting more than 38 million people all over the world causing eventual deaths, with new cases of infection reported each day. Despite the ongoing extensive research on the disease, no effective cure or vaccination have been found till date, with only two cures found based on the experimental trials. A series of treatment methods have been developed which allow the management of disease symptoms and in some cases, an increased lifespan in the affected individuals but is unable to bring permanent cure to the disease. In addition, the current treatment strategies are long term with multiple adverse side effects in the patients. (G.Maartens et al.,2014).

After the existing schemes of treatments for AIDS ranging from synthetic medicines to herb, animal or antibody based drugs, research is now leaning towards a cutting edge discovery of molecular biology- genome editing. It is a technique of addition, removal or modification of specific genome regions by producing splits in targeted nucleotides along the strand by enzyme nuclease followed by DNA repair using natural cellular mechanisms.(X.Liang et al., 2015).

Genome editing tools have been used to produce transgenic cell lines, microorganisms, plants and animals for addition of desired traits or removal of harmful ones, and is lately utilized to find cures of congenital diseases, genetic or hereditary diseases and disorders, cancers and infections such as HIV-AIDS.

Out of the various methods of gene editing such as Zinc Finger Nucleases, Meganucleases and Transcription-activator like effecter nucleases, one of the latest discovered is the CRISPR-Cas system, deemed as a potentially effective tool to diagnose and treat viral infections along with a range of other diseases. (D.S. Strayer et al.,2005)

CRISPR-Cas system forms a part of the prokaryotic adaptive immunity, a RNA-guided endonuclease enzyme able to cleave nucleic acids. (Mojica et al.,2000). As a major barrier to permanent cure of AIDS is the formation of latent viral reservoirs inside infected host cells, CRISPR-Cas system may act as a cleaving agent of viral genomes to damage the pathogen and/or parts of host genome to make them resistant to infection by the virus. On application, the specificity, efficiency, process, ease of delivery of the tool into target host cells and clinical feasibility in treating AIDS are important factors experimented on.

The following review article synthesizes the structure and mechanisms of CRISPR-Cas system and its functions as a genome editing tool particularly in potential cure of HIV-AIDS, describes the relevant experimental results which support the effectiveness of CRISPR-Cas system in treatment of HIV-AIDS, while assessing the limitations and gaps in the approaches being studied that may lead to further potential areas of research in the following area.

Chapter 2: CRISPR-Cas system and HIV-AIDS

2.1: CRISPR-Cas system:

CRISPR-Cas system is a RNA-bound endonuclease enzyme complex forming the adaptive immunity in prokaryotic microorganisms (Mojica et al.,2000).This enzyme is able to break down target DNA and RNA molecules with the guidance of RNA bonded to the endonuclease.

In the year 1987, a group of Japanese scientists studying on a given gene segment in *E.coli* genome accidentally discovered the CRISPR-Cas system without knowing its characteristic structures and functions. Upon further research, it was known that CRISPR-Cas is responsible for the adaptive immune responses in bacterial and archaeal cells as foreign agents such as plasmids, bacteriophages and other foreign components invaded them. (F.J.M Mojica et al.,2000).

CRISPR-Cas system contains small segments of repeat sequences arranged as palindromes clustered close together, with unique spacer sequences called spacer DNA in between that separate the repeat sequences. Frequency of spacer DNA can vary from a few to hundred depending on microbial species. These unique spacer sequences are incorporated into the repeat sequences of CRISPR-Cas from genetic material of foreign agents invading bacteria or archaea. The group of repeat sequences and the spacer DNA are named CRISPR genes, with genes located close to the CRISPR genes known as the cas genes coding for cas proteins, collectively forming the CRISPR-Cas system.

CRISPR locus has adjacent A=T rich regions containing regulatory elements such as promoters and signaling molecules to transcribe CRISPR regions into CRISPR-RNA or crRNA. Transcription of CRISPR regions can also result in trans-activating RNA that helps in maturation of crRNA. When crRNA attaches to tracrRNA, a single-stranded RNA called the single-guide RNA forms, which can guide the Cas protein to excise target nucleic acids.

As host prokaryotic cells are infected by a plasmid or bacteriophage, DNA fragments from these foreign agents combine into host CRISPR-Cas sequences, forming the spacer DNA sequences. If the same infectious agent invades the host cells later, it recognizes similarity between existing spacer DNA within CRISPR-Cas and that of invading species, thus activating the DNA excision mechanisms to damage foreign DNA.

CRISPR-Cas systems are divided into classes, types and subtypes based on differing CRISPR gene sequences and Cas proteins. (P.D. Hsu et al.,2014).

Throughout the different CRISPR-Cas systems, Cas proteins are distributed in different amounts with some protein types present in all systems and others in specific ones. Each Cas protein has specific roles from regulation of bacterial/archaeal genes to maturation of crRNA as target nucleic acids are cleaved.

Class I CRISPR Cas systems include Types I, III and IV, which are composed of crRNA-Cas protein effector complexes with multiple proteins. Class II CRISPR Cas systems involve Types II, V and VI composed of effector complexes with a single multi-subunit protein.

CRISPR-Cas systems cut up target nucleic acids in three phases. In the first phase named Adaptation or Acquisition, specific sequences called PAM that lie close to target nucleic acid sequences are recognized by Cas proteins and target sequences are cut off and joined within CRISPR regions forming new spacer DNA sequences. During the second phase, CRISPR region is transcribed to synthesize crRNA producing precursor crRNA which becomes

mature crRNA upon further modifications. Mature crRNA is composed of repeat DNA sequences and one spacer region. In the final phase, mature crRNA binds to specific Cas proteins to form the protein effector complexes. These complexes recognize the specific PAM sequences in nucleic acids of foreign invaders and crRNA binds to this target region. Bound Cas endonuclease enzyme then becomes activated, cleaving target nucleic acid strands. (P.D. Hsu et al.,2014).

The phases of the following mechanism depend on multiple factors such as the type of CRISPR-Cas system, Cas proteins, recognition of PAM or similar sequences in either single-stranded, double-stranded DNA or RNA region, presence or absence of tracr RNA, type of effector complex formed, target nucleic acids, process of nucleic acid cleavage and enzymes involved in DNA/RNA excision.

These breaks introduced by CRISPR-Cas system in nucleic acid sequences are repaired by either of the two natural cellular processes: Non-homologous end joining and Homology-Directed Repair. Each DNA repair pathway can be utilized for different functions carried out by CRISPR-Cas system.

Non-homologous end joining pathway does not require DNA repair templates. Small pieces of DNA merge with each end of the break in a random manner, thus producing small insertion-deletion mutations in the repaired sequences increasing the probability of more severe changes such as deletions, duplications and frameshift mutations in the future. Therefore, this pathway is more useful for functions such as gene knockouts and formation of non-functional sequences in target regions.

In Homology directed repair pathway, DNA templates with specific sequences are required in which the end regions of these templates must be completely to those of broken ends. Hence,

this pathway is suitable for gene-editing mechanisms such as insertion of single nucleotide or longer bases in target regions.

2.2: Human Immunodeficiency Virus-1:

The contributive pathogenic agent in AIDS belongs to the family Retroviridae and genus Lentivirus. HIV genome is composed of two single-stranded RNAs surrounded by a protein containing capsid and lipid envelope. The nine overlapped genes located in HIV genome are as follows: gag, pol, env, tat, rev, nef, vif, vpr, vpu with two long terminal repeats at both ends. Numerous glycoprotein variants are present on the outer envelope of the virus. (R.Seitz, 2016).

When HIV-1 encounters a suitable human host, it attaches to complementary membrane receptors present on cell surface of CD4+ T-cells, macrophages and dendritic cells of host immune system via viral glycoprotein 120. Upon attachment, structural changes are brought about in viral glycoproteins resulting in binding of virus with CCR5 or CXCR4 receptors on host cell membranes. Viral and host cell membranes combine by fusion between glycoprotein 41 and CCR5/CXCR4, causing entry of viral protein capsids into host cell cytoplasm. Reverse transcriptase enzyme produced by the virus synthesizes a double-stranded viral DNA from its genetic material, utilizing the metabolic machinery and components of host cells. This newly synthesized viral DNA integrates into host cell genome. Transcription and translation of HIV-DNA produce the genetic material and necessary components of the virus, with subsequent assembly of newly synthesized components resulting in new HIV particles. Mature viruses are released from the host cell and enter surrounding healthy cells, progresses the infection and leads to eventual AIDS. (R.Seitz, 2016).

The major approach of treatment to HIV-infection is ART, frequently combined with simultaneous use of anti-viral drugs, combined anti-retroviral therapy or long acting slow effective release Anti-retroviral therapy.

Anti-retroviral therapy works by mechanisms inhibiting viral enzymes and other proteins involved in viral replication cycle, thus disrupting various mechanisms within life cycle of HIV. This approach is effective in controlling viral replication, but as viral DNA remains integrated into host genome, formation of latent reservoirs in host cells cannot be prevented or removed by current treatment approaches. Hence as therapy stops, viral replication is very likely to be reactivated and progress the infection.

Therefore, anti-retroviral therapy is not the right approach to HIV-AIDS cure, as it cannot destroy the latent viruses stored in host cells or inactivate the fused HIV-DNA within host genome. Moreover, it comes with long term expenses and treatment period along with ill side-effects.

CRISPR-Cas system may be programmed to edit host genomes and/or multiple parts of the viral genome to influence the nature of virus-host interactions, leading to potential cure of the disease.

2.3: Features of CRISPR-Cas9 as a gene-editing tool:

Analyzing the crucial structural differences between Class I and Class II CRISPR-Cas system, it is found that Class II CRISPR-Cas system makes a more effective tool in gene-editing mechanisms due to using a single multi-subunit protein to form effector complexes. It

involves Cas9 endonuclease enzymes, CRISPR-RNA and trans-activating CRISPR-RNA to cleave target nucleic acids.

Investigations have showed that CRISPR-RNA can be merged with 5'-end of trans-activating CRISPR-RNA to form a chimer molecule called the guide-RNA or single-guide RNA. This single guide-RNA is combined with Cas9 endonuclease enzymes resulting in the CRISPR-Cas9 system. A 20 nucleotide long sequence segment in the gRNA can be modified to make it complementary to the PAM sequences close to the target molecule. These programmed guide-RNAs and Cas9 proteins are proved to fully effective when experimented in GFP gene of a plasmid, which was pin-pointedly cleaved at right locations. Further experiments have proved that CRISPR-Cas9 system is able to edit genes, create mutations of different types and regulate gene expression by controlling transcription in bacterial cells (P.D. Hsu et al., 2014).

During action of CRISPR-Cas9 complex, as the guide-RNA attaches to complementary PAM sequences upon recognition in the target DNA, Cas9 endonuclease activity is switched on, causing a double-stranded break in target region.

HIV-1 entry in host cells to development of chronic AIDS occurs via mechanisms involving genes and multiple stages of protein synthesis. As cure to the disease would mean permanent changes in specific cell structures such as host membranes and damage to integrated viral genomes in host cells, ability of CRISPR-Cas9 system to target any sequences in nucleic acids, to cleave multiple sequences simultaneously using multiple guide-RNAs and to induce gene editions in target locations are major features of the system as a potential approach in cure of HIV-AIDS (G.Maartens et al.,2014).

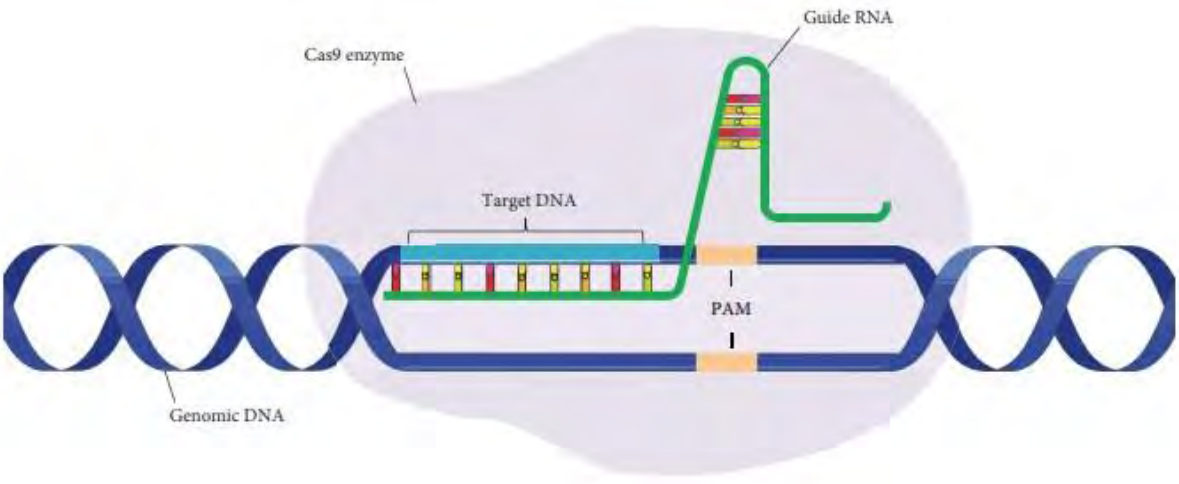

Figure 1: Structure of CRISPR-Cas System

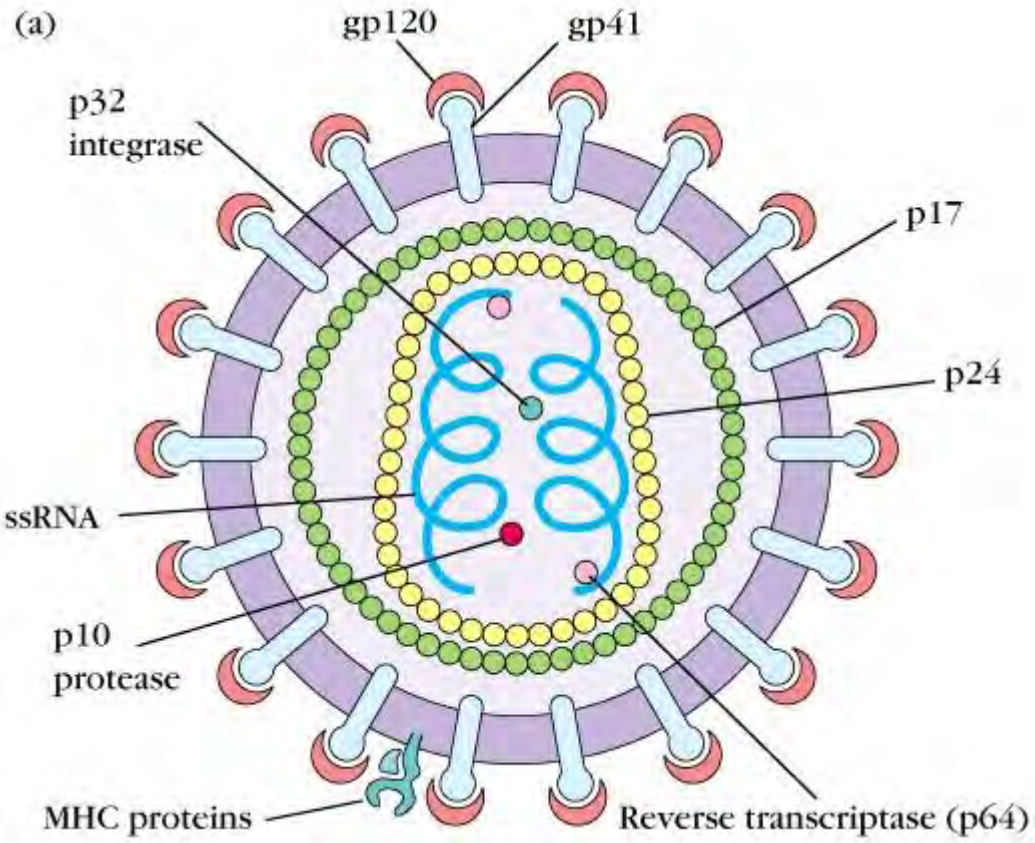

Figure 2: Structure of Human Immunodeficiency Virus

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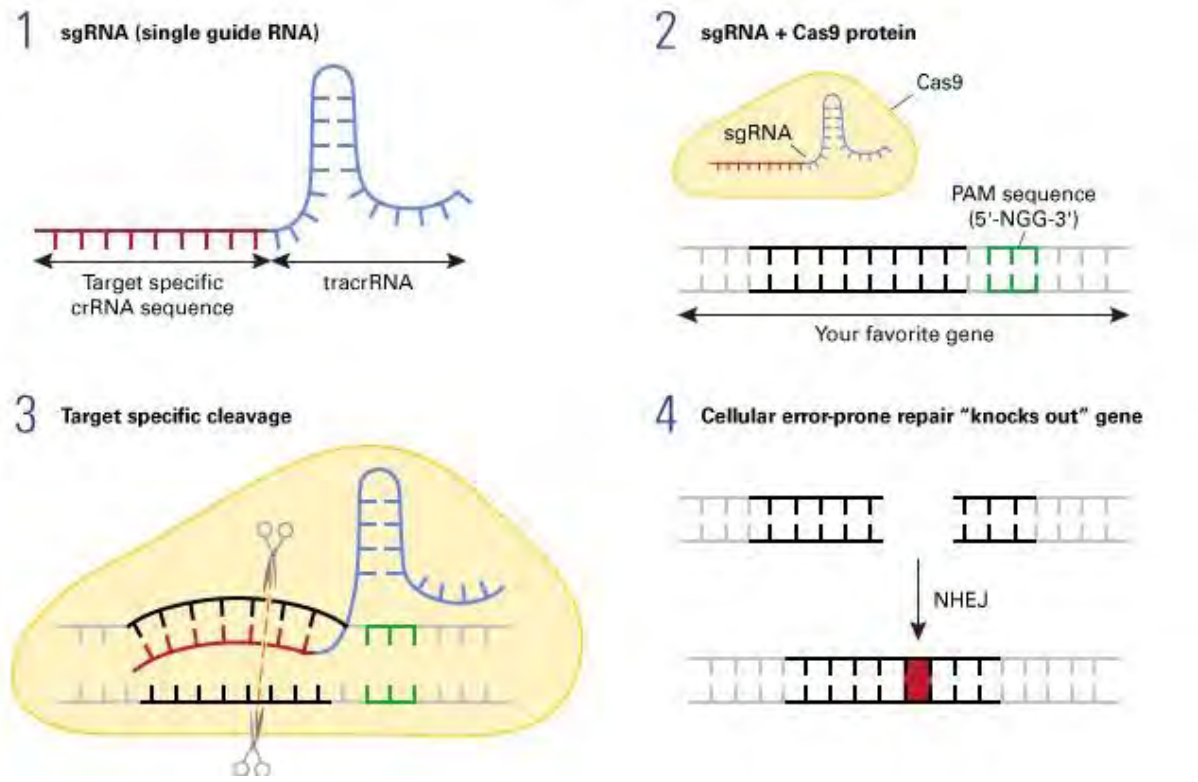

Figure 3: How CRISPR-Cas system works as a genome-editing tool

Chapter 3: Materials, methods and experimental results

3.1: Materials and methods

PREPARATION OF CRISPR-Cas9 REAGENTS, VECTORS AND PLASMIDS:

Lentiviral vectors are packaged in HEK293T-cells by calcium phosphate precipitation method. 15micrograms of transfer plasmid and helper plasmid are transfected simultaneously into HEK293T-cells. Following transfection, viral supernatant is harvested, ultra-centrifuged and stored at -80 C (C.Yin et al., 2016).

For cloning of multiplex gRNA, 'gRNA-scaffold cassette' is amplified using primer and cloned using 'In-Fusion HD Cloning Kit' within 'EcoR1 and Kpn1 linearized pX601-CMV-saCas9-GagD plasmid', producing a AAV delivery vector. Afterwards, sequence specific plasmid is packaged into AAV-9 serotype (X.Qu et al., 2017).

AAV9 vector is used for delivery of CRISPR-Cas9 as this vector has efficient transduction in variety of tissues including those of the central nervous system, which are reservoirs for the virus.

CELL AND VIRAL CULTURE:

Cell culture, preparation and isolation of TZM-Bl cells, Jurkat T-cells and human CD4+ T-cells are carried out. Human blood samples from disease free donors are collected for separating primary CD4+ T-cells. Peripheral blood mononuclear cells are obtained with 'lymphocyte separation medium Ficoll-plaque premium (BD)'. Primary CD4+ T-cells in mononuclear cells are separated by a human CD4+ T-cell isolation kit from Millenyi Biotech. These isolated cells are cultured in RPMI 1640 medium and enhanced by CD3/CD28 in addition to recombinant human interleukin-2 (Z.Liu et al., 2017).

HEK293 T-cells are cultured in 'high-glucose DMEM' in presence of 10% FBS and antibiotics (100U/mL penicillin+100micrograms/mL streptomycin) in humid conditions with 5% CO2 at 37degrees celsius (C.Yin et al., 2016).

TZM-bI reporter cell lines are cultured in high glucose DMEM with 10% FBS and gentamicin of 10micrograms/mL.

Jurkat cells purchased from ATCC are cultured in medium containing 10% FBS and gentamicin.

HIV-1 infectious virus is collected from NIH AIDS Reagent program.

TRANSDUCTION AND TRANSFECTION:

Jurkat T-cells are transduced by spin-transduction at 1200g for 2hours. 8micrograms/mL of polybrene/Sigma is used in cell sample. After centrifugation, supernatant of Jurkat cells transduced by lentivirus is replaced with RPMI containing 10%FBS for culture and analysis in future experiments (Z.Liu et al., 2017).

HEK293T-cells cultured in well plates are transfected with plasmids via ‘standard calcium phosphate precipitation’ protocol with 50microM of chloroquine and 30micrograms of plasmid. Supernatants are then collected, filtered and ultra-centrifuged for two hours with 20% sucrose solution (C.Yin et al., 2016).

293T-cells are also transfected by lenti-X4R5 Cas9/control vector, psPAX2 and pMD2.G using Polyethylenimine (PEI) transfection reagent. Supernatant formed is collected, filtered and stored.

USE OF HUMANIZED MOUSE MODELS AND TRANSGENIC MICE:

Human hematopoietic stem cells injected into mice can form human T-cells which are vulnerable to HIV-1 infection.

Group of mice are exposed to radiation at birth, following which an injection is pushed into mice hepatic vein that carries CD34 cells and hematopoietic stem cells into mice to humanize them. After a specific time length, the humanized mice models are infected with HIV-1 at a specific dose and observed for both acute and chronic symptoms of infection.

In the mice models, human cells are found in brain, lungs, liver, lymph nodes and spleen tissue as confirmed by immuno-histochemical staining. HIV-1 infection in cells is confirmed by HIV-1 p24 staining.

Humanized mice models are collected from Jackson Laboratories, Bar Harbour, ME. Mice are grown under sterile conditions at University of Nebraska Medical Center or at Animal Resources Center by following the ethical rules and protocols determined by National Institutes of Health for care of laboratory animals (Q.Wang et al., 2018).

FLOW CYTOMETRY:

Cells transfected by lentiviral vector Cas9 were collected three days after transfection., washed in PBS, stained with PE-conjugated anti CXCR4 and APC- conjugated anti CCR5 for 15 minutes at 4 degrees celsius. Following incubation, cells are washed thrice. Flow cytometry is used to measure and compare the expression level of CXCR4 and CCR5 membrane receptors on cell surface after transfection (Z.Liu et al., 2017).

CENTRIFUGATION AND ELECTROPORATION:

A specific number (2*10^7) of primary CD4+ T-cells are centrifuged. Following centrifugation, cells are washed with PBS, re-suspended with prepared solution of 100microL EP buffer, Cas9 NLS and chemically modified guide RNA with tracrRNA complex, resulting in a mixture (Z.Liu et al., 2017).

Mixture is shifted into cuvette and electroporated with MaxCyte STX, inserting Cas9 and sgRNA complex into the primary CD4+ cells.

VIRAL INOCULATION:

CXCR4 and CCR5-modified or control cells are obtained and washed thrice. CXCR4-tropic and/or CCR5-tropic HIV-1 is added to the cell culture medium and incubated for 8 hours. Following incubation, infected cells are washed thrice with PBS and RPMI1640 growth medium is added. Viral titer of the supernatant is observed by p24 ELISA at specific time intervals (Z.Liu et al., 2017).

Humanized mice are given anesthesia via inhalation of isoflurane of 2%-2.5% with a 2L/min O2 flow rate. Each mouse is inoculated with HIV-1 by pipetting 20microL of viral supernatant. HIV-infected mice are selected based on latent HIV infection without observable HIV-reporter activity in-vivo for 70 days (Q.Wang et al., 2018).

DNA/RNA/PROTEIN EXTRACTION, PCR, GENOME SEQUENCING AND ANALYSIS, REPORTER ASSAYS:

Blood and cell culture DNA Midi Kit or Qiagen kit (QiAmp DNA mini kit) is used to extract genomic DNA from CXCR4 and CCR5 CRISPR modified or controlled cells, followed by assay using ‘Surveyor Nuclease Assay’ (Z.Liu et al., 2017).

Extraction of nucleic acids is carried out to obtain both positive and negative control cells. Positive control includes total cellular DNA extracted from HIV-1 infected cell line and negative control includes human genomic DNA collected from humanized mice. Nucleic acids are collected from spleen tissue, bone marrow cells, brain, kidney, lung, liver and abdomen.

As an alternative method, tissue samples are collected from selected organs and are mechanically broken using a mortar and pestle. Nucleospin Tissue Kit is used to process the fragmented tissue and Nucleospin RNA/Protein kit is used to extract genomic DNA, RNA and proteins from collected tissue.

Amplification of CXCR4 or CCR5 gene fragment is done via PCR. Following PCR amplification, 400nanograms of PCR product is annealed and digested with T7 endonuclease I of 10 units /microL and analyzed with ethidium bromide (EB) stained 1.5% agarose gel.

Resulting fragments are joined with pGEM-T easy vector (Promega) and analysis of single guide RNA is carried out via DNA sequencing or deep sequencing.

IMMUNOHISTOCHEMISTRY AND IMMUNOCYTOFLUORESCENCE:

Cell samples from lymph nodes, spleen, lungs and liver are immersed into Phosphate Buffered Saline and 4% paraformaldehyde, followed by cell processing to be fixed on parafilms using 0.5% Triton X-100 and 10% normal donkey serum. Alternatively, processed cell sections are incubated using rabbit anti-HA polyclonal antibody in PBS with 0.1% Triton X-100 at 4degrees celsius followed by three consecutive washings. Alexa Fluor conjugated donkey secondary antibody and phalloidin are added to cell sections at rtp (Q.Wang et al., 2018).

Monoclonal antibodies from mice and HIV-1 p24 stains are used to label these layers of cell samples prepared. A polymer-based secondary detection reagent called HRP-conjugated anti-mouse DakoEnVision system is added. Counterstain Mayer’s hematoxylin or Hoechst is added to the cell sections.

Control samples use absence of primary antibodies or mouse immunoglobulin G.

Images of both experimental and control cell samples are obtained using a Nikon DS-Fil Camera fixed to a Nikon Eclipse E800. Images obtained are further analyzed.

USE OF BIOINFORMATICS SOFTWARE TOOLS:

The Broad Institute gRNA designer software tool (website address) has been used to design the guide RNAs. Three single guide RNAs that target long terminal repeat sequences, three single guide RNAs that target gag regions and one single guide RNA which targets pol region of the HIV-genome are selected. In addition, primer sequences required for PCR are designed using the software (C.Yin et al.,2016).

For CCR5 and CXCR4 gene target locations, single guide RNA sequences are designed using the bioinformatics software as mentioned.

STATISTICAL ANALYSIS:

Quantitative data obtained from investigations are tabulated as the ‘mean +/- SD (standard deviation). Statistical analyses are carried out via SPSS software version 16.0. Two groups are compared and analyzed via Unpaired Student’s t-test and multiple groups are compared and analyzed via variance. A value of $p<0.05$ is regarded as statistically significant. In analysis of some experiments, $p<0.01$ is also regarded as statistically significant (C.Yin et al.,2016).

During analysis of infection in mouse models by EcoHIV, comparison between mice infected by only EcoHIV and those infected by both EcoHIV and SaCas9 are made by Student's t-test and linear mixed-effect models (M.E. Karpel et al.,2015).

Analysis of drug levels in tissues, reverse transcriptase activity in HIV-1, HIV-1 staining, levels of T-cells, viral nucleic acids and viral load are done by one-way ANOVA with Bonferroni correction or two-way factorial ANOVA and Bonferroni's post-hoc tests. Outlier values resulting from statistical analysis classified as those above 99%confidence interval of mean and 3-fold higher than standard deviation are left (C.Yin et al.,2016).

For all approaches, around two to four independent experiments have been performed.

3.2: EXPERIMENTAL RESULTS

3.2. A) INHIBITION OF HIV-1 REPLICATION BY CRISPR-Cas9 SYSTEM:

To inhibit viral replication inside host cells, guide RNAs are designed to target different positions such as tat, rev, pol, LTR and gag regions along the HIV-1 genome. In multiple studies (C.Yin et al., 2016), prevention of HIV-replication is attempted by using multiple and then specific combinations of multiplex gRNAs, each targeting a specific region along the viral genome. In the first study, HEK293T-cells are transfected with plasmid containing Cas9 endonuclease and multiplex gRNAs that target LTR regions, gag or pol genes of HIV genome. Results have shown a significant decrease of upto 96% in the expression of luciferase protein by cells, suggesting a marked reduction in viral replication. The second study suggests that targeting specific locations of viral genome such as LTR and tat, have showed greater decrease in reporter genes and gag gene expression. It is found that Cas9 endonuclease enzyme is able to target multiple locations within cells where HIV-genome maybe present.

Two lentiviral vectors containing Cas9 and gRNAs targeting different loci of viral genome are inserted within Sup-T1 cells in two experiments (Q.Wang et al., 2018). The first experiment suggests a decrease in expression of p24 protein in cells, where p24 protein is a marker of HIV-replication. Greater decrease in viral replication has been observed in cells where the target locations in HIV-genome involve conserved gene regions. In the second experiment, results show that specific pairings of gRNAs are more effective in viral replication than random gRNA pairs.

3.2. B) PREVENTION OF VIRAL INTEGRATION INTO HOST CELL GENOME:

Liao et al. and Yin et al. have attempted via their in-vitro experiments that to degrade HIV-1 genome before it integrates into the host genome leads to a reduction in the number of provirus in infected host cells.

Two separate approaches included analyzing whether cleaving of HIV-1 complementary DNA using CRISPR-Cas9 in host cells is viable and transduction of 293T-cells with CRISPR-Cas9 with further observations on early, late and integrated DNA.

Both approaches have showed a marked decrease in HIV-1 positive host cells but exhibit a lesser decrease in early HIV-DNA products.

3.2. C) SIMULTANEOUS AND SEPARATE DISRUPTION OF CXCR4 AND CCR5 GENES IN HOST CELLS:

Simultaneous disruption of CXCR4 and CCR5 genes by CRISPR-Cas9:

To study the effects of simultaneous disruption of CXCR4 and CCR5 genes, a series of experiments are designed using TZM-b1 cells, Jurkat cells, CD4+ T-cells and Alt-R CRISPR-Cas9 system (Z.Liu et al., 2017).

Single guide RNAs are increased in amount by PCR and placed into a plasmid vector. TZM-b1 cells are selected first, because it expresses co-receptors CXCR4 and CCR5 on their cell surface membranes. Transfection of vector plasmid, single guide RNA and Cas9 protein is carried out into TZM-b1 cells. T7E1 assay is carried out to assess the rate of insertions and deletions of CXCR4 and CCR5 genes in modified TZM-b1 cells. Mutation rates of each gene expressed as percentage are as follows: 20.35%, 40.57% in CXCR4 genes and 15.90%, 32.95% in CCR5 genes. Expression of both CXCR4 and CCR5 co-receptors on modified cells are then measured. A gene-knockout efficiency of 23.8% and 23.6% are measured in comparison to none detected in un-modified control cells. As the modified and transfected TZM-b1 cells are infected with HIV-1, it is found that knockout of CXCR4 and CCR5 genes in these cells have caused HIV-1 resistance.

Jurkat cells are cells derived from acute T-cell leukemia, which also express CXCR4 and CCR5 co-receptors on their cell surface. These cells are transduced with lentiviral vector and the gene fragments coding for CXCR4 and CCR5 co-receptors are amplified via PCR. An assay shows that in modified cells, the target gene fragments are disrupted by CRISPR-Cas9 showing significant amounts of insertion-deletion mutations. Changes in CXCR4 and CCR5 protein levels are measured; results suggest a decrease in total levels of CXCR4 and CCR5 proteins on surface of modified Jurkat cells, indication a down-regulation of CXCR4 and CCR5 genes. Modified Jurkat cells infected with HIV-1 show a decreased p24 protein production that indicates a drop in HIV-1 replication compared to un-modified control cells infected with the virus.

Primary CD4+ T-cells in human immune system are a crucial target for HIV-1 replication. T-cells are electroporated to carry out transfection, inserting the plasmid vector carrying sgRNAs and Cas9 protein. When knockout rate of the cells are measured, mutations in both CXCR4 and CCR5 genes are observed. Levels of p24 protein production in HIV-1 infected T-cells are measured at regular time intervals. Modified cells are seen to be resistant to the viral infection in comparison to un-modified control cells.

Alt-R CRISPR-Cas9 system uses spCas9 recombinant protein and a sgRNA composed of tracrRNA and crRNA specific for target CXCR4 and CCR5 genes. This CRISPR-Cas system is inserted into primary CD4+ T-cells to produce modified cells. Knockout efficacy analysis of CXCR4 and CCR5 genes suggest a marked decrease in expression of both genes on modified cell surface, from 88.7% to 54.9% in CXCR4 and 77.1% to 26.3% in CCR5, with an increase in cell populations where CXCR4 and CCR5 co-receptors are absent. Analysis of CRISPR-modified T-cells infected with HIV-1 shows an increase in cell populations from 74.3% to 85.1% where no CCR5 or CXCR4 co-receptors are expressed after being infected.

While simultaneously disrupting both CXCR4 and CCR5 genes, experiments on off-target gene locations are carried out. No off-target effects were found on predicted genome locations. During the experiments, cellular toxicity and rate of apoptosis in modified cells compared to that in control cells are analyzed. No increased cell toxicity or apoptosis is observed in CRISPR-modified cells.

Separate disruption of CXCR4 and CCR5 genes by CRISPR-Cas9:

In the first investigation (Y.Gao et al., 2017), a lentivirus is modified to express sgRNA, spCas9 and reporter gene required for later analysis. Single guide RNA is designed to target DNA regions coding for CCR5 co-receptors on host cell membrane. Human CD4+ lymphoblast cells are transduced with lentiviral vector carrying CRISPR-Cas system. As CCR5 expression is analyzed, it is observed that expression of CCR5 co-receptors significantly decreases in modified cells, with 4.3% CCR5+ cells in modified group and 81.7% CCR5+ cells in control group. Analysis of genomic DNA in modified cells shows a 62% disruption of CCR5 genes with a large number of insertion-deletion mutations at PAM sites of sgRNA and more indel mutations of varying sizes throughout the CCR5 gene. Rate of p24 protein production in modified cells infected with R5-tropic HIV-1 indicate that viral replication is 100times lower in these cells compared to control cells, suggesting HIV-1 resistance.

The second investigation (P.Hou et al., 2015) involves sgRNAs designed to target genes coding for CXCR4 co-receptors and spCas9 protein carried via lentiviral vector. Primary T-cells or Jurkat cells are transduced with lentiviral vector carrying CRISPR-Cas9. Analysis of CXCR4 gene expression shows 15.4% CXCR4+ cells in modified group and 99.3% CXCR4+ cells in control group. Genomic DNA analysis of modified host cells suggests a 30.4% disruption in CXCR4 genes of modified cells. Results indicate significant resistance to X4-tropic HIV-1 infection in CRISPR-modified cells.

Another study (Z.Liu et al., 2017) included human peripheral blood mononuclear cells from NSG mouse model to determine if CXCR4 genes can be knocked out to create resistance against X4-tropic HIV-1 infection by using Alt-R CRISPR-Cas system in-vivo. Results show an increase of CXCR4- T-cells from 2.3% to 20.2% in infected CRISPR-modified mice. An increase in CXCR4 gene disruption has been observed in genomic DNA analysis of infected mice. In addition, these infected mice are shown to have 30times lower levels of HIV-1 in blood plasma compared to control group. Analysis of modified cells collected from mice spleen shows 37% mutations in CXCR4 gene. Results indicate that CRISPR-mediated gene disruption in-vivo can prevent HIV-infection in T-cells.

The studies described above show that simultaneous disruption of CXCR4 and CCR5 genes can provide host resistance against dual-tropic HIV-1 infection whereas separate disruption of either CXCR4 or CCR5 gene can provide resistance against R5-tropic HIV-1 or X4-tropic HIV-1 infection respectively.

3.2. D) PREVENTING REACTIVATION OF HIV-1 REPLICATION IN LATENTLY INFECTED CELLS BY TRANSIENT CRISPR-Cas SYSTEM:

Pro-viral DNA in HIV-1 infected reservoir host cells can be permanently inactivated by modifying the provirus integrated within the host genome via CRISPR-Cas system (W.Zhu et al., 2015). This approach is more likely to be suitable in-vivo conditions and reduce viral escape from host cells. Sup T1 T-cells having latent infection with 'replication-competent' HIV-1 are transfected with different variations of CRISPR-Cas systems, attempting to inactivate via multiple mutations in target locations of viral genome through excision or inversion of viral DNA. In these investigations, CRISPR-Cas reagents used to modify Sup-T1 cells are Cas9 mRNA with gGag1 and GTatRev RNAs, Cas9 protein with gGag1 and gTatRev RNAs and Cas12 protein with crGag1 and crTatRev RNAs. The gRNAs used in these CRISPR-Cas systems target towards highly conserved sequences in the virus as more permanent inactivation of the virus can be obtained by targeting a few crucial and highly conserved domains of HIV-domain. Different combinations of gRNAs are tested on the cells to find the specific gRNA pairs which result in complete prevention of viral escape and permanent virus inactivation in infected host cells.

After the series of experiments with each variant of CRISPR-Cas reagent, p24 and GFP production by SupT1 T-cells, level of infectious viral synthesis, and level of mutations in HIV-1 genome are observed and analyzed.

In cells treated with Cas9 mRNA, a high level of p24 and GFP production is observed in CRISPR-modified cells, indicating no reduction in viral replication. Cells modified with Cas9 protein show marked decrease in production of both p24 and GFP whereas cells treated with Cas12a protein show a slight decrease in levels of these proteins. In control cells, levels of p24 and GFP are observed to remain constant which suggest gene expression and replication oF HIV-1. It can be concluded that Cas9 mRNA has no effects on HIV replication and Cas9 protein is more effective in inactivation of viral gene expression and replication compared to Cas12a protein.

Synthesis of infectious virus particles is observed in both control cells and in cells treated with Cas9 mRNA. In Cas9 protein modified cells, infectious virus production is reduced to zero and in cells treated with Cas12a, viral synthesis has reduced but not to zero, indicating the efficiency of Cas9 protein in inactivation of HIV-1 replication.

Following treatments of SupT1 T-cells with multiple CRISPR-Cas reagents, proviral DNA is analyzed by the size of inversion and excision products in HIV-1 genome. In cells treated with Cas9 mRNA, 96% of proviral DNA is found intact with 1% inversion and 3% excision mutations, wild-type gene sequences with very low proportion of DNA having deletion mutations. This indicates a high rate of HIV-1 proviral gene expression and no inactivation. Cas9 protein modified cells carry 50% intact proviral DNA, 32% excision and 18% inversion products with more insertion-deletion and substitution mutations observed in target locations of HIV-genome. Mutations are seen in highly conserved viral gene sequences which are able to disrupt HIV-1 protein production and interfere with viral replication. These data suggest complete inactivation of HIV-1 by CRISPR-Cas9 system. Cells modified with CRISPR-Cas12a show 94% of intact proviral DNA, 5% excision and 1% inversion products, some wild-type sequences and a low frequency of insertion, deletion and substitution mutations in HIV genome. These suggest mutations formed may disrupt some HIV-1 protein production but still continue to express genes and replicate at a low level.

Deducing from the results, Cas9 proteins are overall more effective than Cas12a or Cas mRNA in PAM recognition and patterns of cleaving target DNA. Therefore, repeated treatment of HIV infected cells with CRISPR Cas9 can cause complete inactivation of HIV-1 and prevent the infection.

3.2. E) IN-VIVO EXCISION OF HIV-1 PROVIRUS BY CRISPR-Cas SYSTEM FROM LATENTLY INFECTED CELLS:

In-vivo excision of provirus by sequential LASER-ART and CRISPR-Cas Treatment:

Integrated HIV-1 DNA is removed from infected cells and tissues using combined treatment of LASER-ART and CRISPR-Cas9 system (H.S. De Silva et al., 2017). The proviral DNA is removed from latent reservoirs by excision of target fragments of HIV genome. LASER-ART treatment involves anti-retroviral drugs which are extremely hydrophobic and lipophilic, able to cross cell membrane barriers at multiple sites and enter latent viral reservoirs in infected cells. This drug produces low side effects, has better availability to living cells and can control viral replication and infection, but cannot remove latent viral reservoirs from infected cells permanently.

A series of experiments is carried out using HIV-1 infected humanized mice by treating them by combined LASER-ART and CRISPR-Cas system. Results show no latent virus in mice bone marrow, lymphoid tissue, blood and brain. When isolated treatment of either LASER-ART or CRISPR-Cas9 system is involved, viral reservoirs can still be detected in infected mice cells.

In the following experiment, disruption of HIV-1 replication by LASER-ART increase the effectiveness of proviral DNA excision by CRISPR-Cas system as found by analyses of obtained results. This suggests as HIV-1 level remains minimal due to the former drug, CRISPR-Cas9 efficacy increases. AAV-9 vector is selected to carry the CRISPR-Cas9 to target cells as it is more bio-available in multiple tissues or organs of model organism. Viral rebound in multiple target cells and tissues of HIV-1 infected humanized mice from both experimental and control groups is measured following the treatment. Evidence of viral rebound indicates infected mice treated with combined LASER-ART and AAV9-CRISPR-Cas9 show no viral rebound, whereas infected mice treated with either LASER-ART or CRISPR-Cas9 show 100% viral rebound once the treatment is removed.

The study uses Cas9 endonuclease protein complexed with multiplex gRNAs which cleave DNA sequences at highly conserved regions within LTRs and gag regions along the HIV-1 genome. The following treatment does not produce mutant HIV strains able to carry out viral escape from infected cells. No significant off-target effects are observed.

It can be concluded from the results that combined treatment of LASER-ART and CRISPR-Cas9 system can be an effective approach to destroy latent viral reservoirs in infected tissues. Moreover, HIV-1 replication in host cells must be limited to a specific level before viral genome excision can be carried out.

In-vivo excision of provirus by saCas9 and multiplex sgRNAs:

In the following investigation (Wang et al., 2018), multiple sgRNAs and saCas9 are delivered using AAV-vector into HIV-infected cells. Studies are conducted on humanized bone marrow/liver/thymus from (BLT) mice with chronic HIV-1 infection and neural progenitor cells from HIV-1 Tg26 transgenic mice.

Three sgRNAs able to target the LTR regions of HIV-genome are selected and paired in multiple combinations. Luciferase reporter assay is carried out to analyze the results. Each combination of sgRNA complexed with saCas9 is able to carry out 95% to 99% excision of HIV-1 genome. When saCas9 is replaced with spCas9, comparison of 'excision efficiency' between saCas9/sgRNA and spCas9/sgRNA are made. Results show a lesser decrease in luciferase activity when spCas9/sgRNA is added, indicating higher excision efficiency of saCas9/sgRNA. This is due to smaller size of SaCas9 which allows more efficient gene delivery and may have better nuclease enzyme activity.

Effective combinations of sgRNA are tested from analyzing the reduction of luciferase reporter activity in HIV-1 infected HEK293T-cells. It is found that when sgRNAs that target LTR regions are combined with sgRNAs targeting structural regions of HIV genome such as gag or pol sequences, highly efficient genome excision is possible. It is also noted that different combinations of sgRNAs targeting LTRs, gag or pol regions can bring about HIV-genome excision to different degrees.

An experiment is designed (C.Yin et al., 2017) to compare HIV-genome excision efficiency between single sgRNA and multiple (duplex/multiplex) sgRNA. A higher decrease in luciferase reporter activity is observed by saCas9 complexed with duplex sgRNAs compared to that of single sgRNAs, indicating higher genome excision efficiency by multiplex sgRNAs.

In a later study, quadruplex sgRNA bound to saCas9 is designed by sgRNA targeting four regions of HIV-genome, LTR-1, LTR-3, GagD and PolB, inserted within saCas9 AAV-vector. Analysis of luciferase reporter activity indicates even higher excision efficiency of saCas9-quadruplex sgRNAs compared to duplex sgRNAs. Further results from PCR genotyping strongly suggest quadruplex sgRNAs to be the most efficient in HIV-genome excision as they are able to produce a wide range of indel mutations and 'fragmental deletions' in target gene regions.

In the experiment designed with transgenic mice (C.Yin et al., 2017), neural stem cells collected from mice are tested for presence of latent viral reservoirs. Latently infected stem cells are then thoroughly infected with both monoplex and multiplex (duplex and quadruplex) saCas9/sgRNA. Analysis and comparison of PCR genotyping results suggest both duplex and quadruplex saCas9/sgRNA are able to excise HIV-genome effectively, with quadruplex DNA having a long-term higher excision efficiency. Effectiveness of HIV-genome excision by multiplex sgRNA-saCas9 is then tested in-vivo. Transduction of CRISPR-Cas system to target tissues was efficient with no toxic side-effects observed. From RT-qPCR, TA cloning and Sanger sequencing result analysis, multiplex sgRNA/saCas9 is proved to deletion mutations in multiple tissue types. Decrease in mRNA expression and HIV-1 structural proteins have been observed in brain, kidney, liver and lungs of infected mice. Data collected indicates effective excision of HIV-genome by saCas9/multiplex sgRNA in target infected tissues and organs with no significant off-target effects.

Another experiment (C.Yin et al., 2017) similar to the one described above has been carried out with humanized BLT mice carrying latent viral reservoirs in human cells. Quadruplex sgRNA/saCas9 is transduced into the infected mice using AAV-vector. Results show high intensities of deletion mutations of HIV-genome in multiple target human tissues of mice, further indicating efficient excision of viral genome in latently infected human cells by multiplex sgRNA/saCas9 delivered via AAV-vector.

Chapter 4: Discussion- Limitations, Research gaps and further research

As the investigations involving HIV-infected cells and CRISPR-Cas systems have used experimental model organisms and biological systems, the actual scenarios of infected and latent reservoirs of human host cells in-vivo and their possible interactions with the CRISPR-Cas system are not reflected with accuracy (R.Bella et al., 2018). As a result, multiple limitations have been noted throughout the series of experiments. These limitations can be used to find the current research gaps, formulate further questions and design experiments for further development of the HIV-AIDS cure by CRISPR-Cas system.

Using animal models in experiments cannot reflect the real degree or level of viral reservoirs and long term infections that develop in human host in-vivo (C.Yin et al., 2017). Transgenic mice models do not show the accurate clinical levels of HIV-latency and infection in terms of HIV-1 pro-viral copies, locations of random viral integration into host cell genomes, various infected cell types and rate of HIV mutation. CRISPR-Cas systems can cause myriads of off-target effects due to lower specificity, which may cause non-target crucial DNA regions to break down and lead to severe cell damage. The extent of off-target effects that may occur in human hosts are not fully understood in cell cultures or experimental models.

In disruption of CXCR4 and CCR5 membrane receptors, no possible cellular toxicity or apoptotic effects by CRISPR-Cas system are studied. It is assumed that the gene disruptions

in-vivo may change the expansion, lifespan or location of some immune system cells in bone marrow and other immune system cells causing severe immune damage in human hosts. Fatality in embryonic mouse models has been observed after CXCR4 gene editing and knockouts along with engraftment issues, suggesting clinical infeasibility.

In investigations involving multiplex sgRNAs (C.Yin et al., 2017), while these RNAs can increase the chances of a large number of InDel mutations on the target gene regions of HIV-1, it may increase the chances of functional loss of host genome. Human grafts in mice models can cause transplantation of graft rejection long term, limiting the chances of studying the long term effects of combined treatment in infected cells. Although the AAV-vector used for transmission of CRISPR-Cas9 produces no side effects on humanized mouse models, effects of this vector or its functional efficiency is not yet known in humans.

Keeping in mind the following limitations mentioned which provides a clear insight into the research gaps, further research areas and questions can be developed and worked upon.

As cell cultures and mice models have been used throughout the investigations, the species involved must be evaluated to study how viral reservoirs develop in these cells and tissues, as observing and analyzing long term infections is important in understanding how in vivo HIV infections develop and progress in human hosts.

CRISPR-Cas systems must be tested on organisms more genetically closer to humans to obtain more accurate information on its efficiency, effectiveness, toxicity, side effects and off-target activities on cells and genomes similar to that of humans, so that it may be used as clinically safe for HIV-infected human hosts with maximum possible effectiveness while reducing unwanted damage and toxicity.

Further carefully designed chemical modifications can be carried out on CRISPR-Cas system as to increase its efficiency and specificity (R.Bella et al., 2018). Sometimes changes caused by CRISPR-Cas in the viral genomes can make them obtain more advantageous traits that increase their chances of survival, replication in host cells and make them resistant to damaging factors. In addition, HIV is known to be frequently mutating hence using a specific set of gRNAs may not be effective in the target sequences present in latent reservoirs. HIV often has large genetic diversity which may prevent the specificity of CRISPR-Cas function in-vivo. Specificity and effectiveness of CRISPR gRNAs can be increased by using more developed bioinformatics tools to identify both conserved and unique target sequences in HIV genome, off-target locations and build specific and multiple gRNAs accordingly, to find the most potentially effective one.

As Cas proteins are foreign agents to the host immune system, possible adverse effects of this treatment on the human immune system have to be studied thoroughly prior to clinical applications.

Current investigations are considering the use of Cas9 mRNA and Cas12 proteins in place of Cas9 protein. Comparisons between their relative effectiveness and side effects must be further checked and analyzed.

During experiments, clinical trials and applications, CRISPR-Cas reagents must be transferred to all cells containing HIV infection, which may not be done using the present delivery methods. More investigations on safe delivery methods that can insert CRISPR into a sufficient number of cells with effective specificity and precision in-vivo are required.

As CRISPR-Cas systems are delivered into infected host cells, they may potentially damage or disrupt many useful regions of host genome hence physiological effects of this disruption on cells and normal functioning of infected humans must be thoroughly studied. A wider variety of cell lines and various gRNA combinations can be experimented on to study their effects. More potential gene editing targets can be found. In-vivo experiments and clinical trials must be carried out on humans to understand proper effectiveness of CRISPR-Cas systems on HIV patients.

More efficient delivery systems and vectors to locations of latent viral reservoirs in host cells in-vivo with higher safety and specificity have to be further developed, as CRISPR systems are needed to be transduced effectively to variety of infected cells and tissues in the host target regions while being resistant to possible host immune responses, without producing toxicity, off-target mutations and adverse effects on cell structures and functions. Different sizes, components and properties of nanoparticles as potentially effective CRISPR-Cas delivery system can be studied.

Potential off-target effects of CRISPR-Cas systems on humans must be studied extensively. Some strategies used to analyze off-target effects in cells are whole-genome sequencing, GUIDE-seq, next-generation sequencing, direct in-situ breaks labeling and di-genome sequencing (X.H. Zhang et al., 2015).

Other potential areas of research can be finding mechanisms to effectively block viral growth in infected host cells and removal of large amounts of infected cells or HIV proviral DNA integrated in host genomes by immune system based mechanisms.

Using CRISPR-Cas based gene editing on humans may violate the rules and regulations of bioethics as it is not yet fully proved safe on humans, even after multiple trials and

experiments in human cells and animal models (Wang et al., 2018). The range of additional off-target harmful effects that may arrive with the therapeutic benefits are not understood yet, therefore both the benefits and potential downsides must be considered and found through further rigorous experiments.

Chapter-5: Conclusive Remarks

The series of investigations reflect the potential effectiveness of CRISPR-Cas systems in treating HIV-AIDS using more than a single approach. During the experiment of inhibiting viral replication, targeting specific HIV genome regions have proved to be more effective at disrupting replication. At particular phases of viral life cycle, preventing integration of the virus or viral products seems to be a viable technique in reducing HIV-infection in host cells. Use of recombinant proteins in CRISPR-Cas system for simultaneous and separate disruption in CXCR4 and CCR5 genes have shown higher effectiveness than using only Cas9 protein. Each combination of gene disruption results in resistance of host cells to different HIV strains. In latently infected host cells containing latent HIV reservoirs, complete reactivation of the virus can be prevented by CRISPR-Cas system. Cas9 protein has proved to be more

useful in breaking down target genomes compared to Cas12a and Cas mRNA. Other treatment approaches such as LASER-ART is combined with CRISPR-Cas9 for successful in-vivo excision of HIV in infected cells. In addition, quadruplex and multiplex gRNAs to excise latent viral resevoirs are a useful approach.

To develop and advance the research further, safer, more efficient and more specific delivery systems suitable to use for human cells must be found. Modifications of the CRISPR structure to introduce various possible gRNA designs and investigating their efficiency in targeting host cells and viral genomes can be helpful in finding the most effective combinations of CRISPR-Cas systems. More target viral genome regions that can be excised or disrupted to treat the infection can be found. CRISPR-Cas system can be combined with other existing lines of HIV-AIDS treatments to check their combined effectiveness.

As CRISPR-Cas system is a genome editing tool and a foreign agent to the human body, possibilities of functional loss of host genome, cell damage, physiological malfunctioning and severely adverse responses of human immune system should not be unexpected. To ensure safety of treatment using CRISPR-Cas tool, henceforth, cell cultures with a longer lifespan and from organisms genetically closer to humans have to be used, including model organisms and designing of clinical trials. Experiments have to be continued for longer periods of time to study the potential long term side-effects, host genome damage, cell toxicity, cellular apoptosis in addition to changes in disease symptoms and progression.

With further experiments, trials and errors, the adaptive immune system in prokaryotes- the CRISPR-Cas system may be developed into an effective and safe tool in treatment and cure of lethal HIV-AIDS and in myriads of other life-threatening diseases and disorders.

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