OPTIMIZATION OF CRISPR/CAS9 TECHNOLOGY TO STUDY HIV LATENCY IN PRIMARY $CD4^+$ T CELLS

by

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STATEMENT OF THESIS APPROVAL

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ABSTRACT

HIV-1 latently infected cells are the major hurdle impeding viral eradication despite the development of ART (Anti-retroviral therapy), which works by inhibiting various viral proteins necessary for HIV-1 replication. Even after years of daily regimens of ART therapy, HIV-1 reemerges once the ART is discontinued. This is because HIV-1 can go latent or quiescent in resting CD4+ cells. These resting CD4+ cells contain integrated HIV DNA within the genetic material in the host cell, but no viral proteins are produced, and they are thus immune to circulating antiretroviral drugs. For that purpose, it is essential to understand the mechanisms and genes involved in the development, maintenance, and activation of latency. To investigate functions of transcripts and pathways critical for biological processes and disease mechanisms, gene knockout is a very useful technique. We propose to use the CRISPR/ cas9 system to knockout target genes and test if these genes are involved in the development of latency or are involved in the reactivation of latently infected cells.

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CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Human immunodeficiency virus-1 (HIV-1) is a common and pathogenic strain of virus responsible for infecting around 34 million people worldwide [1][2]. If HIV-1 infection is left untreated, it advances into a life-threatening illness known as acquired immunodeficiency syndrome (AIDS). HIV is not a particularly infectious virus, nor is it highly contagious like measles. Nonetheless, the world is experiencing an epidemic and the World Health Organization has estimated that 1.1 million people died of HIV in 2015 [3].

HIV-1 is an enveloped positive sense RNA lentivirus comprised of a small genome of about 10kb. It consists of nine structural and nonstructural (regulatory and accessory proteins) coding genes, which are necessary for replication and infection in the human body. When HIV-1 infects, it starts to destroy cells of the immune system, making the body vulnerable to a host of diseases. HIV-1 infects multiple cells including brain cells (like microglia, oligodendrocytes, neurons, endothelia, and astrocytes [4]), T lymphocytes, and macrophages, but its main targets are CD4 lymphocytes, also known as T-cells or CD4+ cells. HIV-1 uses its surface protein gp120 to attach to the cells that have a CD4 receptor on their surfaces. Using the CD4 receptor in conjunction with chemokine receptors (CXCR4 and CCR5), the virus lipid membrane attaches and fuses with the cell membrane [2][5], and the virus core enters into the host cell. During entry, the core un-coats, which allows the genetic material, RNA, and three essential replication enzymes (integrase, protease, and reverse

transcriptase) to enter into cytoplasm. The viral enzyme reverse transcriptase copies the genetic material (RNA) of the virus, so it can be integrated into the host DNA. Reverse transcriptase has a high error rate that makes mutations in the copied DNA. These mutations produce mutant forms of HIV which can help the virus to evolve to evade drug therapies like ART (Anti-retroviral therapy).

ART is a cocktail of several antiretroviral medicines that consist of different nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry inhibitors, and Integrase inhibitors (wedmd.com) [6] [7] . ART works by impeding the HIV life cycle and leads to suppression of viral expression [8] [9]. A patient infected with HIV has a lower level of CD4 count, higher level of RNA viral load, as well as has a higher chance of having opportunistic infections. ART regimens dramatically improve the patient's CD4 count, highly decreasing the level of RNA viral load [10]. Treatment with potent antiretroviral drugs causes plasma viral levels to fall at an exponential rate. Newer antiretroviral drugs are powerful enough to stop all new infections of CD4+ cells [11]. The development of combined antiretroviral therapies ART drugs has thus dramatically extended the life of an HIV infected individual by preventing new infections from arising from latent reservoir of antigen as long as the therapy is being administered [12] . Unfortunately, the lifelong regime of daily pills in ART therapy is not a cure.

The reason ART does not results in a cure is that a small population of infected CD4+ memory T –cells (Tcm) enter a latent or quiescent state [2][13][9]. This state of retrovirus in which it is stored in the resting CD4+ T cells is called latency. Retroviral latency, the state of reversibly non-productive infection of individual cells, should not be confused with the long asymptomatic period between the initial infection and the onset of AIDS [11]. During this

initial period, most infected cells express viral proteins and rapidly replicate. Unlike many other viruses, HIV evades immune responses in the incubation period of the disease by rapid evolution of escape mutations [11]. The latent proviruses are relatively invisible to the immune system, and even a single latent HIV virus can later restart the infection. When ART is stopped, new and often drug-resistant infections arise out of the latent state virus that persists in CD4+T memory cells. The decay rate of the pool of latently infected CD4+ cells is tremendously slow at a half-life of 44 months, which would require over 70 years of treatment to eradicate the latent reservoir [11][14][9]. This inducible reservoir of latent proviruses is thus the major obstacle in the treatment of HIV AIDS, as it may be necessary to eliminate all latent forms of HIV for a successful eradication of the virus. Latency, for HIV, creates a stable reservoir of pathogen for onset of future infections at the level of individual CD4+ T cells. A complete cure of HIV would, therefore, not only require prevention of active infected cells from producing the virus, but also the elimination or complete repression of the latent reservoir. Efforts to achieve this goal would be greatly advanced by better understanding the mechanisms that are relevant to latency.

One of the traditional gene knockout methods is the homologous recombination technique (HR), which has been used to target both alleles in embryonic stem cells of mouse [15]. This method, however, is time-intensive and inefficient [15]. RNA interference (RNAi) is another method that represses gene expression through sequence-specific degradation of mRNA. Although widely used, this method can create off-target effects, and incomplete silencing often does not create noticeable changes in phenotype [15] . Advanced genome-editing technologies like zinc-finger nucleases can modify loci with precision, but are difficult to engineer into large-scale knockout libraries [16] [15]. On the other hand, the new CRISPR/cas9 system is more effective and reliable in both loss-of-function and gain-of-

function screening than other traditional methods of gene knockouts [15].

The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system is a novel tool for gene editing and gene regulation found in prokaryotes. This natural gene regulation, derived from *Streptococcus pyogenes*, protects these microorganisms from invading viruses and plasmids [17][18]. CRISPR-Cas9works through the type II Cas9 nuclease and two individual RNA components, namely crRNA and tracrRNA, which are fused in the recombinant system as an sgRNA [15]. Within the sgRNA, there are both structural RNA elements and 20 residue targeting sequences. The Cas9 protease is directed to the genomic DNA sequence by the sequence encoded in the sgRNA and proceeds to create double strand breaks (DSBs) on each side of the aligned segment. These DSBs recruit a DNA repair mechanisms in the cell, HR, or error-prone non-homologous end joining, which establish insertions and deletions at the target site [15]. The sgRNA directs Cas9 nuclease to the targeted genomic locus through Watson-Crick base pairing to create double strand breaks. To achieve that, Cas9 has to recognize the protospacer adjacent motif (PAM), which resides near the target DNA. This allows for a greater simplicity in the construction of a vector [15]. Cas9 promotes genome editing by making a targeted DSB via a deletion or addition mutation. CRISPR/Cas9 has been engineered to carry out large-scale function-based screening, and is easy to use in mammalian cells [15]. These changes completely alter gene function, which can be advantageous in genome engineering. We hope to use CRISPR/Cas9 to easily target numerous genes and to assay their effects in HIV latency.

CHAPTER 2

SPECIFIC AIMS

In a larger study, we would like to use CRISPR/Cas9 to knock out individual genes and then assay their effect on HIV-1 latency. We will test potential latency regulator genes and perform subsequent mechanistic studies in primary Tcm cells. The genes that are screened are those that are widely implicated in the literature as important in the establishment or maintenance of latency or in its reactivation, as well as the newly proposed genes from unpublished screens performed in many different laboratories. In this study, we are going to validate these targets to see if they are involved in latency by performing experiments in different cell lines. The aim of this pilot project is to develop the CRISPR protocol in conjunction with HIV latency assay. We have chosen five different genes for this pilot project: CD4, Brd4, Hexim1, CDk9, and CXCR4.

2.1 AIM1: To engineer the CRISPR/Cas9 vectors capable of targeting genes involved in HIV-latency

In the original LentiCRISPR.v2 vector, the Cas9 nuclease is followed by self-cleaving peptide and GFP (Green fluorescent protein). We will generate a derivative of the LentiCRISPR vector by replacing GFP with a truncated version of murine heat-stable antigen short (HSAS). The HSAS gene has the advantage of remaining surface exposed and associated with permeabilized or non-permeabilized cells during flow cytometry. Six unique guide RNAs

will be designed against the five target genes and cloned into the modified vector. We aim to conduct knockout mutations in both eukaryotic cell lines - SupT1, and in primary CD4+ cells. The CRISPR vector will be co-transfected with viral helper plasmids to generate lentiviruses that can easily infect primary and cultured cell lines.

2.2 AIM2: To confirm CRISPR in primary cells

While CRISPR/Cas9 has been shown to be effective at single gene knockout in passaged tissue culture lines, it is not known how the technology will work in primary cells. We aim to determine whether the system is capable of successfully knocking out the targeted genes in primary Tcm cells.

2.3 AIM3: Establishment of protocol for primary cells

Using the lentiviruses created in AIM1, and after confirming whether primary cells can be targeted with the CRISPR in AIM2, we would ultimately establish a protocol to examine if these genes play roles in maintaining HIV latency. In Aim3, we will attempt to determine if infection and knockdown efficiencies are sufficient to incorporate CRISPR knockdown into our latency assay. First, we will validate if activation of primary cells at different time points (one-and three-day post isolation) effects the knockout efficiency when CRISPR is administered one day after activation. We will also test various donors to characterize donor to donor variation in CRISPR knockout efficiency.

CHAPTER 3

RESULTS

3.1 Generation of LentiCRISPR-HSAS and lentiviruses

To investigate if the CRISPR- Cas9 system is able to target genes in eukaryotic cells, we generated six different guide RNAs for five genes targets using the online CRISPR tool provided by MIT (crispr.mit.edu) [19] [20]. First, LentiCRISPR-HSAS vector was made by replacing GFP (Green fluorescent protein) tag in LentiCRISPRv2 plasmid, provided by Dr. Ryan O'Connel at the University of Utah. This vector contains two expression cassettes, Cas9 and the single guide sgRNA. Cas9 and sgDNA are operated by two different promoters. sgRNA production is driven by U6 promoter, which is the RNA pol III promoter necessary for production of small RNA. Cas9 production is driven by a constitutive promoter, EF-alpha (human elongation factor-1 alpha), which can be used to drive ectopic gene expression. In our vector, Cas9 is followed by a P2A cleavage sequence and GFP. The HSAS clone is a truncated murine cell-surface protein homologous to human CD24, which has advantage of remaining associated with permeabilized cells during flow cytometry; in contrast, GFP leaks during permeabilization, making it harder to analyze true infected cells during flow cytometry. Furthermore, HSAS has stronger expression and can be used to select positive cells by magnetic sorting to overcome the low frequency of transduction in primary cells. This base clone contains filler sequence, which his flanked by BsmBI site where the gRNA sequence is inserted using the MIT server that identifies 20bp sequences with downstream PAM sequences

(addgene.com) [19] [21].

For our first experiment, we targeted the CD4 gene, as it is a surface receptor that has the advantage of easy detection. Lentiviruses were made from these six constructs by co-transfecting 293FT cells with the CFRISPR vector, a pVSV-G- expression vector, and a packaging vector (pS2PAX) as described in the methods section. Viruses were harvested and concentrated prior to determining the final viral titer. The level of infectivity in Supt1 cells was measured by checking HSAS expression after 2 days of infection. Five out of the six guide constructs displayed a high level of infection by 2 days post infection ranging from 70-100% depending upon the MOI used to infect the cells (Figure 3.1).

3.2 CRISPR-cas9 system can generate knockouts in SupT1 cells

CD4-CRISPR-infected SupT1 cells were monitored for surface-expressed CD4. At 9 days post infection, we observed the level CD4 knockout using a flow cytometer. Two of the six sgRNA constructs showed 20-40% CD4 knockout represented by cells with high HSAS and low CD4 expression (Figure 3.2). The big problem during our experiment was that the level of HSAS was decreasing as the days of infection proceeded. This could be the result of fast growth of uninfected cells in the infected pool, or it could be because of the transcriptional repression of the Cas9-P2A-HSAS gene. Consequently, we isolated HSAS positive cells using magnetic beads after 10 days post infection, then determined the level of HSAS and CD4 before and after sorting. Sorting improved and increased the level of HSAS+/CD4- cells by up to 66%. Due to strong knockout potential, we then chose HSAS-LentiCRISPR antiCD4 constructs A1 and C1 for future testing of primary cells (T_{CM}).

3.3 Testing in primary cells to validate targets

To examine the transduction and knockout levels in primary cells, we first isolated PBMCs from anonymous healthy donors. Naïve CD4+ T-cells were isolated from PBMCs by the Easy Sep negative selection human naïve CD4+ T-cell enrichment cocktail (Stemcell Technologies Inc. Vancouver, Canada). After isolation, naïve CD4+ T-cells were activated using human αCD3/αCD28 antibody-coated magnetic beads in the presence of human α- IL4, α-IL-12 and tumor growth factor TGF-β1. This process will generate NP cells (non-polarized). To establish the protocol, we first checked if activating primary cells for differing amounts of time effects the overall outcome of knockouts (schematic representation is shown in (Figure 3.3)). For this, we activated the primary cells using dynabeads coated with αCD3/αCD28 for 1 or 3 days and infected them with LentiCRISPR virus after removing the cells from the beads. After 2 days post infection, we monitored transduction efficiency via HSAS staining. In the following days, we checked how much knockdown we incurred. We followed loss of CD4+ cells in different donors by staining cells for HSAS (marker) and CD4 protein using flow cytometry. From the two experiments, we concluded that 3-day-activated cells have better transduction rate than 1-day-activated cells shown in Figures 3.4 and 3.5. In contrast, 1 day-activated cells have better knockout CD4 level than the 3-day-activated cells (Figure 3.4). An average over many experiments suggests the total knockout level of CD4 is about 25-35%. We also observed donor to donor transduction/knockout variation (data not shown). These low transductions and knockout rates suggest that conducting the HIV latency assay in primary CD4+ cells will be very difficult as high levels of KO are required to observe an effect from gene loss on the small percentage of latent cells present in the experiment.

We have produced CRISPR/Cas9 vectors capable of targeting genes in AIM1 and are able to show knockout of these targets in SUPT1 cells in AIM2. Unfortunately, transduction

and knockout rates in the primary CD4+ cells were found to below. We will have to improve isolation techniques, CRISPR transduction efficiency, and efficient magnetic sorting in primary cells prior conducting the latency assay. In the meantime, the assay can be attempted in SupT1 cells which obtain transduction rates of 98% and knockout rates of 60%.

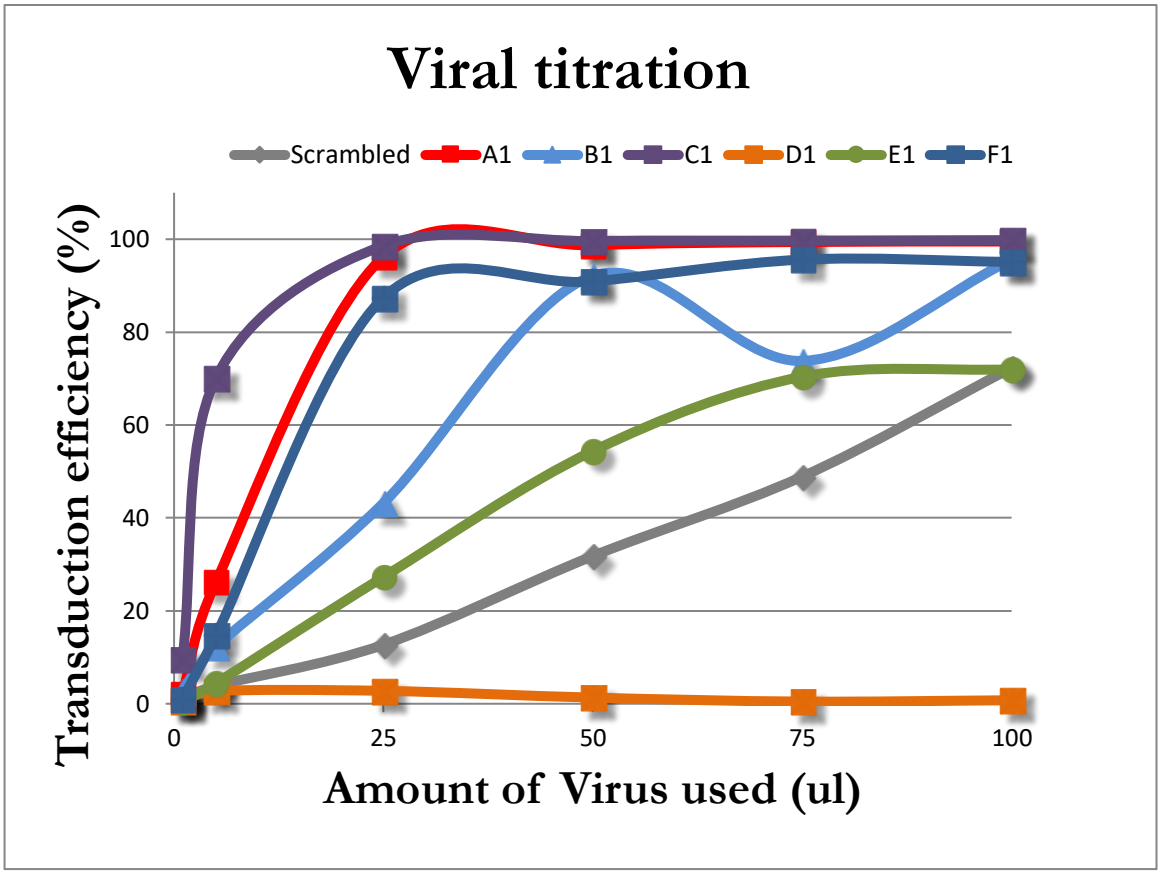


Figure 3.1. Viral titration showing transduction level at day 2 post infection of all five CD4 receptor gRNA.

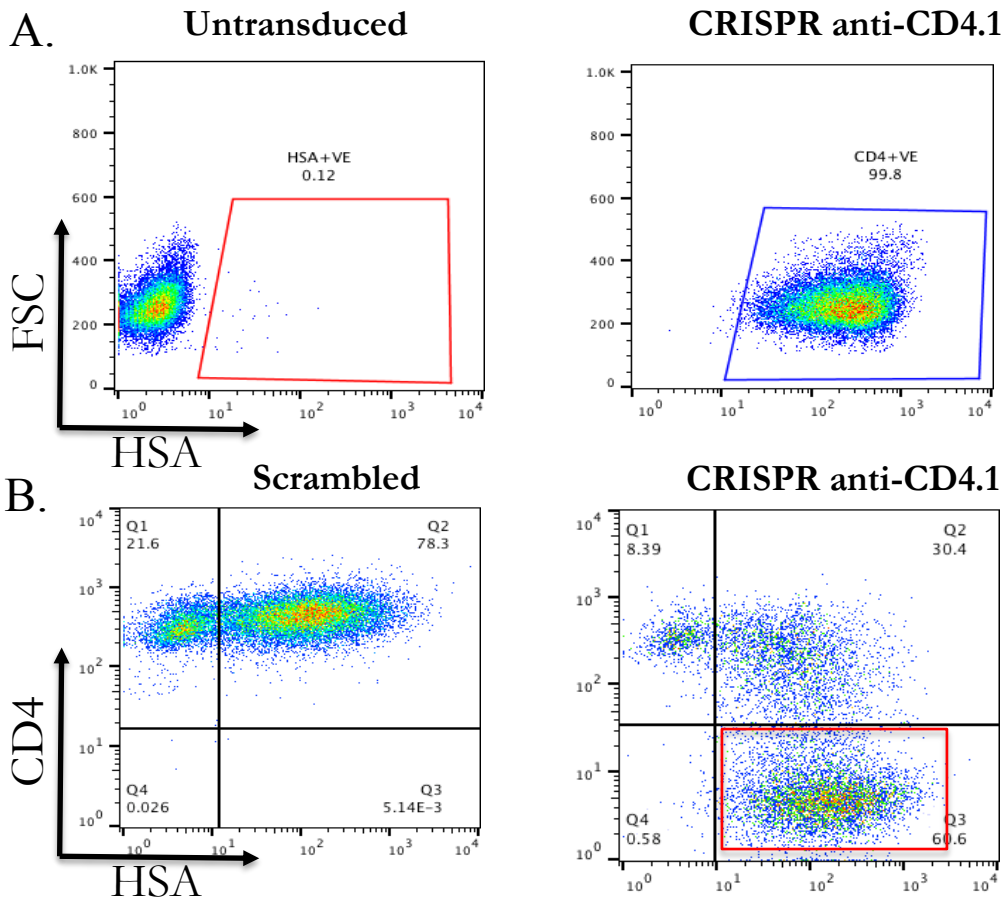


Figure 3.2. CRISPR knockout via CRISPR/Cas9 in SupT1 cells. A. HSAS indicated transduction level (99.8%). B. Showing downregulation of CD4 and HSAS expression in FACS at day 10 post transduction after sorting.

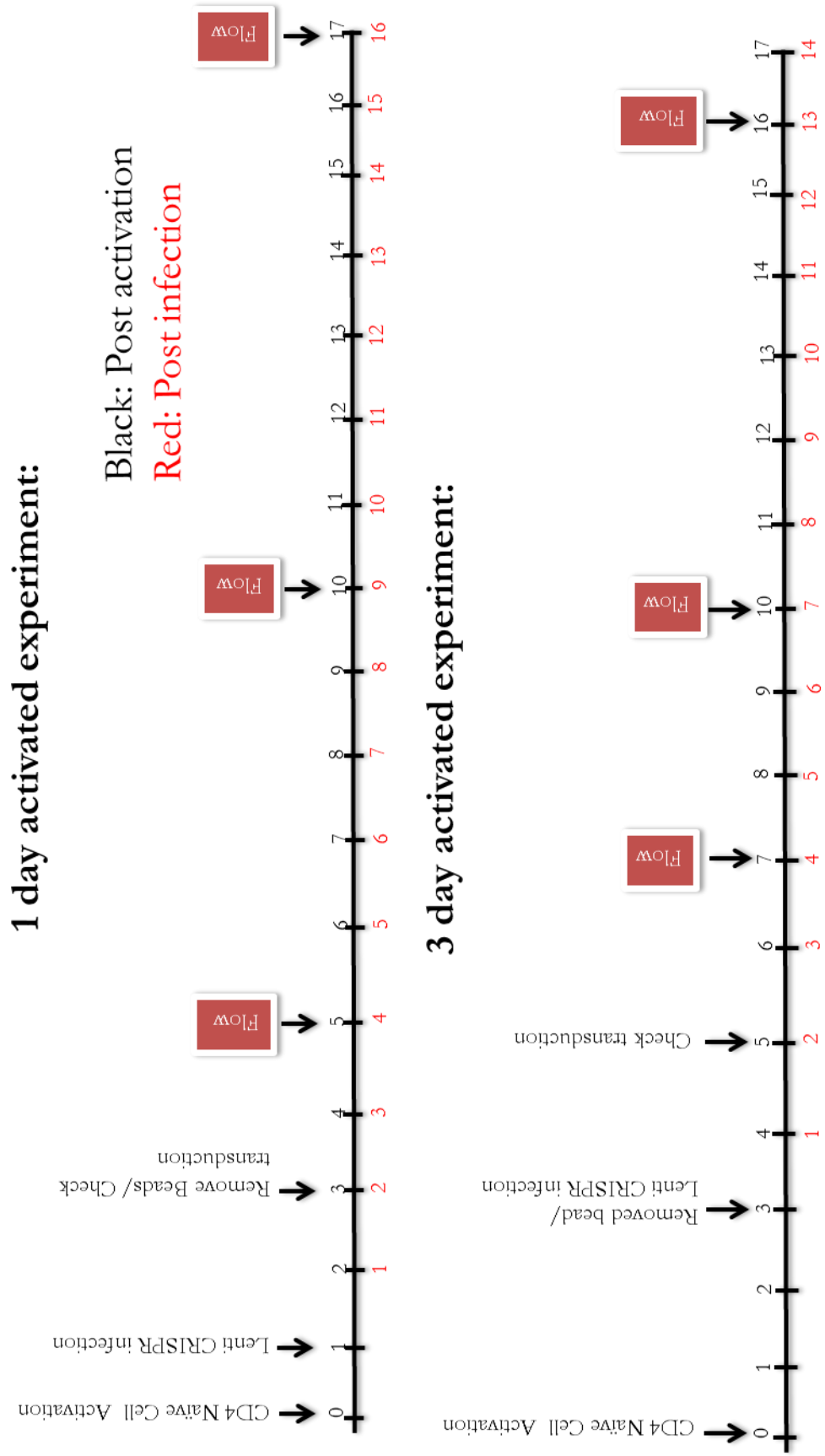
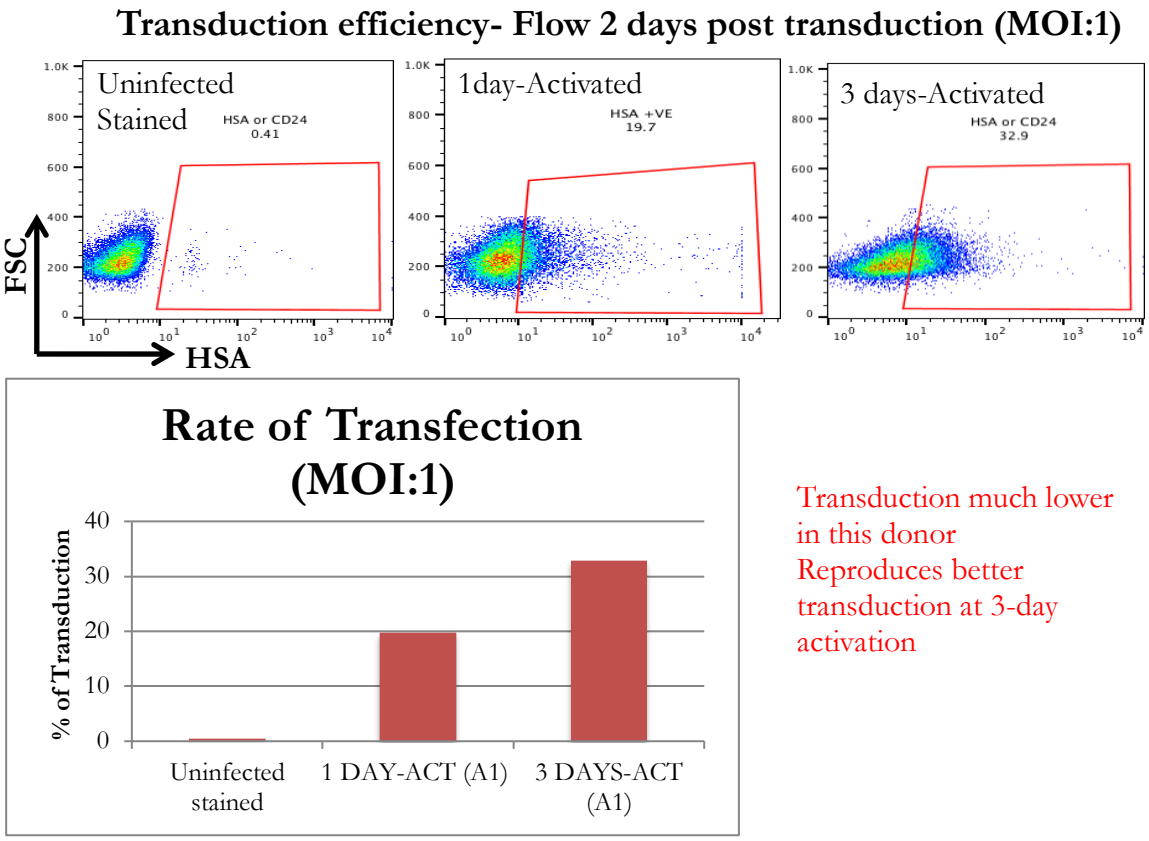


Figure 3.3. Schematic representation for validating targets in AIM3.



Transduction much lower in this donor
Reproduces better transduction at 3-day activation

Figure 3.4 FACS analysis of transduction level in primary cells after 2 days post infection

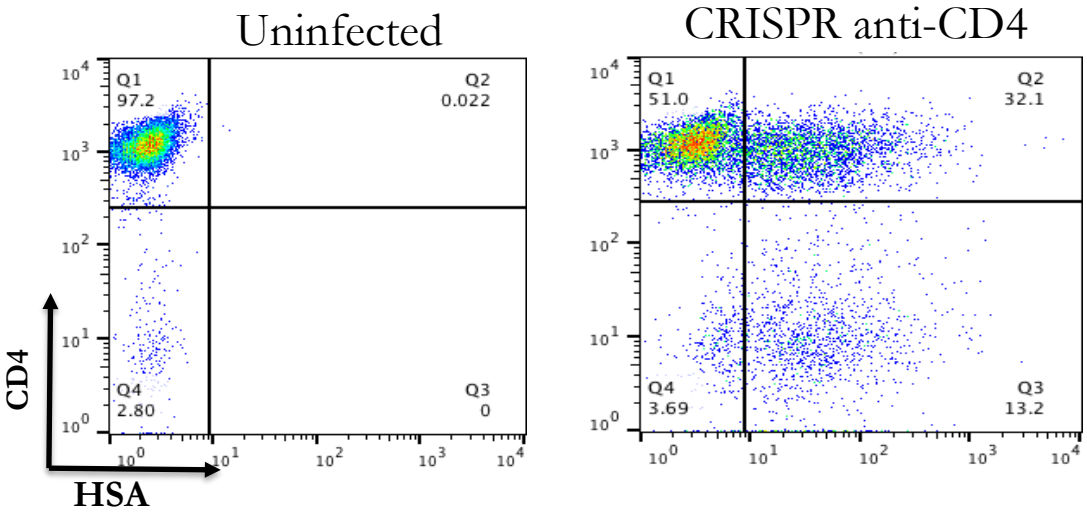


Figure 3.5. FACS analysis of CD4+ T_{CM} expressing HSAS indicating transduction level and knockout of CD4 after sorting at day 11 post infection.

CHAPTER 4

SUMMARY

In our study, we successfully generated CRISPR-containing LVPs with transduction efficiency up to 100% in SupT1 cells and 50% in primary CD4+ t cells. SUPT1 cells were infected with these CRISPR-containing LVPs and we documented the knockout of target genes through flow cytometry analysis. Two out of six anti-CD4 constructs were able to show 20-60% CD4 knockout as represented by cells with high HSAS and low CD4 expression (Figure 3.2). On the other hand, transduction of primary cells ranges from 20%-50% depending on the number of days activated. For transduction, 3-day-activated cells were able to transduce better than 1-day-activated cells, and transducing 1-day-activated cells resulted in higher knockout percentage than 3-day-activated cells. Overall, knockout efficiency in primary cells was 25-35% measured after 10 days.

Low infection and knockout percentage emphasize the need for better isolation and selection techniques for infected cells containing CRISPR knockouts. The numbers are currently too low to continue AIM3 as the HIV latency assay requires the majority of cells to be modified before an effect on latency can be determined. One solution is to continue the validation work in SupT1 cells which do have high infectivity and knockout percentage.

CHAPTER 5

METHODS AND MATERIALS

5.1 Cell lines

HEK293FT, SupT1 cells, and CD4+ T cells

5.2 Cas9/CRISPR constructs design and vector construction

CRISPR guide RNAs (gRNAs) were designed to be 20bp long and immediately preceded by 5'-NGG-3' sequence (PAM) at the 3'end. Cas9 targets for different genes were identified using an online CRISPR design tool (http://crispr.mit.edu/) [19][20]. We selected five unique and nonoverlapping guide sequences in the first or second exon of our target genes (Table 5.1). Synthetic oligos were ordered which contain the guide sequence flanked by four bases which when paired will anneal with an overhang to allow cloning into the Bsmb-I site of LentiCRISPRv2 (addgene.com) [19][21]. LentiCRISPR-HSAS vector was digested with BsmBI and dephosphorylated and the size was confirmed by running 1% agarose gel. The correct band was gel extracted and purified. Each pair of target oligos was annealed, phosphorylated, and cloned into the LentiCRISPR BsmBI site. Clones were transformed into Stbl3 cells (Life technologies) to avoid homologous recombination of the LTR sequences. Finally, positive clones were identified by sequencing (University of Utah core). This way the base filler clone in LentiCRISPR-HSAS vector was replaced by different gRNAs to generate target sgRNAs clones.

We also generated a CRISPR-HSAS-Scrambled sequence as positive control for the HSAS tag and negative control for the gene target using a pair of unique oligos (see Table 5.1).

5.3 Generation of CRISPR-containing LVP

HEK293FT (Human Embryonic Kidney 293 fast T antigen cells) cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% of fetal bovine serum (FBS) and 2mM of L-glutamine. When HEK293FT cells grow exponentially, and are at 70-80% confluent, the cells are ready for transfection. To make lentiviruses, CRISPR-sgRNA (transfer vector) (12.5ug), pSPAX2 (packaging plasmid) (12.5ug), and pVSV-G (pseudo typing plasmid) envelop (5ug) plasmid are mixed and co-transfected in HEK293FT cells using the calcium phosphate method. Supernatants were collected every 12h until the monolayer died. Lentiviruses were concentrated by ultracentrifugation at 25,000 rpm for 2h at 4°C. Most of the supernatant is aspirated and the remaining media plus virus allowed to rest at 4°C overnight before pooling, aliquoting, and freezing at -80 for long-term storage.

5.4 Viral titer determination in SupT1 cells

For the experiment, 5 x 10^5 SupT1 cells were titered by spinoculation using various concentrations from 1-100ul of CRISPR viral stocks in a total volume of 0.5 ml. Then the SupT1-virus mixture is centrifuged at 2,900 rpm for 2h at 37°C. The transduction levels were measured using an PE(XX)-ligated anti-CD24 antibody to determine production of HSAS on the surface by flow cytometry. The level of HSAS production was used to calculate the MOI (Multiplicity of Infection) necessary for further infection in Supt1 cells and Primary cells.

5.5 Isolation and activation of Tcm cells

Peripheral blood mononuclear cells (PBMC) were isolated from healthy donors' peripheral blood using density gradient centrifugation [22][23] [13]. These studies are done following protocols outlined in IRB# 67637 approved by the University of Utah Institutional Review Board. Naïve CD4+ cells were isolated via MACS-microbeads negative sorting using the naïve T-cell isolation kit (Miltenyi Biotech). Cultured Naïve CD4 T-cells were generated and infected as previously described [23][22]. After isolation, naïve cells were activated using human αCD3/αCD28-coated magnetic beads in the presence of human α-IL4, α-IL-12, and tumor growth factor TGF-β1 [23]. Activation of naïve cells was done in two different ways: in the first method, the naïve cells were activated for 1 day and then infected with lentiviral vector, and in the second, they were activated for 3 days and infected with lentiviral vector. The two different ways of activation will provide us information on transduction efficiency of the lentiviral vector. Then, we followed up the experiment to check knockout percentage of CD4 genes in different donors by staining cells for HSAS (marker) and CD4 gene (receptor) using flow cytometry.

5.6 Flow cytometry analysis

To measure the expression of levels of CD4 and HSA in SupT1 and primary cells, 1 X 10^5 cells were stained with human monoclonal antibodies: phycoerythrin-conjugated-(PE)-anti-CD4(Cat#MHCD0405-APC conjugated antibody) for CD4 receptor and mouse monoclonal antibodies Allophycocyanin (APC)-anti-CD24 to stain surface protein HSA. HSA and CD4 fluorescence was measured and collected on a BD FACSCanto flow cytometer and all data were analyzed using Flowjo.

Table: 5.1 List of guide sequences of target genes

	Name Target		Sequence
CD4			
A1	CD4-2-1	For Seq	CACCGCAGGGCTCCTTCTTAACTAA
		Rev Seq	AAACTTAGTTAAGAAGGAGCCCTGC
B1	CD4-3-1	For Seq	CACCGGCGCGATCATTCAGCTTGGA
		Rev Seq	AAACTCCAAGCTGAATGATCGCGCC
C1	CD4-62.1	For Seq	CACCGTGCACTGAGGGGCTACTACC
		Rev Seq	AAACGGTAGTAGCCCCTCAGTGCAC
D1	CD4-62.2	For Seq	CACCGGACCCTCTCCGTGTCTCAGC
		Rev Seq	AAACGCTGAGACACGGAGAGGGTCC
E1	CD4-62.3	For Seq	CACCGGGTGGGTCCCCACACCTCAC
		Rev Seq	AAACGTGAGGTGTGGGGACCCACCC
F1	CD4-62.4	For Seq	CACCGCCTGCTGGAATCCAACATCA
		Rev Seq	AAACTGATGTTGGATTCCAGCAGGC
Hexim1			
A2	Hex1-5	For Seq	CACCGTCGAGAGCGTTCCCCCAGTT
		Rev Seq	AAACAACTGGGGGAACGCTCTCGAC
B2	Hex1-1	For Seq	CACCGCGGACGAGTCGTCCCCATTC
		Rev Seq	AAACGAATGGGGACGACTCGTCCGC
C2	Hex2-9	For Seq	CACCGGACTCCGAGGCCAGTAAGTT
		Rev Seq	AAACAACTTACTGGCCTCGGAGTCC
D2	Hex3-5	For Seq	CACCGAACCGTACTACAAGCTGACC
		Rev Seq	AAACGGTCAGCTTGTAGTACGGTTC
E2	Hex3-13	For Seq	CACCGAACAGAGCCTTCGAGCTTCA
		Rev Seq	AAACTGAAGCTCGAAGGCTCTGTTC
F2	Hex3-1	For Seq	CACCGCGAGCCGAGATGTTCGCCAA
		Rev Seq	AAACTTGGCGAACATCTCGGCTCGC
CDK9			
A3	CDK9-2-1	For Seq	CACCGCTTGGCGAGCTTCTCGTATT
		Rev Seq	AAACAATACGAGAAGCTCGCCAAGC
B3	CDK9-79.1	For Seq	CACCGGCACCGCAAGACCGGCCAGA
		Rev Seq	AAACTCTGGCCGGTCTTGCGGTGCC
C3	CDK9-2-5	For Seq	CACCGGGTGCTGATGGAAAACGAGA
		Rev Seq	AAACTCTCGTTTTCCATCAGCACCC
D3	CDK9-3-1	For Seq	CACCGAAGGATCTTGATCTCCCGCA
		Rev Seq	AAACTGCGGGAGATCAAGATCCTTC
E3	CDK9-79.2	For Seq	CACCGTTCCCCCTATAACCGCTGCA
		Rev Seq	AAACTGCAGCGGTTATAGGGGGAAC
F3	CDK9-79.3	For Seq	CACCGGCTCGCAGAAGTCGAACACC
		Rev Seq	AAACGGTGTTCGACTTCTGCGAGCC

Table 5.1 Continued

	Name Target		Sequence
BRD4			
A4	BRD4-28.1	For Seq	CACCGCAGTTGGTTGGTCTGCCTCT
		Rev Seq	AAACAGAGGCAGACCAACCAACTGC
B4	BRD4-28.2	For Seq	CACCGTAAGATCATTAAAACGCCTA
		Rev Seq	AAACTAGGCGTTTTAATGATCTTAC
C4	BRD4-3-6	For Seq	CACCGGAGCTTCTGCCATTAAGACT
		Rev Seq	AAACAGTCTTAATGGCAGAAGCTCC
D4	BRD4-3-1	For Seq	CACCGAACCGAGATCATGATAGTCC
		Rev Seq	AAACGGACTATCATGATCTCGGTTC
E4	BRD4-4a-1	For Seq	CACCGTTTGGTACCGTGGAAACGCC
		Rev Seq	AAACGGCGTTTCCACGGTACCAAAC
F4	BRD4-4a-3	For Seq	CACCGGGTCTGGACGATGAGGTCCG
		Rev Seq	AAACCGGACCTCATCGTCCAGACCC
CXCR4			
A5	CXCR4-1-2	For Seq	CACCGGGGCAATGGATTGGTCATCC
		Rev Seq	AAACGGATGACCAATCCATTGCCCC
B5	CXCR4-1-1	For Seq	CACCGGAAGCATGACGGACAAGTAC
		Rev Seq	AAACGTACTTGTCCGTCATGCTTCC
C5	CXCR4-95.2	For Seq	CACCGTTGTCATCACGCTTCCCTTC
		Rev Seq	AAACGAAGGGAAGCGTGATGACAAC
D5	CXCR4-2-1	For Seq	CACCGGCCGTGGCAAACTGGTACTT
		Rev Seq	AAACAAGTACCAGTTTGCCACGGCC
E5	CXCR4-95.1	For Seq	CACCGGTAGCGGTCCAGACTGATGA
		Rev Seq	AAACTCATCAGTCTGGACCGCTACC
F5	**CXCR4-2-3**	For Seq	CACCGAGGTGGTCTATGTTGGCGTC
		Rev Seq	AAACGACGCCAACATAGACCACCTC
Scrambled			
SC	**Scrambled**	For Seq	CACCGGCACTACCAGAGCTAACTCA
		Rev Seq	AAACTGAGTTAGCTCTGGTAGTGCC

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