CCR5 gene knock-out mediated TALEN technique and its effects against HIV infection on lymphocytes and macrophages

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Abstract

Most strains of HIV-1-1 use CD4 and CCR5 as receptors for cell entry. A naturally occurring CCR5 gene mutation known as CCR5-Δ32 results in CCR5 dysfunction and makes the cells resistant to HIV-1-1 infection. Previously, two patients who received hematopoietic stem cell transplantation which carried homozygous CCR5-Δ32 have had their HIV-1-1 infection functionally cured, suggesting that gene-editing targeting CCR5 could make it possible for curing HIV-1-1 infection. Currently, the transcription activator-like effector nucleases (TALEN) system provides the highest accuracy on gene editing with good efficiency. In this study, I aim to establish a TALEN-based gene-editing platform targeting CCR5 for HIV-1-1 cure. Previous publication has indicated that mRNA has more advantages than DNA when delivered to the cells. Thus, firstly in this study, I optimized electroporation delivery of mRNAs to the cells and then the protocol of *in vitro* transcription of mRNA. After transfecting primary T cells with TALEN mRNAs, the expression of surface CCR5 was downregulated compared to the non-edited cells. The knock-out of the CCR5 gene was confirmed on the genomic level by DNA sequencing. After HIV-1-1 challenge, the CCR5 gene-edited primary T cells showed increased HIV-1-1 resistance compared to their non-edited controls. Macrophages also express both CD4 and CCR5, which makes them another target of HIV-1 besides CD4+ T cells. Moreover, HIV-1-infected macrophages are more resistant to immune attack and are a key reservoir of HIV-1. Thus, I tested this TALEN system on monocytes derived macrophages (MDMs). After transfection of the TALEN mRNAs, the surface CCR5 expression on the MDMs was also downregulated. The CCR5 gene knock-out was confirmed on the genomic level as well. The TALEN-treated MDMS

showed increased resistance to HIV-1 infection compared with the un-edited controls after HIV-1 challenge. The result of this study provides evidence and support for using TALEN to knock out CCR5 as a method for curing HIV-1 infection.

Declaration of Originality

I, Sai Liu, declare that all the work presented in this thesis is my own work, and that any information used here from other published, unpublished sources or collaboration is correctly referenced.

Copyright Declaration

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Abbreviations

Anti-reverse cap analog	ARCA
Combined anti-retroviral therapy	cART
Complementary DNA	cDNA
Clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9	CRISPR/Cas9
Dendritic cell	DC
Dulbecco's modified Eagle's medium	DMEM
Dulbecco's Phosphate Buffered Saline	DPBS
Double-strand break	DSB
Escherichia coli	E. coli
Enhanced green fluorescent protein	eGFP
Fetal bovine serum	FBS
Genomic DNA	gDNA
GHOST-CXCR4-CCR5-CXCR4 cell line	GHOST-Hi5
Guide RNA	gRNA
Graft-versus-host disease	GvHD
Homology direct repair	HDR
Human immunodeficiency virus	HIV
Hematopoietic stem cells	HSCs
Hematopoietic stem cell transplantation	HSCT
Human T-cell leukemia virus	HTLV
Interleukin-2	IL-2
in vitro transcription	IVT

Lipid nanoparticles	LNP
Macrophage-cluster stimulation factors	M-CSF
Monocyte-derived macrophages	MDMs
Multiplicity of infection	MOI
Mononuclear phagocyte system	MPS
Men who have sex with men	MSM
Macrophage	Mφ
Next-generation sequencing	NGS
Mon-homologous end joining	NHEJ
Mucleoside reverse-transcriptase inhibitors	NRTIs
Nucleoside triphosphates	NTPs
Protospacer adjacent motif	PAM
Polymerase chain reaction	PCR
Phytohemagglutinin	PHA
Cas9 ribonucleoprotein	RNP
Roswell Park Memorial Institute Medium	RPMI1640
Rev Response Element	RRE
Sterile alpha motif and histidine/aspartic acid domain-containing protein 1	SAMHD1
Tris-acetate-EDTA	TAE
Transcription activator-like effector nucleases	TALENs
Transcription activator-like effectors	TALEs
Tris-Borate-EDTA	TBE
Median tissue culture infectious dose	$TCID_{50}$
Transfer RNA(lysine 3)	$tRNA^{lys3}$

Untranslated region	UTR
Zinc finger nucleases	ZFNs

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Chapter One. Introduction

1.1 Global AIDS/HIV epidemic

Being a global public health issue for more than three decades, the first case of human immunodeficiency virus (HIV) infection leading to AIDS was observed and medically examined in the United States in 1981(1). It was found that these patients with rare opportunistic infections have severely suppressed cellular immunity without a known cause, and later, it was discovered that there was a dominant percentage of intravenous drug users or men who have sex with men (MSMs) among the patients. As cases accumulate, several cases suffering from Kaposi's sarcoma also appeared which attracted the interest of the Centre for Diseases Control (CDC) of the United States(2), which was the beginning of this disease to be properly investigated.

At the very early stage of the investigation, the cause of this disease was unknown, and several names were given to it. The early epidemiology investigation indicated that this disease mostly co-relates to homosexuals, heroin users, hemophiliacs, and Haitians. However, not after long that it was found that this disease was not isolated in these four communities and displayed a tendency to spread to other communities. Finally, the CDC assigned the final name to this disease, acquired immunodeficiency syndrome, AIDS.

The investigation to find the cause of the disease continued. In 1983, it was firstly reported

by Barré-Sinoussi *et al* of Luc Montagnier team in Pasteur Institute in Paris, France, that they isolated a virus from the lymph node of a man at risk of AIDS, they believed it can be the cause of AIDS and named it lymphadenopathy-associated virus(3). One year later, Gallo *et al* have isolated a retrovirus from AIDS or pre-AIDS patients, which they found to have significant similarity to the human T-cell leukemia virus (HTLV) and named it HTLV-III(4). Further research on these two strains of pathogen continued, and in 1986, it was finally confirmed that they are the same virus, which is different from HTLV immunologically, and named it human immunodeficiency virus (HIV-1)(5). By the end of that year, a total of 38,401 AIDS cases had been reported by 85 countries to the World Health Organization.

3 years after the discovery of HIV-1, another subtype of HIV was also isolated and reported from a patient in Cape Verde(6). Further investigations have shown that HIV-1 and HIV-2 are both descendent of the simian immunodeficiency virus (SIV), but from different SIV subtypes(7, 8).

With the confirmation of the pathogen, AIDS can be better understood. Currently, AIDS is regarded as is a spectrum of conditions caused by the infection of HIV(9). 2-4 weeks after the contraction of HIV-1, 40-90% of the patients develop an influenza-like illness or a mononucleosis-like illness, which can exhibit fever, swollen lymph nodes, throat ache, a rash, headache, tiredness. This stage is called an acute infection, which lasts for around one month, and opportunistic infections can happen in this stage(10). During this stage, the acute adaptive immunity against the infection would be established, which causes the

dynamic changes of the serum level of HIV components. Fiebig *et al* have established a staging system to describe the timepoint and duration of each stage, which helps to determine the length of infection in acute infection, as well as the diagnosis protocol for acute HIV infection(11). Notably, acute symptoms usually last for less than a month, however, it can cause up to 154 days for the serum marker of HIV-1 to achieve the balance.

Table 1.1 Fiebig stages of early HIV infection

Fiebig stage	Cumulative duration (days)	HIV RNA	p24 antigen	Immunoassay	Western blot
1	5	+	–	–	–
2	10	+	+	–	–
3	14	+	+	+	–
4	19	+	+/–	+	Indeterminate
5	88	+	+/–	+	+ (p31 band-)
6	Open-ended	+	+/–	+	+ (p31 band+)

After the acute phase of HIV-1 infection, this disease will step into the chronic phase, where there will usually be no symptoms to experience, and the viral load in the blood will maintain a steady but relatively low level. However, during this stage, the level of the CD4+ T cells of the patient will go through a slow depletion, when this depletion reaches lower than 350 cells/μl blood, it is defined as the pre-AIDS phase, and some minor symptoms as mild but persistent fatigue can appear. And when the CD4+ lymphocytes reach 200 cells/μl of blood, then the patients step into the AIDS phase, as this level of CD4+ T cells is not enough to work as the centre of adapted immunity, which allows opportunistic infection and cancers to emerge.

Infection of HIV-2 causes similar symptoms. However, the symptoms, physiopathology and disease progression are much milder or slower than HIV-1 infection(7, 8). To date, HIV-2 infections are mainly recorded in West Africa, with cases sparsely separately globally(12). All through the HIV pandemic, most HIV infections are caused by HIV-1 infection.

Currently, there is no cure for HIV infection via medication. However, with the combined antiretroviral therapy (cART), the HIV infection can be controlled by suppressing the viral replication entirely for the lifetime. But the cART requires daily taking of medication with punctuation, and adherence to the medication must be precise (>95% on-time taking of pills) and life-long, thus cART puts a considerable burden on the healthcare system of every country, also on the patients without access to the medication either/or physically and financially. In light of this situation, research on HIV-1 cure has never stopped its steps.

1.2 HIV-1 structure

To develop a cure for HIV-1 infection will be impossible without the understanding of the structure and life cycle of HIV-1, let alone the pathogenesis of it. HIV-1 belongs to the genus of Lentivirus and is a member of the retrovirus family. HIV-1 has an average diameter of 120nm, and is in a roughly spherical shape, it is a single-stranded, positive-sense, enveloped RNA virus (Figure 1.1).

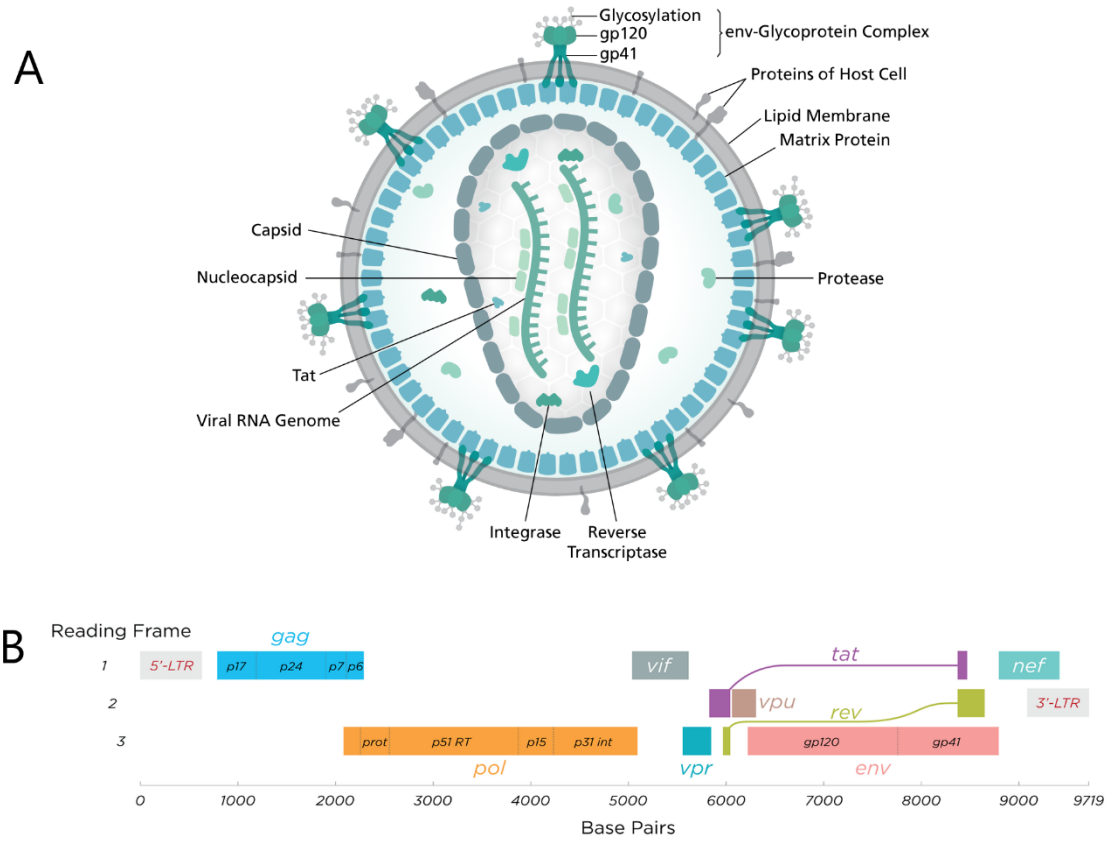

Figure 1.1. The structure and genome of a mature HIV-1 particle. A) the structure of the HIV viral particle. B) the genome organization of the HIV genome. The credit of both illustrations: Thomas Splettstoesser, unmodified, under CC BY-SA 3.0 license.

The outmost layer of the virus is a lipid bilayer taken from the cell membrane when the virus particle is released. Under the lipid bilayer is the envelope matrix protein p17 of HIV-1 working as the backbone of the bilayer, which encapsulates the genomic RNA and other proteins(13). On the envelope, there are two different proteins exposed to the environment(14), one of them is the protein taken and attached from the host cell, the other is the envelope protein complex of gp120 and gp41. This complex is shaped like a tall glass, the top of it is gp120 which is a trimer. Under gp120 is gp41 which shapes like a stem to connect gp120 and the envelope.

The gp120 protein, which is encoded by the *env* gene of HIV-1 around 480 amino acid(14), is the key component of HIV-1 to interact with the surface molecule of its target cells, inducing the attachment of HIV-1 to a target cell which leads to virus entry. Primarily, the env gene product is gp160, after translation, gp160 will be cleaved into gp120 and gp41, then gp120 will be non-covalently bonded to gp41 and gp41 will be anchored on the envelope(15).

The envelope encapsulates the Vif, Vpr, Nef and viral protease, along with the capsid of the viral genome. The capsid of the genome is composed of viral protein p24 and inside it, the integrase and reverse transcriptase can be found, along with the noncovalently linked two copies of positive-sense single-stranded RNA.

1.3 HIV-1 infection and life cycle

There are three main routes for the transmission of HIV-1, which are sexual contact, significant exposure to infected body fluids or tissues, and vertical transmission (mother to child transmission including pregnancy, delivery, or breastfeeding)(16). Sexual contact transmission can happen during heterosexual along with male homosexual intercourse, which in its nature is an exchange of body fluid via the small to micro-injury to the sexual organs during intercourse. The significant exposure to infected body fluids or tissues is mainly caused by sharing used needles for intravenous usage of narcotics, and it can also be from blood transfusion of contaminated or infected blood. HIV-1 can't be transmitted

through common day-to-day physical contact, or most externally secreted body fluid, such as saliva, sputum, sweat(17).

Currently, sexual transmission is accounted as the primary cause of new cases(18). Globally, heterosexual transmission takes the largest percentage. However, this percentage can vary depending on countries. For example, as of 2018, male homosexual anal intercourse was accounted for 86% of the new cases(19). Intra-venous exposure to blood or blood product is the second major cause of HIV-1 infection. In resource-rich countries, screening the blood product for HIV-1 has made the risk of HIV-1 contamination very unlikely, but in a less developed country, it can still be a problem. Moreover, intravenous drug abuse is still a global issue, sharing a needle to inject the recreational drug still introduces the risk of HIV-1 transmission to these people. This specific route can be managed by clean needle exchange programs. Unfortunately, it is not very financially and logistically feasible in low resources countries(20).

1.3.1 Viral entry, life cycle and release

Given the transmission route of HIV-1, it is clear that HIV-1 infection starts with the exposure of the targeted immune cells to HIV-1. Although the site of the exposure can vary depending on the route, the new life cycle of an HIV-1 particle starts with attaching to its target cell. The target cell of HIV-1 is the CD4+ cell, which includes CD4+ T cells and mononuclear phagocyte system (MPS) cells(21). All the target cells need is to express CD4

molecule and a chemokine co-receptor (CCR5 or CXCR4) for HIV-1 to initiate the viral entry(21, 22). When meeting the target cell, the gp120 starts to interact and bind to the CD4 molecule, which will lead to a structural change of gp120 to expose the chemokine receptor binding domains, which will bind to the chemokine co-receptor(22, 23). When this process is completed, gp41 will be activated and penetrate the cell membrane to fuse the virus envelope with the cell's lipid bilayer and subsequently release the viral enzymes and capsid into the cell(22, 23).

After the release of viral enzymes and the capsid into the cell plasm, the capsid proteins of HIV will be degraded. However, what remains there will start the next step of HIV's life cycle. The released components include an isotype of transfer RNA for lysine, the $tRNA^{lys3}$ which is acquired and packed into the viral particle from the previous viral host. The $tRNA^{lys3}$ will bind to the 5' end of the viral RNA genome and prime the follow-up retro-transcription. The retro-transcriptase from the virus then catalyzes the retro-transcription of the viral RNA genome into a double-stranded complementary DNA (cDNA) (24). This viral cDNA will subsequently be translocated to the host genome, where the viral integrase will integrate the cDNA to a random site of the host genome(25), and the integrated viral cDNA is then named the HIV-1 provirus. The reverse transcriptase lacks the correction function thus is highly prone to introducing errors to the cDNA, which is the major reason for the very high rate of mutation of HIV-1(26). After this, the viral DNA can stay dormant, or get transcribed with the cell's activity with the presence of certain cellular transcription factors, for example, NF-κB(27).

The transcription of the provirus will produce full-length viral genomic RNAs. Most of these gRNAs remain intact and encode the *gag*/*pro*/*pol* genes of HIV, but a few of them will be subsequently spliced into shorter RNAs. Through the primary and second splicing process, the full-length viral RNA will be cut into shorter viral RNAs of various lengths. The full-length and partially spliced viral RNA would retain in the cell nucleus, as they all contain the Rev Response Element (RRE), while the fully spliced RNA that has the RRE removed will mature into mRNAs and be transported out of the nucleus. These fully spliced mRNAs are responsible for the translation into auxiliary proteins of TAT, REV, Nef, Vif and Vpr. The partially spliced viral RNA will be processed into mRNA for Vif, Vpr and Vpu/Env, or a longer isoform mRNA for TAT. The rev protein translated from one of the spliced mRNAs will be translocated back into the cell nucleus, where they will bind to the secondary structure of RRE and activate the alternate CRM1-mediated nuclear export pathway, which transports all the HIV viral RNAs out of the cell nucleus(28). All these viral mRNAs will translate into the viral proteins or the precursor proteins.

The proteins translated from the mRNAs mentioned above are not necessarily the final proteins to assembly an infectious new viral particle. Some of the proteins can be first translated into a precursor protein or polyprotein. These polyproteins can be modified through a series of biochemical reactions and cleaved by the original viral protease introduced by the viral particle that infected the cell(15). For example, the gag-pro-pol mRNA can translate into standalone gag protein, or the gag-pro-pol polyprotein through the ‘occasional ribosomes slip backward one nucleotide’ mechanism. Through this process,

it produces roughly a 20 gag to 1 gag-pro-pol ratio, to ensure the abundance of gag protein and significantly less pro and pol protein. The polyprotein needs to be further cleaved individual functional protein, along with other mature viral proteins, to catalyze and assemble an infectious HIV-1 particle. For example, the gag protein (p55) will then be cleaved into matrix protein p17, capsid protein p24, p15 by the viral protease and biochemically modified. The p15 protein will be further cleaved into nucleocapsid protein p7, p6 and p1 of unknown function. Along with other mature-at-the-beginning enzymes and proteins, they will pack some of the full-length HIV-1 RNAs and assemble a new virus particle(29). During this process, it is possible that some of the viral structural or functional proteins have not been successfully modified, which may result in failure of assembling a new viral particle or loss of infectivity(30). The final assembling and maturation of the virus happen on the plasma membrane, where the newly synthesized Gag protein is transported to. After this, the new virus particle is ready to bud from the cell membrane(31, 32).

1.3.2 Pathogenesis of HIV-1 infection

In the early years of HIV-1/AIDS research and clinical identification, the first characteristic of acute HIV-1 infection is the rapid CD4+ T cell depletion within the period of fast and massive viral replication. During the acute phase, the viral load can reach several million copies of HIV-1 per milliliter of blood(33). However, the adaptive immunity against HIV-1 also starts to build, and from a little later than halfway of the acute phase, the cytotoxic T lymphocytes (CD8+ T cells or CTLs) already acquire the ability to kill the infected CD4+ cells.

This killing effect leads to the rapid control of viremia and rebound of the CD4+ T cell level(34). By 4-6 weeks after primary infection, the humoral immunity will be established with HIV-1 antibody production to the level of detection, which is known as seroconversion. During the acute phase, 50-90% of the patients develop a set of influenza-like symptoms, which start to emerge usually at day 10-14 of infection and last for 1-4 weeks(35, 36). The CD8+ T cells attacking the infected cells as the main mechanism, along with the apoptosis of the infected cells, can deplete the CD4+ T cells to a very low level that allows the opportunistic infection to develop. This effect is found not only in the circulating immune cells but mostly in the mucosal CD4+ T cells, which will be massively depleted during the acute phase and explains the digestive tract symptoms, due to the higher level of CCR5 expression on them than the circulating counterpart(37)

After the acute phase, the balance of immunity and HIV-1 replication will be established. The CD8+ T cells and humoral immune response can not eliminate the infection, but only control it to a certain level. On the other hand, the continuous replication of HIV-1 maintains the activation of the immunity against HIV-1(38). Moreover, the loss of mucosa CD4+ T cells would not be restored much after the acute phase, which compromises the immunity surveillance in the digestive tract and contributes to microbial translocation, which is another major mechanism of persistent systemic immunity activation(39). With the immunity activation, the immune cells are more prone to apoptosis, and the persistent activation will also eventually cause functional exhaustion of immune cells. The depletion and exhaustion of immune cells will finally lead to the collapse of immunity(40)

1.3.3 The treatment of HIV-1 infection and HIV-1 reservoir

In the early days of HIV-1 medical management, this infection was a death sentence to the patients. Following the acute phase, the chronic phase of HIV-1 infection usually lasts around 8 years. When developed into the AIDS phase, the life expectance is shorter than a year. Research on HIV-1 treatment has never stopped. In 1987, the first anti-HIV-1 medicine, zidovudine, was introduced for clinical use. However, previous unofficial trials conducted none-publicly had shown it did not control HIV-1 long-termly and drug resistance came up very soon. Nevertheless, drug development continued to develop more options of medication effective by different mechanisms. Finally, it was in 1996 that two trials began and the result published in 1997 that two different nucleoside reverse-transcriptase inhibitors (NRTIs) plus one protease inhibitor can successfully suppress the viremia without short term rise of drug resistance(41, 42). This treatment strategy has been from that time extensively investigated and revealed that two NRTIs as the backbone combined with a drug of a different mechanism can suppress the viremia and restore the immunity function, to achieve a near-normal to normal life expectance of the patient if managed in time. Nowadays, this treatment is named the combined antiretroviral therapy (cART), which now makes HIV-1 infection a manageable chronic infection rather than a death sentence(43).

However, cART is not a cure. This treatment requires great adhesion to it, it must be taken on time, every day, for the lifetime. For resource-rich countries, it can be a burden on the health care system. For resources limited countries or countries that do not provide free

HIV-1 treatment to the people, it will be a burden on the patients. Once the treatment is stopped, the viremia will relapse very soon.

The reason for this is because of the latency of the virus, the withdrawn of cART's suppression effect on the viral replication will soon lead to the rebound of the viremia. The cells that are latently infected are called the HIV-1 reservoir, which includes memory CD4 T cells, macrophages (Mφs), dendritic cells (DCs), microglia, epithelial cells and more to be discovered(44, 45). With the cART, the provirus that has been integrated into these cells is kept inactivated. However, these cells are also long-living cells that remain viable for years if not decades. During this period, the latency means no production of a virus component, let alone viral particle, that these cells will remain unrecognized by the immunity surveillance(46).

The reservoir cells are not only an issue of HIV-1 latency, but these cells also play a part in the spreading of the infection through different compartments of the body. For example, in the early days of HIV-1 research, it was already found that the macrophages can migrate through the blood-brain barrier, which helps to spread the virus to the central nervous system(47). The newly replicated virus from these macrophages can further infect the parenchymal microglia, meningeal macrophages and other intra-cranial cells that express CD4 and the co-receptor(48, 49). All these cells can become viral reservoirs after cART, and due to the existence of blood brain barrier, the design of new cART drugs and future cure strategies have to take penetrating the barrier into consideration.

In light of this, other strategies to manage HIV-1 infection are constantly under investigation, in the hope to find a cure. For example, a group of patients called long-term non-progressors (LTNP) has been shed light on, because although they have been infected and maintained a viremia while they have their CD4+ T cell count in the normal range without medication for significantly longer than the common HIV-1 infection progression(50). Several studies showed that several special genetic features can collectively or individually contribute to this phenomenon(51, 52). However, most of the LNTPs will eventually progress to AIDS, showing these genetic backgrounds can only slow down the progression, but not provide full control. In search of a cure, the research continues.

1.4 CCR5 and HIV-1 cure

Another group of people also caught the attention of academics. These people, although living a high-risk lifestyle, maintained infection-free. Further investigation on these people has found that a prominent group of them has a rare natural homologous mutation on their CCR5 gene which disrupts the function of the gene product, the CCR5 molecule(53, 54).

CCR5, the C-C chemokine receptor type 5, also known as CD195, locates on the cell surface of a variety of cells. CCR5 is a G protein-coupled receptor and a member of the beta chemokine receptors family of integral membrane proteins(55). Expressed on a variety of immune cells, it is also expressed on a subpopulation of breast and prostate cancer cells and works to block the metastasis of these two cancers (56).

Currently, there is a CCR5 inhibitor medicine developed to treat HIV-1 infection(57). Most importantly, there are two cases that functionally eradicated their infection of HIV-1(58, 59) and one presumed to have done that(60), based on CCR5 dependent medical procedures. To understand this, it is important to discuss the HIV-1 tropism then how to use CCR5 as a target.

1.4.1 HIV-1 tropism

As previously described, HIV-1 needs to recognize and interact with the CD4+ and a chemokine co-receptor to enter the target cell. Most HIV-1 strain recognizes either CCR5 or CXCR4 molecule as the co-receptor(61). Between these two chemokine receptors, CCR5 is the major one used, and almost by all primary HIV-1 strains regardless of genetic subtype(62). The HIV-1 strains that only uses CCR5 as the co-receptor are called an R5 strain of HIV-1, formerly known as the macrophage (M-tropic) strain. And the strain that uses CXCR4 is called an X4 strain. Worth noticing, there are strains that can use both co-receptor, known as R5X4 dual tropic strains.

The sequential difference of the gp120 protein determines the tropism of the virus. The gp120 protein has 3 variable loops, named V1 V2, and V3. There is also a bridging sheet under the V1-V2 region linking the C4 region of gp120(63). Previous studies have shown that the bridging sheet and the base of the V3 loop together is the key region responsible for the interaction of gp120 and the co-receptor and mutation in this region changes the

specificity of the co-receptor recognition from CCR5 to CXCR4 (64, 65).

Natural isolates of HIV-1-1 strains are almost all R5 tropic, and more than 90% of the primary infection are with the R5 virus(66-69). However, during the course of infection, it happens that the occurrence of mutation on the V3 loop of the virus switches the tropism of the original virus(70). It is worth noticing that, the duration of infection is not necessarily a factor to drive the tropism to shift, it is caused by multiple factors, for example, strong immune pressure and inadequate use of CCR5 inhibitor(71). These findings suggest that using CCR5 as a target needs an extensive assessment before commencing, but it is possible to be used at various stages of HIV-1 infection.

With the different tropism of HIV-1 strains, the role of dendritic cells is crucial. Currently, the sexual transmission route of HIV accounts for the highest percentage of new cases, and DCs are located in the skin, genital epithelia and subepithelia of genitalia(72). Because DCs express a high level of both CCR5 and CXCR4, they are the first target cells to be exposed to the virus. After the antigen process of HIV by the DCs, T cells that have contacted the infected DCs can be activated to T helper cells and subsequently get infected and disseminate the infection. Furthermore, the infected DCs still retain the ability to migrate to the nearby lymphoid tissues such as lymph nodes, where the infected DCs would contact the HIV target cells massively(73). With the dual expression of CCR5 and CXCR4, DCs plays a central role in the initiation and dissemination of HIV infection, regardless of the viral tropism. This factor needs to be considered when using CCR5 as a therapeutic target.

1.4.2 CCR5 based treatment and the three cases of HIV-1 functional cure.

The population who has the homozygous 32 base pairs deletion of their CCR5 gene (CCR5-Δ32) remained infection-free living their high risky life has inspired quite a lot of research. Many CCR5 related events happened in 2007. Firstly, a small molecule drug to inhibit CCR5 called Maraviroc was approved by the US FNA for clinical use. However, with the possible underlying X4 or R5X4 strain circulating in some individuals, these patients can fail the treatment(74). Nevertheless, Maraviroc was still effective in quite a large percentage of patients and also provides a tool for CCR5 related scientific research. Also in 2007, the Berlin patient received his bone marrow transplantation which carried the CCR5-Δ32/Δ32 mutation, years later after very tight monitoring, proved to be the first functional cured patient of HIV-1 infection, if not a completed viral eradication(58).

The CCR5-Δ32 mutation is a 32-base-pair deletion causing a premature stop codon on the CCR5 gene, as a result, the CCR5 molecule translated from that gene is dysfunctional. This mutation attracts attention from academics and the research on it showed that this mutation originated from northeast European Caucasians(75). This mutation appeared rather late in history around only 2250 years old, thus did not have enough chance to further spread to different people or races. Due to these facts, only 2-5% of the population in Europe, the Middle East and the Indian Subcontinent has homozygous CCR5-Δ32(76). When it comes to heterozygous, the percentage is bigger but still small, for example, 10-20%

of European Caucasians processes at least one allele with CCR5-Δ32(77, 78). It is easy to deduce that with homozygous CCR5-Δ32 the individual is very likely to be immune from R5 tropic HIV-1 infection, because of the dysfunctional CCR5 molecule. Even with heterozygous CCR5-Δ32 +/-, the individual will have lower CCR5 expression on the cell surface which will also work as a protective factor against R5 strains of HIV-1(79). The protective effect of CCR5 disruption was proved too *in vitro* by using siRNA to completely block the CCR5 mRNA and this procedure led to full resistance to laboratory adapted R5 tropic HIV-1 strain(80).

In light of all these findings, when the 'Berlin patient' who had HIV-1 infection and acute myeloid leukemia went to Dr Gero Hütter, Dr Hütter decided to perform bone marrow transplantation to treat the patient's leukemia but at the same time, CCR5 came into his mind. The medical team selected one donor among 60 matching donors because this particular donor was CCR5-Δ32 +/+. The Berlin patient stopped his cART after this treatment immediately. He also suffered graft-versus-host disease, leukoencephalopathy, and other complications, and even had a relapse of leukemia so had the same transplant from the same donor one year later, and three years later, his medical team reported he's HIV-1 viral load in the blood was constantly undetectable, no virus replication was found in the biopsy of multiple compartments of HIV-1 latency, and his HIV-1 specific antibody was in constant decline, suggesting the 'Beilin patient' was very likely to have a functional cure of HIV-1 infection(58).

The ‘Berlin patient’ was such an inspiration to the fight against HIV-1 infection, but the scarcity of homozygous CCR5-Δ32 donors severely limits the application of this treatment. More importantly, bone marrow transplantation alone carries high or even lethal risks irrelevant to CCR5. Most of the severe complications that the ‘Berlin patient’ suffered can kill the patient and this led to the research to advocate against using bone marrow transplantation to solely treat HIV-1 infection. But HIV-1 infection and hematological malignancy can happen together and that’s why the International Collaboration to guide and investigate the potential for HIV-1 cure by Stem Cell Transplantation (IciStem, https://www.icistem.org) has been established. This European collaboration has screened hundreds of thousands of hematopoietic stem cell donors to pool the donors with homozygous CCR5-Δ32 (up to 22000 donors identified by 2019)(81), if any patient comes up with HIV-1 infection and hematological malignancy that needs hematopoietic stem cell transplantation (HSCT), the patient can be matched to the donor’s pool and have a test to confirm if he only has R5 strain virus infection. If affirmative to both questions, then he can be a candidate to have HSCT with CCR5-Δ32 +/+.

The IciStem has performed the HSCT on 39 HIV-1+ patients by 2019 with hematopoietic stem cells from their donor’s pool, among the 16 patients who had blood samples available, 4 were transplanted with homozygous CCR5-Δ32 hematopoietic stem cells and two patients showed clear pieces of evidence of a functional cure of HIV-1 infection(82).

The first one, known as the ‘London patient’ (IciStem cohort #36) was a patient who

suffered from HIV-1 infection and Hodgkin's lymphoma and had the HSCT from IciStem's CCR5-Δ32 +/+ donor. His cART was interrupted 16 months after the HSCT and viremia didn't rebound ever since. At 46 months post-HSCT, his plasma, semen, and cerebrospinal fluid sample still didn't show any HIV-1 RNA trace. Biopsy samples from gut, lymph nodes were tested for HIV-1 provirus, only incomplete provirus was found at an extremely low level, indicating they were only the remanence of his immune system before the HSCT, all the productive provirus integrated cells had all been killed. HIV-1-specific CD4+ and CD8+ T cell activities were also undetected, although he still had HIV-1-specific antibodies, the antibody level was very low and in constant decline(59). All these indicated a successful replicate of the 'Berlin patient'.

The other HIV-1+ patient from IciStem project's CCR5-Δ32 +/+ HSCT who survived was called the 'Düsseldorf Patient' (IciStem#19) who suffered acute myeloid leukemia. His cART was interrupted 70 months after HSCT and 14 months later, his viremia has not rebound which was reported on The annual Conference on Retroviruses and Opportunistic Infections (CROI) 2019, a year later on the CROI 2020, the team further reported that like the 'London Patient', the 'Düsseldorf Patient' also showed HIV-1 provirus trace on only some components but not in his lymph node sample, suggesting the 'Düsseldorf Patient' is very likely on the same path as the 'London Patient'.

1.4.3 Other strategies of HIV cure

Although the homologous CCR5-Δ32 carrying HCSTs is the only strategy that has been proved to cure HIV infections, other strategies are under investigation. These strategies fall into several genres.

Firstly, the broadly neutralizing antibodies (bNAbs) based strategy. With current technologies to modify the antibody structure, affinity and specificity, the latest bNAbs has shown promising effects in delaying the viremia rebound, which performs both prophylaxis and therapeutic effects against acute infection, cell-free transmission and cell-to-cell transmission(83, 84)

Secondly is the shock-and-kill strategy. The core of this strategy is to use the latency reverse agents (LRAs) which is a group of medicine to inhibit the latency-inducing enzymes or activate viral replication. After the reversal of the HIV latency, the effector component would be introduced, such as natural immunity, anti-viral therapy, bNAbs, specificity-redirected CTLs, chimeric antigen receptor T cells (CAR-T). Unfortunately, the data published now suggest that the LRAs need improvements to achieve a stronger latency reversal, and the 'kill' effector needs to be further optimized(85, 86).

The third is the immune modulation strategy. The most predominant part of this strategy is the immune-checkpoint blockers. The immune-checkpoint markers are inhibitory receptors

found to be expressed on T cells after long-term immune activation, which result in the inhibition of the response to chronic viral infection. Later investigation has suggested that these exhausted CD4 T cells harbour enriched provirus(87), revering the exhaustion using antibodies against these markers such as PD-1, CTLA-1 can not only enhance their anti-viral function, but also reverse the latency and decrease them as the viral reservoir(88, 89).

1.5 Gene editing technologies and gene therapy

The three patients who had a functional cure of HIV-1 infection clearly indicate replacing the immune system with a CCR5-Δ32 +/+ is a possible path to eradicate R5 strain HIV-1 infection. However, there are several obvious obstacles to prevalently use this treatment.

Firstly, the scarcity of CCR5-Δ32 +/+ donors makes a significant factor. As described before, there are only 2-5% of the population among Caucasians who carry this homozygous mutation, which severely limits the prevalence of a potential immunological matching. Moreover, the CCR5-Δ32 +/+ is race-specific, which means it is almost impossible for a match across different races, which prevents the application of this treatment to people that are non-Caucasian.

Secondly, bone marrow/HSC transplantation carries tremendous risks, several of the risks can be lethal. To perform this treatment, the first step is to completely ablate the original hematological/immune system (myeloablation) of the patient by chemotherapy/radiation, in

order to ensure the engraftment of the donor's hematopoietic cells from the attack of the original immune system. The donor's adhesion to the donations must be ensured before the procedures. Because once the procedure is done, the patient can survive only for days without the new hematopoietic cells to establish a new hematological system. Even after transplantation of the donor's cells, it still needs on average two weeks and can go up to several weeks, to have the engraftment of the new cells then produce enough immune cells to battle infections and red blood cells to maintain oxygen supply(90). During this course, a lethal infection can rise and eventually kill the recipient(91). Other complications directly from the new HSC can happen, including hepatic veno-occlusive disease, mucositis, hemorrhagic cystitis, and most importantly the graft-versus-host disease (GvHD)(91-94). The GvHD might play a role in the controlling and even eradication of the remnant infected immune cells from the original immune system, based on the experiences from the 'Boston patients' who had prolonged remission of HIV-1 viremia after HSCTs that does not carry CCR5 mutation (CROI 2007). However, their viremia relapsed that indicate the HSCTs and the GvHD helps in maintaining control of HIV-1 replication and the HIV-1 reservoir but only for a period of time and not enough for the long-term control, since the extremely small remanent of virus can ignite the new infection to the non-resistant new cells(95-97). Moreover, the GvHD alone has the potential to kill the patient, it can be managed to downregulate its level, but impossible to precisely control its severity. Controlling of the GvHD needs the administration of steroids and immune-suppressors, but these medicines, especially on high dose, can damage the engraftment of the new hematopoietic system and raises the risk of the development of severe infection or cancer, then lead to a clinical

dilemma(98). GvHD was a severe complication that the 'Berlin Patient' suffered, and one major reason that Dr Hütter advocated against using HSCTs with CCR5-Δ32 +/+ as an eradicating treatment for HIV-1 infection unless the patient also has other life-threatening diseases that require an HSCT.

However, with the development of gene editing techniques, it is now possible to artificially disrupt the expression of CCR5 permanently. The disruption by gene editing is called a gene knockout or knockdown, current development allows the disruption to happen on the site of CCR5-Δ32 to mimic the natural one, or another site of CCR5 to finally translate to dysfunctional CCR5 molecule to prevent the entry of HIV-1 to the cells. With gene editing, it opens up the possibility to generate CCR5 disrupted allogenic or even autologous hematopoietic cells for transplantation, which brings the potential to overcome the shortage of donors with natural CCR5-Δ32 +/+.

1.5.1 Gene editing tools that are programmable

Gene editing is a series of biochemical procedures and reactions to insert, delete, modify or replace DNA sequences in the genome of a living organism. The development of gene editing techniques started in the late 1990s(99). In the beginning, meganucleases were discovered and used in gene editing. Meganucleases are one category of DNA restriction enzymes, they are characterized by a large DNA sequence recognition site (12 to 40 base pairs), and process the function of both endonucleases and deoxyribonucleases. A library of

meganucleases recognizing different sequences has been identified in the late 90s, and then used in some gene editing studies. However, meganucleases have a significant shortage, they are natural enzymes, the repertoire of the recognizable sequence is limited. This issue makes its application very limited. Since the target gene might very likely not have any of the recognizable sequence or the sequence at a site that does not fit the purpose of the work. However, this problem has been solved as three major classes of programmable gene-editing tools have been developed, which are zinc finger nucleases (ZFNs, Figure 1.2), transcription activator-like effector nucleases (TALENs, Figure 1.3) and the clustered regularly interspaced short palindromic repeats/ CRISPR-associated protein 9 (CRISPR/Cas9) system (Figure 1.4).

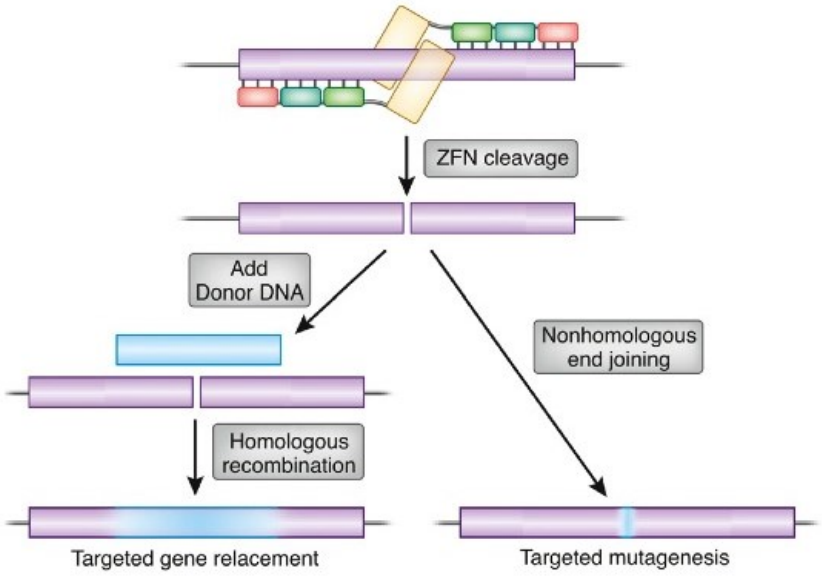

Figure 1.2. Structure and the function mechanism of zinc finger nucleases. Image credit to Dana Carroll, image unmodified, under CC BY-SA 3.0 license.

Zinc finger nucleases are artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain. The zinc finger DNA-binding domain

consists of 3 to 6 individual zinc finger repeats, each of the repeats is designed to recognize 3 base-pairs of DNA sequence. Combining several zinc finger repeats, it can be programmed to recognize desired genetic sequence(100). The DNA-cleavage domain is a type IIs restriction endonuclease FokI, when forming a dimer, the FokI endonuclease can cleave and cut the double-strand DNA to create a double-strand break (DSB)(101). In order to form the dimer, another strand of ZFN is needed to recognize and bind to the opposite strand of DNA, providing another FokI in the opposite orientation to dimerize. Given this mechanism, the two sequences that the sense and antisense arm of ZFN recognize need to have a gap between them to give a space for the FokI enzymes to dimerize, which is usually 5-7 base-pairs long(102).

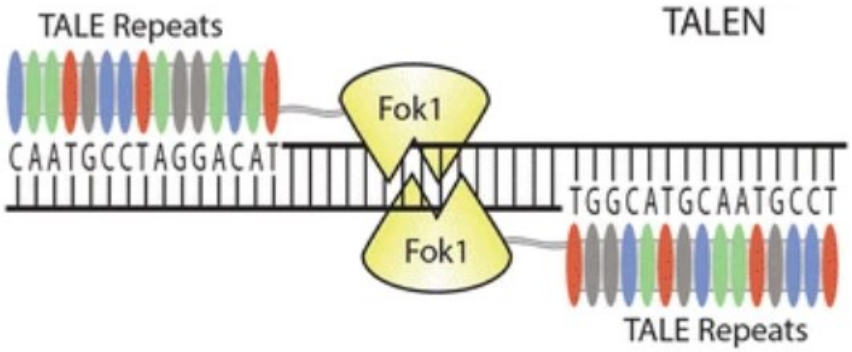

Figure 1.3. Structure of transcription activator-like effector nucleases. Image from the publication of Aimee Malzahn et al.(103) , image cropped, permission to use under CC BY-SA 3.0 license

Transcription activator-like effector nucleases are similar to ZFNs. They consist of the same FokI DNA cleavage domain fused to a TAL effector DNA-binding domain. The DNA-binding domain is formed by several transcription activator-like effectors (TALEs), TALEs are proteins secreted by some β- and γ-proteobacteria that can recognize and bind to one single

specific DNA base-pair, firstly found to work as a defense/immunity system against foreign DNA invasion(104). TALEs are characterized with a central repeat domain containing usually 34 amino acid residues, the 12th and 13th positions of amino acid residues are highly variable and determine the specificity of the recognition(105). By combining different TALEs, it gives one arm of TALEN the function of recognizing any desired DNA sequence, in the manner of 1 TALE to 1 base-pair, which is different from ZFN's 3 zinc fingers repeat to 3 consecutive DNA base-pair manner. The DNA cleavage domain of TALEN is the same as ZFN, consisting of one FokI endonuclease, for the FokI endonuclease to heterodimerize, the two arms of TALEN need to recognize two DNA sequences on the two strands with a gap of 12-30bp length(106). The simpler structure of the individual unit and the more straightforward relationship of each unit in the DNA-binding domain increase the accuracy and efficiency of making a TALEN construct(107).

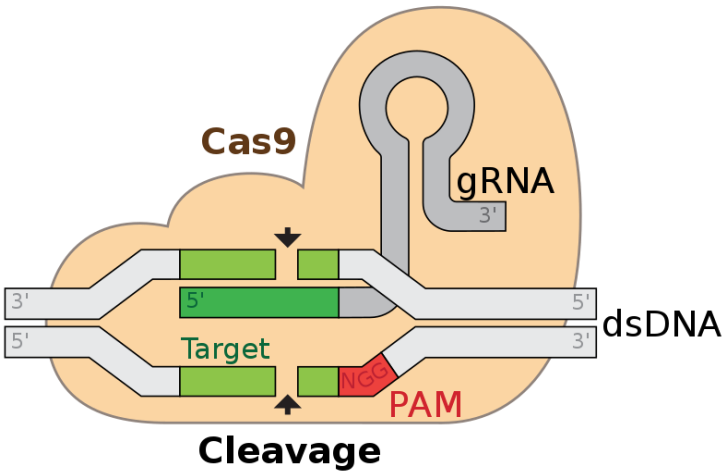

Figure 1.4. Structure of CRISPR/Cas9 system. Image credit to Marius Walter, image unmodified, under CC BY-SA 4.0 license.

The clustered regularly interspaced short palindromic repeats system/CRISPR-associated

protein 9 system is significantly different than ZFN and TALEN in the mechanism. CRISPR is a family of DNA sequences found in the prokaryotic organisms' genomes, eg, bacteria and archaea(108). These sequences are used to detect DNA sequences injected by bacteriophages thus work as a form of acquired immunity. Cas9 is an endonuclease, when the CRISPR sequences are complementary to the foreign sequence, the Cas9 will be activated by the CRISPR sequence to recognize and cleave that foreign sequence and finally destroy it(109). Several types of natural CRISPR/Cas9 systems have been found, among them, the one type that uses a guide RNA (gRNA) transcribed from the CRISPR DNA sequence as the guiding sequence to activate Cas9 has been investigated and modified, then engineered version is now prevalently used as a powerful gene-editing tool in gene-editing related research(110). This engineered CRISPR/Cas9 system consists of Cas9, crRNA and tracrRNA to perform DNA sequence deletion. The crRNA works as the sgRNA and the DNA recognition and binding domain, it is linked to the tracrRNA. The Cas9 enzyme has the function of DNA helicase and endonuclease. Cas9 can interrogate the whole DNA sequence and specifically recognize a 2-6 bp sequence called the protospacer adjacent motif (PAM)(111), the PAM sequence that spCas9 uses is 5'-NGG-3' that usually happens every 8-12 base pairs in the human genome(112). Once PAM is recognized, Cas9 can anchor to the PAM site and separate the two strands of the DNA double helix. If the Cas9 carries the sgRNA, then the crRNA part can screen the decoupled double strand. If recognition is successful, the tracrRNA will activate the Cas9 to cleave and introduce a double-strand break(112).

1.5.2 How the gene is disrupted

As described, the last step in all three gene-editing tools is to cut the DNA double-strand to introduce the double-strand break (DSB). When the DSB happens, the innate mechanism of cells would start to repair the break. Two different pathways can perform this function, the non-homologous end joining (NHEJ) and the homology direct repair (HDR). Both pathways involve a series of enzymes to ligate the break through multiple steps. The NHEJ pathways will firstly hold the ends of the break together then process the ends, if the ends are not compatible with each other, several enzymes will come in to remove the mismatch and damage, and then resynthesize the base pairs and finally use other enzymes to ligate the break, whereas if the end is compatible and the nucleotide structure is intact, then break can be ligated(113-115). The cleavage of DNA by the DNA-cleavage domain of ZFNs and TALENs, or Cas9 is usually leading to incompatible overhangs at each end of the DSB, thus the NHEJ pathways will repair the DNA but introduce errors, which can result in deletion, insertion, base-pair change, and ultimately change the amino acid sequence of the product to result in a dysfunctional protein. For the HDR pathway of DNA repair, a homologous piece of DNA is required as a template for the DNA repair, thus a certain sequence of DNA will be introduced and integrated into the genome(116). Because of this, the HDR pathway is the crucial mechanism for gene knock-in.

1.5.3 Comparison between gene editing tools

The accuracy and efficacy are the top two considerations in gene editing work, the three gene-editing tools differ in these two considerations. The accuracy is also known as the on-target and off-target effect. Take the CCR5 gene as an example, it has the same origin in the evolution of CCR2, thus share a similarity in the gene sequence(117). The choice of the target sequence needs to be carefully examined to avoid too much similarity with other genes, but that does not necessarily eliminate the chance of a mismatch to the target gene. When this mistake happens during the screening of the genome by the gene-editing tool, it is then called the off-target effect and results in collateral damage to another gene. When the off-target effect happens extensively, it will jeopardize the work and result in low efficiency on the target, unwanted phenotype changes to the cells and possible extensive apoptosis due to excessive cleavage of the DNA(118).

When it comes to the efficiency of gene editing, a great number of studies have shown that CRISPR/Cas9 exhibits the highest among all three programmable gene-editing tools. This difference is majorly rooted in the mechanism of the tools, especially the DNA recognition process. Unlike ZFNs and TALENs, CRISPR/Cas uses a single RNA strand instead of a protein as the DNA recognition and binding domain, the Watson–Crick complementary rule used in CRISPR RNA-DNA binding exhibits significantly higher binding efficiency than nucleic acid-protein interaction. Plus, the Cas9 protein screens the whole genome to anchor to the PAM sequence that allows a better efficiency too(119). Previous study has only shown that TALEN can out-compete CRISPR/Cas9 in a very limited scenario, eg, heterochromatin target sites(120). When comparing TALENs with ZFNs, many studies have provided evidence of a

better efficiency from TALENs(121, 122).

When it comes to the accuracy of the gene-editing effect, or more specifically the off-target effect, the scenario changes. The nature of the zinc finger repeat and its three-unit-to-three base-pairs mechanism lead to possible target site overlap and crosstalk between individual zinc fingers considerably complicate the production of sequence-specific ZFNs (123, 124). This 'context dependent' effect can be more apparent when targeting a gene with a shorter sequence, which makes the options of sequence segment too few to discover an optimal target sequence. For the CRISPR/Cas9 system, early-stage studies have already found significantly more noticeable off-target effects compared to TALENs(125-127). Further investigation has revealed that the 12bp sequence immediately upstream of the PAM sequence is the most important determinant to the recognition of DNA sequence(128, 129), thus the traditional CRISPR/Cas9 construct can tolerate up to 5 mismatches, especially beyond the 12bp upstream of PAM(125). Comparing to the conventional design of ZFNs and TALENs that recognize 18bp and 24bp minimal length of sequence, the specificity of CRISPR/Cas9 will be severely limited. Although several methods have been developed to specifically enhance the specificity of CRISPR/Cas9 including truncated sgRNA(130), using other Cas9 variants(131-133), using heterodimers Cas9 nickase to mimic ZFN and TALEN's double-strand DNA recognition to prolong the target sequence(134). However, although a decrease in off-target effects has been observed, a decrease in efficiency to around TALEN's level has also been observed. Thus generally to date, TALEN is considered the most precise one in gene editing in general.

1.6 Hypothesis and Objectives

Hypothesis

TALEN technique mediated CCR5 gene knock-out is highly efficient and results in low and tolerable cytotoxicity, leads to increased resistance to HIV-1 entry in different lineages of immune cells *in vitro*.

Objectives

1. Optimize the protocols for *in vitro* transcription of mRNA from plasmids
2. Build up a platform for gene editing mediated by TALEN system, including cell culture methods and mRNA-to-cell delivery by electroporation.
3. To knock out the CCR5 gene by TALEN technique in human primary T cells and macrophages, then test the resistance to HIV-1 of these cells *in vitro*.

Chapter Two. Materials and Methods

2.1 Molecular Biology techniques

2.1.1 TALEN-CCR5 and eGFP construct plasmids

The TALEN constructs are designed, synthesized and provided by Professor Linqi zhang's team from QingHua University, China. In their publication, it was shown that this TALEN design, which was named TALEN-CCR5-515, was the most efficient one among all they have designed. Upon transfection to cell line, this TALEN-CCR5-212 plasmid complex was able to perform CCR5 gene disruption(135).

The genomic sequence that the left arm of this TALEN construct recognizes is GCTGGTCATCCTCAT, and for the right arm is AAGGCTGAAGAGCATG. The site of CCR5 protein that these two sequences encode is at the first transmembrane domain of the CCR5 molecule.

The nucleic acid sequence encoding either one arm of the TALEN complex is cloned to a plasmid containing a bacteriophage SP6 promoter followed by a 5'UTR sequence. Following the TALEN sequence, there is a Poly(A) signal sequence and then a NotI enzyme restriction site for linearization of the plasmid. The plasmid also contains an ampicillin resistance gene.

The pCS2-GFP plasmid is also provided by Professor Linqi zhang's team, which encodes the sequence of the enhanced green fluorescent protein (eGFP). The backbone of the eGFP plasmid is the same as the one of TALEN's.

2.1.2 E.coli transformation and plasmids extraction for expansion of plasmids

To expand and harvest plasmids, Escherichia coli (E. coli) transformation and culture were performed, followed by extraction and purification of the plasmids. Materials for E.coli transformation are as follow:

1. LB Broth powder and LB agar plate powder and Ampicillin solution (Sigma-Aldrich, USA)
2. Subcloning Efficiency™ DH5α Competent Cells (Invitrogen, USA)

For extraction and purification of a large dose of plasmid (>200ug for IVT or plasmid expansion), the Plasmid Plus Midi Kit (Qiagen, Germany) was used. For a small dose of plasmids used for Sanger sequencing, the QuickLyse Miniprep Kit (Qiagen, Germany) was used.

General protocol for restriction enzyme digestion

Restriction enzyme digestion has been used for plasmid linearization, plasmid double digestion authentication, cloning the products from a polymerase chain reaction (PCR) to

plasmids. All restriction enzyme was purchased from New England Biolabs (USA), appropriate 10 X buffer was shipped with every enzyme bought.

For enzyme digestion, firstly the total reaction size and plasmids dosage were decided, eg, 100ug plasmids in 100ul reaction size for plasmid linearization used as the template for the transcription of mRNA. Then, an appropriate 10 X buffer of 1/10 volume of total size was pipetted to a nuclease-free capped Eppendorf tube or a 200ul PCR tube. Plasmids in water solution containing the targeted dose were pipetted into the tube, then nuclease-free water was added to the total reaction size minus enzyme. Finally, an appropriate amount of enzyme was gently pipetted into the tube and mixed gently by pipetting till no visual turbulence. The reaction then would be incubated at 37℃.

All the enzymes here were high fidelity enzymes, so when performing plasmid double digestion authentication, cloning PCR product with enzyme sites to plasmids, the reaction was incubated for 30min, with a 1ul enzyme to 1ug plasmid ratio. 0.5 ul enzyme was the minimum dose of restriction enzyme used in the reaction condition. To generate the mRNA transcription template by linearizing the plasmids, the incubation time was 4 hours, with a 1ul enzyme to 5ug plasmid ratio.

Related protocols:

Making LB bacterial culture medium and plate

- Dissolve LB Broth powder or LB agar plate powder (Sigma-Aldrich, USA) in a bottle of

double-distilled water, in concentration according to the manufacture's instruction. Vigorously shake the bottle till the powder has been completely dissolved.

- Screw the lid of the bottle till near open, tape the lid with a piece of autoclave heat-sensitive tape.
- Place the bottle into an autoclave and run the autoclave procedure.
- After autoclave, check if the autoclave tape has shown the black strips indicating appropriate disinfection. If so, screw the lid of the bottle tight and take the bottle out. Let the bottle cool down at room temperature.
- When the LB agar medium is cooled down to under 50℃, then add ampicillin solution (Sigma-Aldrich, USA) to the medium with 1:1000 (v/v) dilution.
- After adding ampicillin solution, quickly pour ~30ml of the medium to 10cm Petri dishes in a biosafety cabinet. Let the dishes cool down to room temperature and the medium has congealed. Put the lid on the Petri dishes and put the dishes into a 4℃ refrigerator. They can be used after 24 hours.

E.coli transformation

- Thaw a vial of DH5α Competent Cells (Invitrogen, USA) on ice.
- Immediately after the bacteria is thawed, take 50ul of it into a prechilled clean capped Eppendorf tube, add 5ul of target plasmid in water solution containing ~4ng of the plasmid by very gentle pipetting. Then very gently flick the bottom of this tube three times to mix the plasmids and the bacteria. Place the tube on ice for 20 minutes.
- After 20 minutes, place the tube into a 42℃ water bath for 20 seconds to perform heat

shock of the E.coli. After this, place the tube on ice for 2 minutes. Till the end of this step, all procedures must be conducted in a very gentle fashion, in order not to agitate the competent E.coli, so as to not induce closure of the pores on the cell membrane.

- Add 500ul of LB medium into the tube, place the tube into a shaker/incubator. The incubator should have already been warmed to 37℃ and set the shaker to rotate at the speed of 220 rounds per minute. The incubation will last for 30 minutes.
- After incubation, take 100ul of the bacteria suspension and spread it on an LB/Agar/Ampicillin plate. Place the plate into a 37℃incubator overnight.

E.coli culture

1. From the whole process of E.coli culturing, when doing procedures on a bench, every procedure was conducted within a 20cm radius around an already lit gas torch to ensure air sterilization.

- Pick a single colony of E.coli on the LB/Agar/Ampicillin plate, and place it into a sterile capped tube containing 4ml of LB medium supplemented with 1:1000 (v/v) ampicillin.
- Screw the cap of this tube half loose, restrain it with a piece of tape, and place it into a 37℃ incubator for 6 hours.
- After the incubation, if the culture appears viscous, take ~1ml of it and centrifuge to pellet the E.coli, which can be used to authenticate the plasmids by electrophoresis or Sanger sequencing.
- After authentication, the suspension can be poured into a sterile flask containing 50~60ml LB medium supplemented with 1:1000 (v/v) ampicillin for further culturing.

This flask will be incubated in a 37℃ incubator overnight. After it, the suspension is ready to be used for plasmid extraction and purification.

For a small dose of plasmids used in verifying gene knockout work by Sanger sequencing, the CCR5 gene segment amplified from the genome DNAs extracted from the treated cells needs to be first cloned to backbone plasmids, then have these plasmids transformed and expanded to acquire enough quantity for Sanger sequencing. The quantity required by the sequencing service provider was 1ug and tens of E.coli colonies were needed in case not to miss the colonies that contain the disrupted CCR5 gene segment.

In this work, 30 colonies were picked up for each of the target samples. Each colony was seeded into 1ml LB/ampicillin medium contained in each chamber of a deep 96 well plate. Then the 96-well plate was sealed with an air permissible film as the lid and put into incubation overnight. After it, the suspension was ready to be used for plasmid extraction and purification.

Plasmid extraction and purification

1. For the large amount extraction of plasmids (>200ug), Plasmid Plus Midi Kit (Qiagen, Germany) was used.
2. For the small amount extraction of plasmids (1-5ug) used for plasmid authentication, Plasmid Plus Mini Kit (Qiagen, Germany) was used.
3. For the plasmids expanded in the deep 96 well plate to be used in the confirmation of

gene knockout by Sanger sequencing, QuickLyse Miniprep Kit (Qiagen, Germany) was used.

All procedures of the plasmid extraction and purification were conducted in accordance with the manual of the kit.

2.1.3 Electrophoresis for DNA and RNA

Electrophoresis has been used for the verification of the plasmid or PCR product length, gel purification of PCR products and the verification of mRNA length.

If the electrophoresis was run for DNA, then agarose gel was used. For mRNA, MOPS/agarose/formaldehyde denaturing gel was used.

Recipe for gels

1. Agarose gel

1%(w/v) agarose gel is made by disolving 1g agarose (Sigma-Aldrich, USA) per 100ml 1x Tris-acetate-EDTA (TAE) buffer (ThermoFisher, USA).

1. After adding the agarose into the buffer contained in a flask, the flask will be put into a

microwave and heated till boiling.

2. When boiling, the flask will be taken out immediately and shaken in a swirling manner then put back into the microwave and repeat the process for 3 times.
3. 10ul SYBR safe DNA gel stain (Invitrogen, USA) per 100ml gel will be added to the gel liquid, shake till thoroughly mixed, and pour into gel tray with appropriate lane ruler.

After complete cooling down, the liquid will be congealed. The electrophoresis will be conducted in the same 1X TAE buffer

Alternatively, TAE buffer can be replaced with Tris-Borate-EDTA (TBE, ThermoFisher, USA), and the electrophoresis will be run in 1X TBE buffer.

DNA samples used here are all mixed with 6X DNA Gel Loading Dye (ThermoFisher, USA) before loading to the gel. 1 kb DNA Ladder or 100 bp DNA Ladder (New England Biolabs, USA) depending on the length of the targeted DNA was used to indicate DNA length.

2. MOPS/agarose/formaldehyde denaturing gel

1. Add 1g of agarose powder is to 72 ml of deionized water.
2. Melt the agarose by a microwave, and then add 10 ml of 10X MOPS buffer (Invitrogen, USA) and mix.
3. When the agarose solution cools to 60°C, add 18 ml of fresh formaldehyde (37%, Sigma-Aldrich, USA) in a fume hood and mix thoroughly. Then the solution can be

poured into a gel tray.

The electrophoresis will be run in 1X MOPS buffer, with mRNA sample mixed in equal volume to 2X RNA Gel Loading Dye (ThermoFisher, USA) before sample loading, and Millennium™ RNA Markers (ThermoFisher, USA) was used as length indicator.

2.1.4 *In vitro* transcription for messenger mRNA

In vitro transcription (IVT) is an enzymatic reaction that uses RNA polymerase to transcribe the DNA template into RNA. This reaction requires a DNA template that has a bacteriophage promoter sequence (e.g. from the SP6 coliphage) upstream of the sequence of interest. The corresponding RNA polymerase can bind to the promotor then read and transcribe the sequence of interest in the form of RNA. The DNA template can be a PCR product or a plasmid. In the case of using plasmid as the template, the plasmid must be linearized using a restriction enzyme at an immediate site downstream of the targeted sequence, otherwise, the RNA polymerase will circulate the template and transcribe the whole template continuously.

In this work, all the template plasmid has a NotI site following the coding sequence, and restriction enzyme digestion using NotI enzyme was used. After digestion, the reaction went through sodium acetate-ethanol precipitation for purification. The procedures were:

1. Add 1/10 volume of 3M sodium acetate (Sigma-Aldrich, USA), 2 volumes of 100% Ethanol (VWR, USA) to the reaction, mix well by vortex.
2. Centrifuge the tube at top speed for 15min in a desktop centrifuge. After centrifugation, the linearized plasmids should form a pellet.
3. Pipette out the supernatant, and let the pellet dry at room temperature for 15min.
4. Dissolve the pellet in nuclease-free water by repeatedly pipetting.

Alternatively, the purification of the linearized plasmid can be performed by using the PCR product purification kit, which gives better yield and purity. Kit purification was done according to the manufacturer's manual.

With the DNA template, the mRNA can be produced in the co-transcriptional capping method or the transcription then capping method. The co-transcriptional capping method can produce capped RNA in one step, but in its essence, the RNA polymerase recognizes and uses the cap analogue as guanine and prolong the sequence after it. Although this method reduces the duration of the reaction and the possibility of contamination from multiple manual procedures, it also means the guanines contained in the reaction are significantly reduced to ensure a better production of capped RNA which results in lower yield. The alternative method is the transcription then capping method which caps the RNA sequence afterward of the transcription by using enzymes like vaccinia virus capping enzyme. However, this method needs two steps of transcription and increases the possibility of RNase contamination and is more expensive. Thus, in this work the capped RNA is

synthesized by the co-transcriptional method.

In the early works, the IVT was conducted with mMESSAGE mMACHINE SP6 kits (Invitrogen, USA). This kit has all 4 kinds of Nucleoside triphosphates (NTPs) and mRNA Cap analogue premixed together as a single vial of reagent, and the cap analogue in the reagent is m7G(5')ppp(5')G, the conventional structure. Since this kit uses the co-transcriptional strategy, which means the cap analogue is incorporated with individual NTP in a one-by-one manner by the RNA polymerase to form the following mRNA sequence, instead of incorporating the cap to the already transcribed sequence. It is possible to incorporate the m7G(5')ppp(5')G cap analogue in the reversed orientation, and thus makes this kind of mRNA untranslatable.

Upon further study, there are several modified cap analogues commercially available. The 3´-O-Me-m7G(5')ppp(5')G cap analogue, also known as anti-reverse cap analog (ARCA), has its molecular structure modified to make it only in the correct orientation to be incorporated. In that case, the ARCA was used from the second year of my work. Since the ThermoFisher kit has the NTPs and cap analogue premixed, RiboMAX Large Scale RNA Production Systems kit (Promega, USA) or HiScribe SP6 RNA Synthesis Kit (New England Biolabs, USA) together with ARCA (Jena Biosciences, Germany or New England Biolabs, USA) was used for IVT later in this study. Each kit has 4 kinds of NTP provided individually but the cap analogue is not included, which makes it possible to use ARCA.

For the procedures of IVT, it is similar when using either of the three kits. The procedures are as follow:

1. Mix the buffer and NTPs/analogue to the concentration instructed by the manufacturer.
2. Pipette the appropriate amount of linearized plasmid template to the reaction.
3. Pipette adequate amount of nuclease-free water to round up to the required reaction size.
4. (Optional) RNase inhibitor can be added to this reaction (ThermoFisher kit has RNase inhibitor contained in its enzyme reagent).
5. Add the SP6 RNA polymerase to the reaction and mix by gentle pipetting. By far, all reagents should be kept on ice.
6. Then incubate the reaction under 37℃ for the instructed amount of time.
7. After incubation, add the DNase provided by the kit to the reaction to degrade the DNA template.

These procedures only make capped mRNAs. It is crucial to polyadenylate the 3' end of the mRNA to add a poly(A) tail, which ensures the stability and translation of the mRNA in the cell. To perform this, Poly(A) Tailing Kit (ThermoFisher, USA) or E. coli Poly(A) Polymerase kit (New England Biolabs, USA, USA) was used. Both of the kits use E. coli Poly(A) Polymerase (EPAP) for the 3' polyadenylation with the presence of Mg^{2+}, Mn^{4+} and ATP. Procedures can be summarized as mixing the templated degraded IVT reaction with the poly(A) buffer and ATP then add the EPAP and incubate under 37℃ for an appropriate amount of time. Then a

mature mRNA is synthesized. To verify the mRNA, a small amount of reaction before and after Poly(A) tailing can be used to be electrophoresed to test the length and make sure of successful polyadenylation.

To purify the mRNA, LiCl precipitation or silicone membrane RNA-biding purification kit (Monarch RNA Cleanup Kit, New England Biolabs, USA, USA).

For the LiCl precipitation

1. Add 3/5 reaction size of 7.5M LiCl Precipitation Solution (ThermoFisher, USA) to the reaction, mix well by gentle pipetting. Incubate at -20℃ for 30min, then centrifuge in a desktop centrifuge at top speed for 15min at 4℃.
2. Remove the supernatant and rinse the pellet by gently adding 1ml of ice-cold 70% ethanol then pipette out the ethanol.
3. Resuspend the RNA pellet in nuclease-free water, test its concentration by nanodrop and aliquot. Store the RNA at –80°C.

For silicone membrane purification, all procedures were done according to the manufacturer's instructions.

2.1.5 Genomic DNA extraction and polymerase chain reaction (PCR) amplification of CCR5 or other gene segments

Genomic DNA (gDNA) of all kinds of cells used in this work were extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany). In summary, an appropriate number of target cells was centrifuged to pellet and washed then re-suspended in PBS. Proteinase K and RNase are then added to the reaction along with cell lysing buffer and incubated for 10min under 56℃. After that, DNA was precipitated with isopropanol and silicone membrane biding buffer was mixed with it, then the reaction was loaded to a silicone membrane column. By applying vacuum to the column, the liquid of the reaction went through the silicone membrane and gDNA would bind to the membrane. Upon repeating wash once again, nuclease-free water was added to the membrane to dissolve gDNA, then by centrifugation, the gDNA solution was collected.

For PCR amplification of the gene, firstly primers were designed using SnapGene software. GC pair percentage and hence annealing temperature were primarily taken into consideration. The primers designed were synthesized by Sigma-Aldrich (USA).

For the PCR, Q5 Hot Start High-Fidelity 2X Master Mix (New England Biolabs, USA) was used. Per instruction, each reaction consisted of the Q5 DNA polymerase reagent, DNA template (100ng gDNA or 30ng plasmid) and water in a 50ul reaction. The PCR condition was (Table 2.1):

Table 2.1. The parameters of PCR amplification process for the CCR5 gene

Initial Denaturation	98°C	30 seconds
30 Cycles	98°C	5–10 seconds
	Appropriate annealing temperature °C	10–30 seconds
	72°C	20–30 seconds/kb
Final Extension	72°C	2 minutes
Hold	4–10°C	

The PCR product was purified by a PCR product purification kit (Qiagen, Germany) as mentioned above.

2.1.6 Cloning PCR product to plasmids for sanger sequencing

This work was performed for the verification of CCR5 gene knockout. Firstly, the forward and reverse primer were designed with a restriction enzyme site at the 5' end of the forward primer and the 3' end of the reverse primer. A 5nt abundant sequence was added to the upstream of the enzyme site for forwarding primer and downstream of the reverse primer, to ensure restriction enzyme's binding. These two primers scaled the sequence of the CCR5 gene, in which the TALEN binding sequence and CCR5-Δ32 location were included.

After PCR amplification of the CCR5 gene segment, the purified PCR product went through restriction enzyme digestion to create sticky ends in order to be incorporated into the backbone plasmid. Then the PCR product went through gel purification. The gel run was conducted in the way described above, then the gel was put onto Safe Imager Blue-Light Transilluminator (ThermoFisher, USA) to visualize the PCR product band. A small piece of gel containing the band was sliced by a disposable surgery lancet and went through DNA purification by QIAquick Gel Extraction Kit (Qiagen, Germany) in accordance with the manufacturer's instructions.

The backbone plasmid, which is called pOPINJ, contains a lacZ gene was used in this work for further white-blue screening. The pOPINJ was a gift from Mr. Qiao Gao (Addgene plasmid # 26045; http://n2t.net/addgene:26045; RRID: Addgene_26045). In the LacZ gene, there is a PvuII enzyme site. Thus, primers for CCR5 gene segment amplification were designed as containing a PvuII site at the 5' end of the forward primer and at the 3' end of the reverse primer. The PCR amplicon of the CCR5 gene by these primers, along with the pOPINJ plasmid was digested by the PvuII-HF restriction enzyme (New England Biolabs, USA) to create sticky ends. After gel purification, the PCR amplicons were ligated with the backbone plasmid by T4 ligase (New England Biolabs, USA). The procedures are as follow (Table 2.2):

Table 2.2. List of the components and the dose of each of them used in the T4 ligation reaction. All reactions were assembled on ice in an Eppendorf tube.

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COMPONENT	20 ML REACTION
T4 DNA LIGASE BUFFER (10X)*	2 µl
VECTOR DNA	0.020 pmol
INSERT DNA	0.060 pmol
NUCLEASE-FREE WATER	to 20 µl
T4 DNA LIGASE	1 µl

T4 DNA Ligase should be added last. The reaction was mixed gently by pipetting and incubated at 16°C overnight. After that, it was heat-inactivated at 65°C for 10 minutes. After chilling ice, 5ul of the reaction was used for E.coli transformation, then seeded on an X-gal plate. Upon successful insertion, the lacZ gene would be interrupted, thus the E.coli would not be producing β-galactosidase encoded by the lacZ gene. The β-galactosidase can hydrolyze X-Gal, resulting in 5-bromo-4-chloro-indoxyl, which dimerizes to form a blue pigment. Thus, all blue colonies are not with the CCR5 amplicon insertion, and white colonies were picked for expansion. The recipe for the X-Gal plate is as follow:

- Add 40 µL of the X-Gal Solution (ThermoFisher, USA) on the surface of a LB-Agar gel.
- Add 40 µL of 100 mM IPTG Solution (ThermoFisher, USA).
- Spread evenly and let it dry for 30min. then it is ready to use.

30 colonies were picked from each plate, and each colony was cultured in LB with ampicillin medium, in a deep well 96 well plate. After seeding, the plate was sealed with an air permissive membrane and incubated in a shaker/ incubator overnight at 37℃. After incubation, plasmids were extracted using QuickLyse Miniprep Kit (Qiagen, Germany) according to the manufacturer's manual. Then all the plasmid samples were sent to Genewize inc. lab for their Sanger sequencing service.

2.1.7 T7 endonuclease I (T7EI) assay for mutation detection.

T7EI assay is used here to verify CCR5 knockout on the genetic level. The T7 endonuclease I used here is bought from New England Biolabs, USA, USA. This endonuclease recognizes any mismatch of a double strand DNA (dsDNA) and cuts the dsDNA at the site of the mismatch. To perform this assay, the CCR5 gene segment containing the region of the knockout was amplified by PCR, and the PCR product was purified and mixed with T7EI buffer, then went through slow annealing to create heterogenous dsDNA. After that, the dsDNAs were treated with T7 endonuclease I and then electrophoresed. The mechanism and expected result is summarized in Figure 2.1.

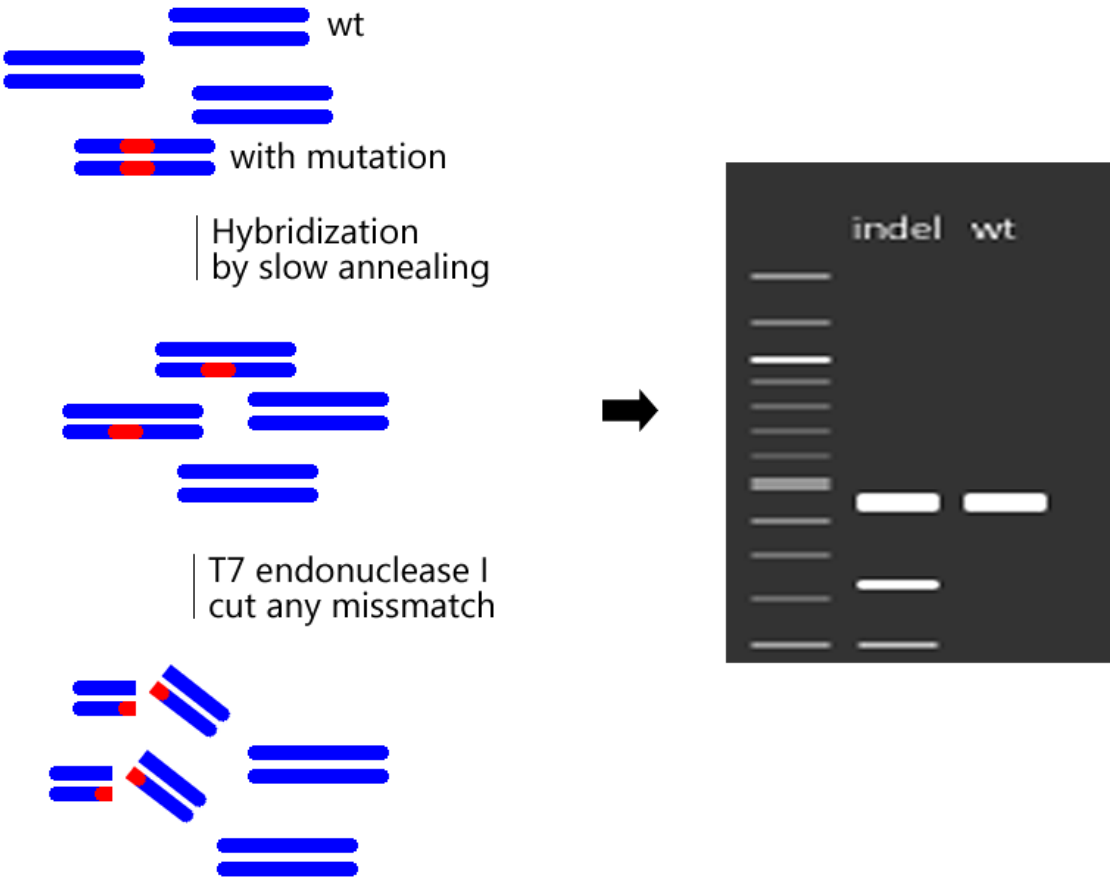

Figure 2.1. How T7EI assay detects mutation. In the detection of gene-editing effect. PCR reaction was firstly performed to acquire amplicons of the target gene, and the products would be a mixture of wildtype (wt) and indel-carrying amplicons. After purifying the PCR product, it will go through high temperature denature and then a slow cooling down process to allow the single-strands to hybridize. After these, there will be double strand DNAs of wt, indel, and indel-wt hybrid. The wt and indel segment on one double strand will cause a mismatch and treating the product with T7 endonuclease would cause a double strand break at the mismatch site. In that case, after electrophoresis, this sample will have three bands. The highest is the double strand DNAs that do not have a mismatch, the other two are the two fragments from the cut at the mismatch site.

2.1.8 Next-generation sequencing (NGS) for mutation verification

In the late stage of the work, HIV-1-infected lymphocytes have their gDNA extracted and CCR5 segment amplified by PCR, the PCR products were sent to GENEWIZ company for its

NGS service to test if there was enrichment of CCR5 knockout after HIV-1 infection at different time-point. GENEWIZ NGS service was conducted using the Illumina chip sequencing technique, and the result was analyzed by their software. The result was returned as an HTML webpage file demonstrating the percentage of indel on the PCR product, comparing to the original consensus sequence. In the analyzed result, the percentage of all indels, indels causing frame-shift mutation, frequency of each indel shown in different methods were shown.

2.2 Cell cultures

2.2.1 Cryopreservation and recovery of cell-line and primary cells

For long-term preservation, all cells should be kept in liquid nitrogen. And to recover cells from cryopreservation, cryopreservation tubes containing the cells were taken from liquid nitrogen and immediately put onto dry ice and transferred to a 37℃ water bath. All the tubes were put onto a floater and then put into the water bath and agitated in a swirling fashion to optimize the distribution of heat. Immediately after the ice in the tubes is thawed, a pasture pipette was used to gently aspirate the cells and add them drop-wise to 37℃ medium containing fetal bovine serum (FBS, Sigma-Aldrich, USA, lot 15C177). During the process, the tube containing the medium was gently swirled to ensure fast mixing. The amount of medium used was 10 volumes to the amount of the cryopreservation buffer of

the cells added. Finally, ~ 1ml medium was used to rinse the cryopreservation tube to ensure maximum recovery.

Immediately after these procedures, the tube is centrifuged for 5 min at 500g. Then the supernatant was discarded, and the pellet of the cells was loosened by gently flipping the bottom of the tube with a finger. The cells were washed by prewarmed medium once again and then resuspended in desired medium and ready for the next application.

For cryopreservation, all cells will be washed twice by PBC, all supernatant discarded completely and suspended in FBS containing 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich, USA). The concentration of cells can be 1-50x10^6 cells per 1 ml of cryopreservation buffer. All the cryopreservation vials were put into a 4℃ prechilled Mr. Frosty container and then put into a -80℃ refrigerator overnight (Max. 3 days). After that, the vials were ready to be transferred to liquid nitrogen.

2.2.2 GHOST-CXCR4-CCR5 cell line maintenance.

In this work, the cell line used was the GHOST-CXCR4-CCR5 cell line (GHOST-Hi5), provided by Prof. Linqi Zhang's team of Tsinghua University, China. This cell line originates from the HOS cell line, which is a human osteosarcoma cell line and is an adherent cell type. Upon modification, the original cells over express CD4, CCR5 and CXCR4 molecules, thus is

used as the reporter cell line for this work.

After recovery of the cells, they were seeded at $3x10^5$/ml in the culture medium to a flask. The medium for maintaining this cell line is Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FBS 2 mM L-glutamine (Invitrogen, USA), 100 units/mL penicillin G (Sigma-Aldrich, USA), 100 µg/mL streptomycin(Sigma-Aldrich, USA), 500 µg/mL G418 (Gibco, USA), 100 µg/mL Hygromycin (Gibco, USA) and 1 µg/mL Puromycin (Gibco, USA), in a 37 °C, 5% CO2 incubator.

Passages were done every three days. Firstly the medium was poured out and rinsed 3 times with Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich, USA) to ensure no residue of FBS, then 2 ml per 25cm2 of flask well of 5% trypsin solution was added to the flask and incubated at 37℃ for 20min. By the end of incubation, all cells should be already rounded up and detected, then an equal volume of FBS was added to neutralize the trypsin, then 1/8 amount of the cells were washed with PBS twice and then used for passage.

2.2.3 PBMC isolation from fresh blood or blood cones.

In the first year of this project, the PBMCs were isolated from whole blood. The blood used was donated by certain members of the lab I work in. In the following years, the PBMCs were isolated from the leukocyte reduction system chambers, also known as the blood

cones. The blood cones were acquired from the NHS blood transfusion unit, which is a by-product of machine separation of the whole blood donated for transfusion. The machine separation would isolate red blood cells, white cells, platelets, plasma and white cells, all of them except the white cells have clinical use, whereas the white cells can be provided for research use. The blood cone is a cylinder that contains some leftover red blood cells, plasma and nearly all the white cells from each blood donation. To protect the privacy of the donors, the blood cones would not be provided the donor's personal details, thus for most of my work, the race, gender and age of the donors were unknown.

The procedures of isolating PBMCs are as follow:

3. Add 15 ml of room temperature Histopaque-1077 (Sigma-Aldrich, USA) To a 50mL conical centrifuge tube.
4. If using a blood cone, spray the blood cone and a pair of scissors with ethanol. Cut the cords of the blood cone and collect the containing to a 50ml sterile centrifuge tube. Dilute the whole blood/ blood cone 1:2-1:3 with DPBS
5. Carefully layer 25 mL of whole blood onto the Histopaque-1077 by a long pipette.
6. Centrifuge at 800g for 20 minutes at room temperature. The brake of the centrifugation is set at the lowest of the machine.
7. After centrifugation, carefully aspirate the opaque interface containing mononuclear cells with a Pasteur pipette to a clean 50mL conical centrifuge tube.
8. Wash the cells by adding 10 mL DPBS and gently mix by a Pasteur pipette, then top up the tube with DPBS.

9. Centrifuge at 300g for 10 minutes to wash off the Histopaque-1077 and platelets. A small volume was used for total cell counting and repeat the wash once.
10. Discard the supernatant, gently flip the bottom of the tube to loosen the cells, re-suspend them in the appropriate amount of cryopreservation buffer for further liquid nitrogen storage.

2.2.4 T cells activations

In the early stage of the work, only around $10x10^6$ PBMCs were used for lymphocyte activation, thus I used anti-CD3 and anti-CD28 antibodies as the activation method. After thawing the PBMCs, they were cultured in Roswell Park Memorial Institute Medium (RPMI1640) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptavidin, 2 mM L-glutamine for (together as R10 medium) for 1 hour in a 37℃ 5% CO2 incubator to rest the cells. The culture was 5ml medium in a T25 flask. Then, interleukin-2 (IL-2) to 200IU/ml, anti-CD3 antibody (clone HIT3a, Biolegend) to 50ng/ml, anti-CD28 antibody (clone 28.2, biolegend) to 1ug/ml was added to the medium, gently shake the flask to mix and incubate in the same condition for 3 days.

In the later stage of the work, a large number of cells were needed, thus using antibody stimulation was too expensive. In that case, I switched to phytohemagglutinin (PHA) stimulation. The protocol was, after resting the cells at the density of 2 million cells/ml, IL-2

(same concentration as above) as PHA-P (Sigma-Aldrich, USA) was added to 5ug/ml concentration. The incubation condition was the same as above.

2.2.5 Magnetic beads negative depletion of CD8+ cells and positive selection for CD14+ cells

To enrich CD4+ T cells for HIV-1 infection assay, or to isolate CD14+ cells for macrophage differentiation, magnetic beads separations were performed. The magnetic beads are antibody-linked micro magnet beads, that can bind to the correspondent receptor on the cell surface to label the cell. Then the cell will go through a separation column attached to a magnet. The tail of the column is filled with cis-magnetic beads that trap the beads but let the unlabeled cells go through. If it is the unlabeled cells that are wanted, then it is called a negative selection, usually used for the depletion of a certain type of cells. If it is the other way around, then it is called positive selection, usually for the enrichment of a certain cell type. The magnetic beads, magnet and separation columns were purchased from Miltenyi Biotec, Germany. Summary of the protocol:

1. After thawing cryopreserved PBMCs or directly after isolation from blood cone cells were firstly rested for 1 hour in a 50ml centrifuge tube under 37℃ and then count the cells.
2. Wash and re-suspend the cells in beads buffer (DPBS with 0.5% bovine serum albumin

and 2 mM EDTA) and magnetic beads were added to the concentration of the manufacturer's instruction. EDTA was purchased from Sigma-Aldrich, USA.

3. Incubate the reaction at 4℃ in darkness for 15min, then add 1ml per 10^7 cells amount of beads buffer, centrifuge the tube at 300gfor 10min, then pour the supernatant out.
4. Resuspend the cells with 500ul beads buffer, then transfer the cell suspension to the magnetic separation column and let the suspension go through it.
5. The column should be washed twice with an appropriate amount of beads buffer to wash off the unlabeled cells.
6. For negative selection, the column can be discarded. For positive selection, then take the column off the magnet, added the beads buffer to the column and use the plunge provided to flush the labeled cells to another clean centrifuge tube.

2.2.6 *In vitro* differentiation of monocytes to macrophages by cluster stimulation factors

To acquire monocyte-derived macrophages (MDMs), firstly monocytes were isolated from PBMCs by CD14 magnetic beads positive selection. Then, the monocytes were cultured at $1*10^6$ cell/ml density in a flask. The medium used was R10 medium supplemented with 20ng/ml macrophage-cluster stimulation factors (M-CSF, Miltenyi Biotec, Germany) and the culture was incubated under 37℃, 5% CO2. Medium is completely changed every 3 days. Multiple total incubation periods were tested in this work.

On the last day of the culture, the culture medium would be poured out, and fresh sterile DPBS would be used to gently rinse the flask for 2 times to rinse out all residue culture medium. Then, 1ml/cm2 500units/ml accutase solution (Sigma-Aldrich, USA) was added to the flask to detach the cells. The process would take ~1hour in a 37℃ incubator and all cells would round up, part of them would already be floating in the solution. A cell scrapper would be used to gently scrap the cells off the flask wall.

2.2.7 HIV-1 infection assay

For lymphocytes, spin-inoculation of HIV-1 was performed. On day 3 after electroporation, part of the lymphocytes was firstly tested by flow cytometry of their CCR5 expression. If downregulation of CCR5 has been observed, then an appropriate amount of lymphocytes would be transferred to the containment level 3 laboratory, then resuspended in 200ul R10 medium containing HIV-BaL variant at the multiplicity of infection (MOI) of 0.01. The HIV-BaL was obtained through the NIH HIV-1 Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus Type 1 (HIV-1-1) Ba-L, ARP-510, contributed by Dr. Suzanne Gartner, Dr. Mikulas Popovic and Dr. Robert Gallo. The virus-cell suspension is contained in a 12ml round bottom tube and centrifuged at 1000g for 2 hours at 25℃. After this, the tube would be removed from the centrifuge and the cells washed twice by 10ml of fresh 37℃ R10 medium. Then each sample would be cultured in a well of a 6 well plate in R10 medium supplemented with 200IU/ml IL-2, medium changed every 3 days. On day 4

and day 12, an aliquot of the cells would be used for p24 intracellular staining, another aliquot for gDNA extraction.

The MOI in this work is an estimated value, converted from the median tissue culture infectious dose ($TCID_{50}$) on primary CD4+ T cells that is already indicated by the provider of this virus stock. The $TCID_{50}$ is the dilution of a virus required to infect 50% of a cell culture. Host tissue cells are cultured on a well plate titer, and then varying dilutions of the testing viral fluid are added to the wells. After incubation, the percentage of infected wells is observed for each dilution, and the results are used to calculate the TCID50 value. The conversion formula under the ideal situation is MOI = 0.7 x $TCID_{50}$. As a working estimate used in this protocol, the formula is MOI = 0.5 x $TCID_{50}$.

For MDMs, $1x10^6$ MDMs will be seeded to a well of a 12-well plate after the CCR5 expression flow cytometry test. In each of the wells, there is 1ml prewarmed to 37℃ R10 medium. The MDMs would be cultured overnight to ensure adherence and being well-fed. On the day of infection, the medium in the well will be gently aspirated out and replaced with 200ul prewarmed R10 medium containing 20ng p24/ml of HIV-ADA (obtained through the NIH HIV-1 Reagent Program, Division of AIDS, NIAID, NIH: Human Immunodeficiency Virus-1 ADA, ARP-416, contributed by Dr. Howard Gendelman) and cultured under 37℃ for 4 hours. Every 30min the plate will be gently hand-rocked. After the infection, the medium will be aspirated out and each well very gently washed with 1ml prewarmed DPBS twice. Then 1ml prewarmed R10 medium will be added to each well and cultured under 37℃ for 3

days. On day 3, pour the medium out, and gently wash the wells with DPBS twice, then treat the cells with accutase for 1 hour under 37℃. At the end of it, each well will be gently pipetted to ensure the detachment of cells. The cells of each well will then go through intracellular staining of p24 and go through flow cytometry.

2.3 Electroporation

2.3.1 General background

Electroporation, also known as electropermeabilization, is to use an electric pulse to shock the cell membrane a singular or multiple times to create pores. These pores increase the permeability of the cells for chemicals, proteins, or nucleic acids. The electrical field applied to the cells will inevitably impact the cell viability, thus parameters for electroporation must be optimized on the target cells to achieve a balance of cell death and transfection efficiency.

Electroporation can be conducted via a cuvette, or a plate, even *in vivo* on experimental animals. In this work, electroporation was performed using a cuvette for each sample.

The electroporation can be done in two different modes, the square wave pulse or the

exponential decay wave. The square wave pulse means the electrical field applied to cells is fixed at the set voltage during the set period, whereas the exponential decay wave has its electrical field decreased exponentially. Most mammalian cells get better transfection efficiency with square wave pulse, yet some certain mammalian cells prefer exponential decay waves. Also, the cells are suspended in the electroporation buffer, and the choice of buffer can greatly impact the efficiency. Moreover, the electroporation process in its essence is electricity going through a resistance made of cell suspension, thus heat will be produced that has the potential of damaging the cells. In that case, the cells might need pre-cooling to better be preserved.

In this work, before the optimization of electroporation for each cell type, a literature review was done to seek previous protocols. Electroporation buffer, cuvette type and cell density in suspension were chosen according to previous protocol. Then, minor change of the protocol was tested, e.g., 50V gradient changes of the voltage set for both of the modes, 250μF gradient changes for the capacity set of exponential decay wave mode. eGFP mRNA was used as the reporter for the transfection efficiency. 18-24 hours after electroporation, the viability of the cells was checked by cell counter or confluency of adhesive cells was viewed under a microscope to examine the impact of electroporation on cell viability. Then, cells were harvested to be tested by flow cytometry to see the positive rate of GFP to examine the efficiency of electroporation.

All the optimization and TALEN complex transfection works were performed mainly on the Gene Pulser Xcell electroporator (Bio-Rad, USA), Gemini twin wave electroporator (VWR,

USA) was used for the GHOST-Hi5 cell line, as the Gene Pulser Xcell electroporator performed poorly on this cell line.

2.3.2 Electroporation for GHOST-CXCR4-CCR5 cells lines, Lymphocytes and Monocytes/Macrophages

The GHOST-CXCR4-CCR5 (GHOST-Hi5) cell line was used for confirming the gene-edit function of the TALEN Plasmids and mRNA. Thus, the cell line went through optimization for both DNA and RNA optimization. The electroporation of both types of nucleic acid was conducted using a 4mm gap cuvette, $2x10^6$ cells were washed and re-suspended in 600ul neat OPTI-MEM medium (Gibco, USA) and the cuvette was kept on ice. 4ug pCS2-GFP plasmids or 2ug of the CleanCap Enhanced Green Fluorescent Protein mRNA (Trilink Biotechnologies, USA) were mixed in the cell suspension for optimization. Exponential decay mode was used according to established protocols for the HOS cell line, which is the progenitor cell line of GHOST-Hi5. According to the protocol, capacity was set at 1050uF, then multiple voltages were tested for the optimization.

Under the optimized parameters, 16μg of each arm of TALEN plasmids or 4ug of each arm of TALEN mRNA was used for authenticating the function of the TALEN constructs and the mRNA transcribed from them.

For the lymphocytes, the electroporation parameter was optimized in a 2mm gap cuvette. T cells were activated by antibodies as mentioned above for 3 days, then $2x10^6$ cells were washed and re-suspended in 100ul of neat OPTI-MEM medium. The cuvette was also pre-chilled and kept on ice when pipetted in with the cell suspension. Square wave mode was used according to other established protocols. 5 sets of parameters were tested, which were 500V,0.5ms; 500V,1ms; 400V,0.5ms; 400V,1ms; 360V,1ms and an untreated sample was used as the negative control. Each parameter test had been triplicated.

For monocytes/macrophages, the optimization was initially tested on monocytes. The monocytes were isolated by magnetic beads from blood cones then went through cytokine differentiation for 3 days. $5x10^6$ cells were washed and re-suspended in 250ul neat OPTI-MEM medium (Gibco, USA) containing 5ug in-house made eGFP mRNA (with ARCA cap analogue) and pipetted into a pre-chilled 4mm gap cuvette then kept on ice. 950uF, 500uF and 250uf capacity with 300V voltage was tested for optimization, an untreated sample was used as the negative control. Tests of each parameter set were triplicated.

As the progress went on which will be reflected in the result chapter V, it was also necessary to verify the condition on macrophages, the cell suspension was in the same condition as the monocytes, 500uF and 250uf capacity on 300V voltage settings were tested, each condition test was triplicated too.

2.3 Flow cytometry for phenotype test of cells

2.4.1 Flow cytometry panel for cell line, lymphocytes and Monocytes/Macrophages

Each sample to be stained by chrome conjugated antibody will be first pipetted in a FACS tube in the early stage of the work. In the second year of my PhD, we bought an electric aspiration machine to fully remove the supernatant, thus the staining was conducted in an Eppendorf tube since then. The sample will be firstly washed by DPBS for once, then resuspended to 100ul volume. The FACS staining buffer can be fresh DPBS, or DPBS already mixed with antibodies that were used in all samples as the buffer-antibody master-mix. After adding all the antibodies needed in each sample, the tube will be incubated at room temperature in a dark environment for the recommended duration from the manufacturer of the antibody. If the incubation period differs from the different antibodies, then the ones with longer incubation were added first, incubated till the short incubation ones were due to be added, then incubated till every antibody or dye reaches the recommended time.

The following antibodies and dyes have been used on the cell line, lymphocytes and monocytes/macrophages (Table 2.3):

Table 2.3 Chrome conjugated antibodies and cell dyes used in this project. The CD4 and CD8 antibodies with different chromes were used in a different stage of the work, but the clone of them was consistent. The GHOST-Hi5 cell line is an adhesive cell line, the cells will detach when dead and rinsed out before trypsin treatment, the harvested cell viability was >98%, thus no need for dead cells staining. Lym=lymphocytes, M φ =monocytes/macrophages.

	BRAND	CLONE	DILUTION	ISOTYPE/FMO	NOTE
CD4	BD	OKT4	1:100	none	Cell line, lym, Mφ
CD8	BD	HIT8a	1:100	none	Lym
CCR5-BV421	BD	2D71:50		BV421 Mouse IgG1, k	Cell line, Lym, Mφ
HLA-DR	eBioscience	L243	1:100	FMO	Lym
CD14-APC	BD	M5E2	1:100	FMO	Mφ
AQUA DEAD CELL STAIN	ThermoFisher, USA		1:1600	none	Lym
NEAR-INFRARED DEAD CELL STAIN	ThermoFisher, USA		1:1000	none	Mφ

2.4.2 Flow cytometry for p24 intra-cellular staining

Recipe for all reagents needed

1. 20 g/ml lysolecithin in 1% paraformaldehyde:
    - Dissolve 5g of paraformaldehyde in 50ml of 1N NaOH, add DPBS to 400ml in total.
    - Titrate the solution with 4N HCL to pH 7.2 +/- 0.1.
    - Add DPBS to 500ml in total.
    - Dissolve 10 mg of lysolecithin in the solution, mix well and store at 4℃.
2. 50% methanol by diluting 100% absolute methanol with an equal amount of PBS, Store at -20℃.
3. 0.1% NP-40 (Nonidet P-40): Add 0.5 ml NP-40 to 499.5 ml of PBS. Mix and store at 2-

80℃.

Intracellular Staining protocol

1. Wash the cells with DPBS in a FACS tube. Centrifuge the cells at 500g at 4℃ for 5 minutes and resuspend cells in 100ul DPBS/antibody/dye master-mix. incubate for 30 min at room temperature.
2. Wash cells with 5 ml PBS and centrifuge at 500g at 4℃ for 5 minutes. Pour out the supernatant then decant the excessive supernatant onto a paper towel.
3. Fix by adding 1ml of 20 g/ml lysolecithin in 1% paraformaldehyde. (reagent must be brought to room temperature before use). Vortex and incubate for 2 minutes at room temperature then centrifuge for 5 minutes 500g at 4℃.
4. Decant or aspirate supernatant. Vortex and then add 2 ml of -20℃ 50% methanol, Vortex then incubate in a 4℃ refrigerator for 15 minutes. Centrifuge as above.
5. Decant or aspirate supernatant. Vortex and add 1 ml of 4℃ 0.1% NP-40. Vortex vigorously then incubate in a 4℃ refrigerator for 5 minutes. Centrifuge as above.
6. Pour out the supernatant then decant the excessive supernatant onto a paper towel. Add 150ul DPBS containing 1ul of p24-FITC antibody (clone: KC57, Beckman Coulter). Vortex and incubate for 15 minutes at room temperature. Then wash by 5ml of DPBS, centrifugation speed as above.
7. Decant or aspirate supernatant. Vortex then dilute the sample with 300ul PBS. The flow cytometry test must be done on the same day.

2.5 Statistic Analysis

Statistical analysis was performed using GraphPad Prism software version 7.0 (GraphPad software). A two-tailed unpaired Student's t-test was used to compare data between two groups. *P < 0.05, **P < 0.01, ***P < 0.001. All the data with error bars are presented as mean values ± standard error of the mean (SEM). A P value of less than 0.05 was considered significant.

Chapter Three, Verification of plasmid constructs, optimization of electroporation, *in vitro* transcription, verification of TALEN function on the cell line.

3.1 Verification of plasmid constructs

Firstly, restriction enzyme double digestion was performed to verify the length of the two TALEN construct plasmids. Since the difference of these two constructs occurs at the recognition motif of the TALEN sequence, NcoI and EcoRI enzymes were used as they scale the whole targeted sequence. A single NcoI enzyme digestion was used as the control of this test. A software simulation was performed to indicate the appropriate appearance of the gel image. As indicated in the gel image (Figure 3.1), the lower band of the TALEN left arm plasmid showed an ~100bp short than the TALEN right arm, which corresponds to their genetic sequence.

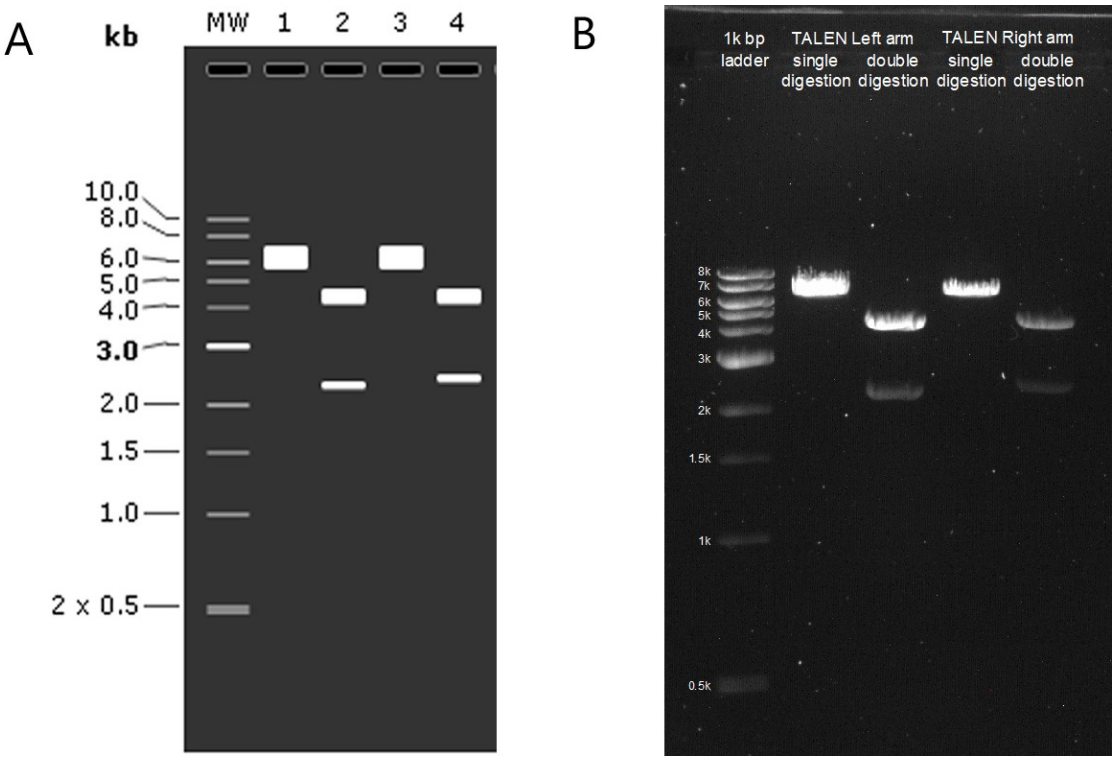

Figure 3.1. Electrophoresis image for the verification of the TALEN plasmids by single and double restriction enzyme digestion. A) software simulation to indicate the correct site of each band of the single and double enzyme digestion. B) actual gel photo of the electrophoresis, the band for both arms appeared on the indicated site from the software simulation.

3.2 Optimization of electroporation

3.2.1 GHOST-CXCR4-CCR5 cell line

3.2.1.1 Optimization of plasmid electroporation.

The primary parameters for electroporation on the GHOST-CXCR4-CCR5 (GHOST-Hi5) cell line were in reference to the established protocol for the HOS cell line for transfecting plasmids. Cuvette type, cell density, buffer choice, electroporation mode and capacity under

exponential decay mode have all taken the same value of the protocol. In summary, the electroporation was under exponential decay mode, 1050uF capacitance, 50Ω resistance, in a 4mm cuvette. $2x10^6$ cells were suspended in 600ul OPTI-MEM medium containing 4ug GFP plasmids in each cuvette. 4 different voltages were tested, which were 250V, 300V, 350V, 400V. Immediately after electroporation, the cells were transferred to a well of a 6 well plate. In each well, there is 3ml of pre-warmed to 37℃ D10 medium. The cells were cultured overnight then subjected to tests. Each test was duplicated (Table 3.1).

Table 3.1. The result of each repeat of all the parameter set for plasmid electroporation on GHOST-Hi5 cell line

	%GFP+			***Confluency***
	No.1	No.2	mean	mean
250V	41	49	45	~60%
300V	52.9	64.3	58.6	~40%
350V	56.2	69.1	62.65	~20%
400V	58.8	70.7	64.75	Very low

Cell confluency showed voltage higher than 300V group caused significant cell death after electroporation, whereas the 300V had acceptable cell viability and GFP positive rate, so this group was chosen as the optimal condition for the next step of work to verify the CCR5 knockout function of the TALEN construct plasmids.

3.2.1.2 Optimization of mRNA electroporation.

The non-Voltage parameters used in the mRNA electroporation optimization were the same as in the plasmid electroporation work. 2ug of eGFP mRNA was added to the cell

suspension instead of eGFP plasmids. The voltages tested in this work were 300V, 350V, 400V (Table 3.2). Procedures were the same as the DNA electroporation set-up, if not otherwise specified.

Table 3.2 The result of each repeat of all the parameter set for mRNA electroporation on GHOST-Hi5 cell line

	%GFP+			***Confluency***
	No.1	No.2	mean	mean
300V	48.5	68.3	58.4	~50%
350V	54.8	74.9	64.85	~30%
400V	57.1	80	68.55	Very few

300V condition in the mRNA electroporation setting achieved better confluency and GFP positive rate than the DNA setting, so it was retained as the parameter for mRNA electroporation.

3.2.2 Activated Primary T cells

Activated primary T cells electroporation is a well-developed platform, thus the optimization work was essentially a comparison between minor modifications of the protocol provided by the manufacturer of the electroporator. Using the same cuvette (2mm), pulse mode (square wave), buffer (OPTI-MEM) and cell density ($2x10^6$ cells in 100ul buffer) indicated in the protocol, 5 different voltages have been tested, which were 500V,0.5ms; 500V,1ms; 400V,0.5ms; 400V,1ms; 360V,1ms. 2ug per $2X10^6$ CD3/CD28 soluble antibody activated T cells were used in each of the samples. An untreated sample was used as the negative

control. The cuvette had been pre-chilled on ice and was kept on ice after pipetting in the cell-mRNA suspension in order to minimize the heat damage caused by electrical shock. Immediately after electroporation, the suspension was aspirated out of the cuvette and pipetted into 37℃ R10 medium. The samples were cultured overnight and tested by flow cytometry. The result was as follows detailed in Table 3.3.

Table 3.3. Result of every sample tested by FACS for the mRNA electroporation optimization on the activated primary T cells.

	%GFP+			MFI			%GFP+	MFI	%Viable
	#1	#2	#3	#1	#2	#3	mean	mean	mean
Untreated	0.6			477			0.6	477	91.2
500V,0.5ms	78.4	74.2	72.7	1857	1821	1785	75.1	1801	83.3
500V,1ms	61.3	59.2	47.2	2287	2104	1942	55.9	2111	40.0
400V,0.5ms	76.6	77.6	81	1797	1866	1917	78.4	1860	88.2
400V,1ms	83.2	90.3	80.6	2598	2664	2390	85.3	2414	88.9
360V,1ms	81.4	83	79.2	2445	2501	2437	81.2	2461	76.5

Among all the samples, the 400V,1ms setting has the best mean eGFP positive rate and cell viability. Although the mean fluorescence intensity was lower than the 360V1ms group, the difference was so small therefor it was considered the same as the 360V1ms group. Thus the final electroporation parameters chosen for delivering mRNA to the activated T cells was $2x10^6$ cells in 100ul OPTI-MEM medium, 2mm cuvette, electroporate using square wave mode, under 400V and one single wave for 1ms.

3.3 *In vitro* transcription of mRNA from the eGFP and TALEN plasmids

3.3.1 Electrophresis verification of the mRNA

The length of untailed and tailed mRNAs was both tested for verifying if the untailed mRNA corresponds to the correct length and confirm tailing. To do this, a very small sample before and after poly(A) tailing reaction was taken and went through electrophoresis on a MOPS-formaldehyde denaturing gel electrophresis (Figure 3.2). Upon successful IVT, the band of untailed RNA appeared at the anticipated size indicated by the RNA ladder maker (GFP~950nt, TALEN-left ~3000nt, TALEN-right~3100nt). The polyadenylation reaction should add a poly(A) tail with the length of 150-200nt, claimed by the manufacturer. The figure shows that the RNA electrophoresis showed the untailed and tailed mRNA appeared at the expected site, indicating this IVT method is capable of synthesizing the desired mRNA for GFP and TALEN.

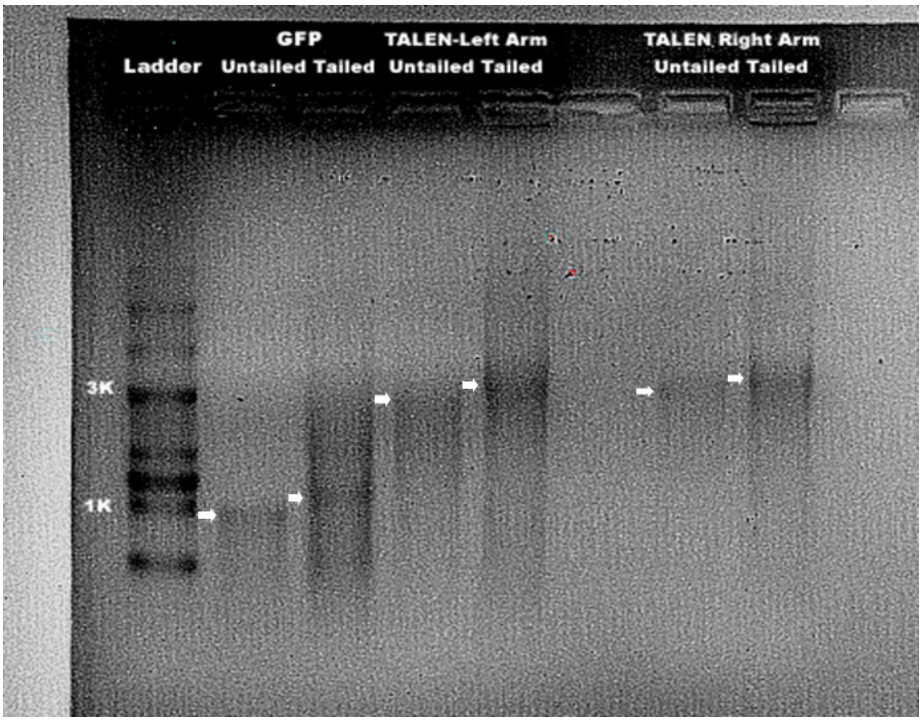

Figure 3.2. Electrophoresis result of the IVT mRNAs before and after polyadenylation for eGFP, TALEN left arm and TALEN right arm. All three bands of uncapped mRNA appeared at the correct site of length, which are ~950nt, ~3000nt and ~3100nt, respectively. The Poly(A) tailed mRNAs were slightly longer than the untailed counterparts, corresponding to the poly(A) tailing kit's claim, that 150-200nt of poly(A) tail would be added to the capped mRNA.

3.3.2 Comparison of different IVT kits/components

At the early stage of this project, the mMESSAGE mMACHINE SP6 kits (Invitrogen, USA) was used for the *in vitro* transcription of mRNA for both eGFP and TALEN arms. This kit uses the conventional mRNA cap analogue m7G(5')ppp(5')G, thus makes it possible that the cap analogue is incorporated into the mRNA reversely, renders the mRNA untranslatable. This effect clearly indicated that, under the optimized electroporation parameters which were determined by transfecting the CleanCap Enhanced Green Fluorescent Protein mRNA (Trilink Biotechnologies, USA), the average GFP positive rate was 85.3%, occasionally 95% in later works. However, using the eGFP mRNA transcribed by this kit, the average positive rate

was only 54.48%.

Optimization of the IVT work was needed to achieve a better quality of the mRNA synthesized. In that case, I looked for a better component, so I started to use the ARCA cap analogue. As mentioned previously, the ARCA cap analogue can only be incorporated into the mRNA sequence in the correct orientation, which significantly improves the translation of the mRNA produced. From this stage, the mRNA was transcribed using the RiboMAX Large Scale RNA Production Systems kit (Promega, USA) which provides NTPs, buffer and enzyme separately. The ARCA cap analogue was purchased from Jena Bioscience and added to the reaction. The eGFP mRNA transcribed by this method achieved a significantly better GFP+ rate of CD4+ T cells of 79.68%, which was closer to the GFP+ rate to the CleanCap Enhanced Green Fluorescent Protein mRNA (Figure 3.3), used in the electroporation optimization described in the latter sections.

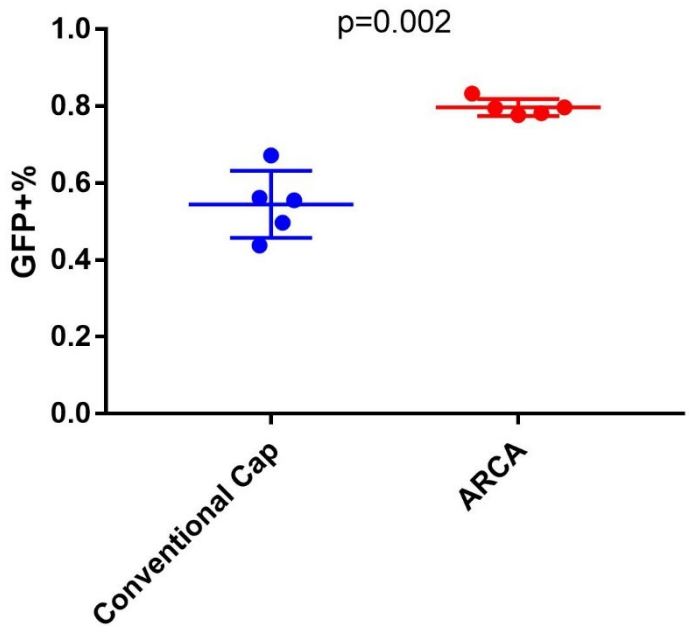

Figure 3.3. Comparison between the eGFP mRNA capped with the conventional cap analogue and the ARCA. The mean eGFP+ rate in the conventional cap group and ARCA group was 54.48% and 79.78%, respectively (p=0.002).

3.4 Verification of TALEN function on cell line

3.4.1 TALEN plasmids transfection and CCR5 gene knock-out

8ug of TALEN plasmids of each arm and 2ug of GFP plasmids were electroporated to $2x10^6$ GHOST-Hi5 cells. After electroporation, the cells were cultured for 3 days then went through flow cytometry to test for their CCR5 expression. Another cuvette was only electroporated with GFP plasmids as a no-gene-edit control. This work has been duplicated, the result is shown in Figure 3.4.

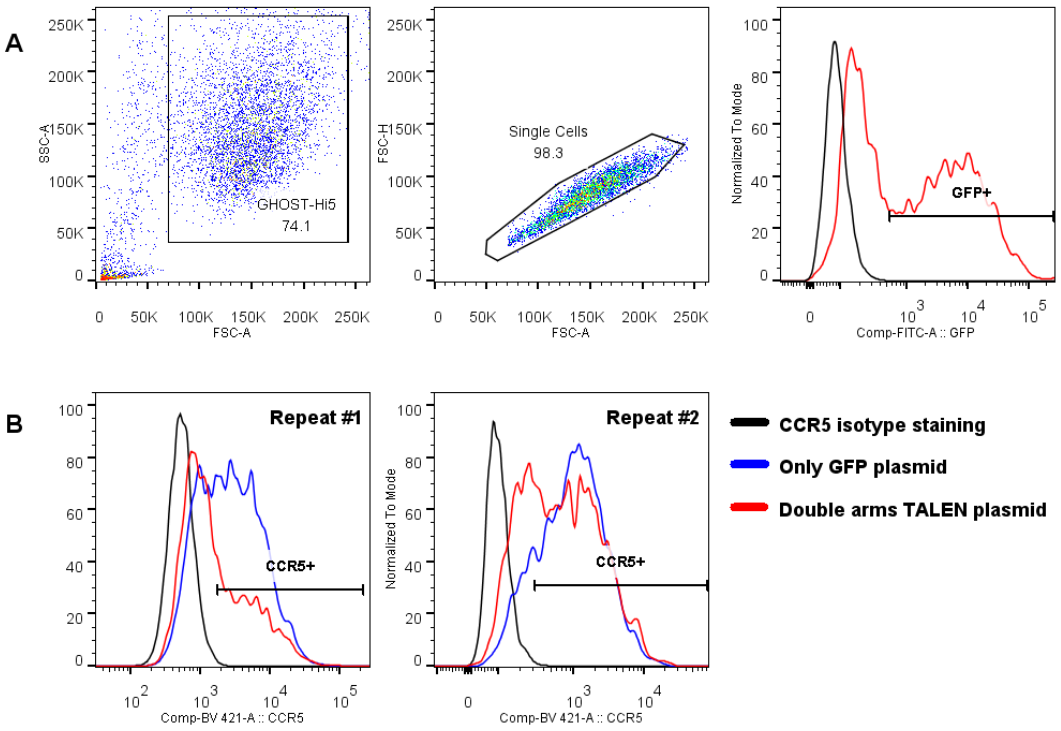

Figure 3.4. The downregulation of CCR5 after treating the GHOST-Hi5 with the eGFP plasmids and TALEN plasmids. A) Gating strategies to scale the GFP+ cells. B) After 3 days of culture post-electroporation, the GFP+CCR5+ population was downregulated in the treated cells of both repeats (39.4% and 15.4%, respectively).

Although a variation of CCR5 expression rate happened in the two groups, a downregulation of CCR5 expression has been observed in both groups. The CCR5 expression rate in repeat #1 was 36.8% vs. 61% between TALEN double arms plasmids sample and only GFP sample, in repeat #2 was 72.6% vs 85.8% between TALEN double arms plasmids sample and only GFP sample. The CCR5 downregulation rate was 39.4% in the first repeat and 15.4% in the second repeat. This result shows that both arms of the TALEN construct together can perform CCR5 knockout in the plasmid form.

3.4.2 TALEN mRNA transfection and CCR5 gene knock-out

To confirm the CCR5 knockout function of the mRNA *in vitro* transcribed from the TALEN construct plasmids, 4ug of each arm of TALEN mRNA has been electroporated to $2x10^6$ GHOST-CCR5-CXCR4 cells. 2ug of GFP mRNA was used in the test group and the No-TALEN group as a reporter. This work was also duplicated (Figure 3.5).

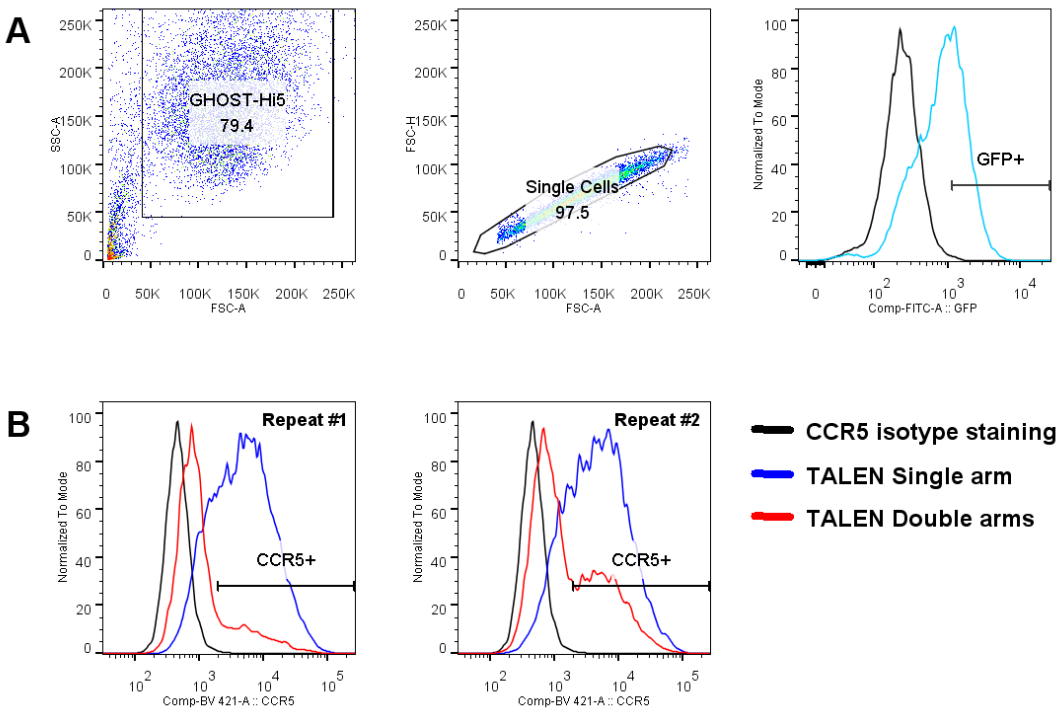

Figure 3.5. The downregulation of CCR5 after treating the GHOST-Hi5 with the IVT mRNAs of eGFP and TALEN. A) Gating strategies to scale the GFP+ cells. B) After 3 days of culture post-electroporation, the GFP+CCR5+ population was downregulated in the treated cells of both repeats (73.0% and 48.9%, respectively).

In the two repeats, the CCR5 expression rate by FACS in the TALEN double arms mRNA treated samples was 21.0% and 40.0%, respectively. In the GFP mRNA only samples, it was 77.8% and 76.9%, respectively. The CCR5 knockout downregulation rate was 73.0% in repeat

#1 and 48.9% in repeat #2. This result indicated that this TALEN design in the form of mRNA could also knock out the CCR5 gene, resulting in downregulation of CCR5 expression and the downregulation was stronger than the plasmids-mediated ones.

3.5 Discussion

The works described in this chapter were the preparation for using the TALEN constructs to perform the CCR5 gene disruption on primary cells and reflected many aspects of the considerations on the detailed methodology of a gene-editing project.

Firstly, the TALEN constructs were designed by our collaborator, Prof Linqi Zhang's team from Tsinghua University, China. In their work, published, they have designed a variety of TALEN constructs targeting different segments of the CCR5 gene. The TALEN-CCR5-515 construct stood out from their many designs, to have the best efficacy in disrupting the CCR5 gene(135). This reflects that the design of TALEN needs to consider the sequence targeted, not only if the sequence is indeed unique that is not causing off-target effect, but also the methylation of DNA can affect the recognition function of TALEN(136), thus, upon the initial design of TALEN for a specific gene, multiple target gene sequences need to be tested if using traditional TALEN scaffold. Further investigation has been carried out to address this issue. A new scaffold of TALEN has been developed to avoid the negative impact of DNA methylation, which facilitate in simplifying the designing process of a new TALEN construct(137-139)

In their work using the TALEN-CCR5-515 plasmid constructs, the disruption of the CCR5 gene both in the GHOST-CXCR4-CCR5 cell line and primary T cells has been confirmed, and further work on the CCR5 partial knockout cell line against HIV-1 infection has also been confirmed, but not on primary cells, which needs to be further confirmed.

Upon Prof Zhang team's findings, indeed more questions and aims can be raised. Firstly, is the form of TALEN to be delivered to the cells. Delivering plasmids has certain benefits regarding the simplicity of the research. A TALEN design is first assembled in the form of plasmid, carried by a plasmid, it's already ready to be transformed, expanded and then transfected to the cells. However, as a foreign sequence of DNA, a plasmid transfection to cells carries many risks as well. First to be noticed is the cytotoxicity of foreign DNA which will induce apoptosis as a defense mechanism(140, 141). Secondly, the introduction of plasmids to the cell nucleus can potentially cause rearrangement and integration into the host's genome(142).

Both issues can be addressed to use mRNA as the carrier. Plus, the plasmid needs to be transported to the cell nucleus to be transcribed into mRNA and released to the cytoplasm to be translated into protein, this step will be avoided by delivering mRNA to the cells and it will increase the efficiency of transfection(143). To acquire both optimal efficiency and viable cells, transfecting mRNA instead of DNA is indeed a better option. The benefit does not stop here. Plasmid exists more stably in the cells, leading to a significantly longer half-life than RNA. This stability leads to excessive and prolonged exposure of the cell genome to

the programmed nuclease and exposes the genome to a higher possibility of off-target damage(144, 145). On contrary, the nature of the very short half-life of mRNA can solve the problem, its transient presence leads to highly controlled production of proteins that shortens the exposure of cells to gene editing. This effect is more significant ZFNs and CRISPR/Cas9 who have a higher off-target effect, but it can also be a consideration on TALENs. Thus, in my work, mRNA of TALENs were used as the nucleic acid carrying the TALEN genetic sequence to the cells.

The delivery of nucleic acid to the cells is also a significant component in the design of a gene-editing project. Three major categories of transfection methods have been widely used nowadays, which are chemical transfection reagent, electroporation, viral vector. Each of them has its own advantages and disadvantages(146).

Chemical transfection reagents are chemical molecules to form a particle to encapsulate the nucleic acid inside. Upon meeting cells in a liquid phase suspension, the chemical capsule can fuse with the cell membrane or activate endocytosis, to directly release the nucleic acid contained or interact with the endosome and finally escape the endosome attack and be released into the cytoplasm(147).

Electroporation uses electricity pulse to perforate the cell membrane, leading to a transient increase of cell membrane permissibility to allow the nucleic acid in the cell suspension to flow into the cytoplasm directly(148).

A viral vector is basically a recombination of the target nucleic acid with the viral structural gene. After the transfection of the complete construct, the vector-producing cell will synthesize the outer structure of the original virus but contain the target nucleic acid inside the vector particle instead of the original genome. In that way, the viral vector is still able to infect the target cells and release the nucleic acid inside the vector to the cells, but it cannot replicate since it does not contain the original genome(149).

Each transfection method has its pros and cons. Chemical transfection reagent is relatively easy to use, but its cytotoxicity is also significant. The efficiency and cytotoxicity of chemical reagent is largely cell type and condition-dependent, the pH value, cells stage, cells health can greatly affect the final result, especially for the liposome-based reagents. The new generation of chemical transfection reagents based on polymers or lipid nanoparticles (LNP) has been developed recently, largely decreasing the sensitivity to the chemical environment (146). Reagents based on LNP or polymer are not thoroughly developed as a research tool so far, but their application in vaccine development has already shown great promise in the COVID-19 era. Viral vector requires expertise and further complicates the research process and the encoding capacity of the viral vector can be a limitation. Among the commonly used viral vectors, the adenovirus vector has a coding capacity for the cargo of 4.5kb, Adeno-associated viruses vector has 5kb, lentivirus and retrovirus vector has ~9kb. Notably, the relatively new generation of 'gutless' adenovirus vector has a cargo capacity of 37kb(150), all these indicate that the choice will be limited to encode both arms of TALEN. Although three types of viral vectors can still be utilized, the nucleic acid type is restricted.

All these vectors deliver DNA ultimately, with the lentivirus and retrovirus vector integrating the delivered gene to the host genome. Given that foreign DNA is more cytotoxic and stable, the off-target effect of gene editing will be enhanced due to prolonged exposure to the gene-editing complex. Whereas electroporation is purely physical and the procedure is quick. It can be widely used across cell types and under optimized parameters, both high efficiency and cell viability can be achieved(151). Thus, this project was designed to use electroporation to deliver the TALEN mRNA to the target cells.

In this part of the work, various aspects of the work have been investigated or optimized. At the beginning of the whole work, the TALEN constructs and eGFP plasmids have been verified regarding their function. In this process, it helped me to get familiar with the equipment, devices, procedures, and establish laboratory work principles through teaching and manual practice. The double digestion verification of the plasmids and further cell line transfection authenticated the eGFP and TALEN plasmids both structurally and functionally.

After authentication of the plasmids, *in vitro* transcription work (IVT) was conducted to make the mRNA transcripts of both TALENs and eGFP. Different parts of the mRNA can affect the stability and translation efficiency of the mRNA. Firstly, the untranslated region (UTR) of the mRNA can affect the efficiency and stability of the mRNA(152). The UTRs are located immediately upstream and downstream of the protein-encoding sequence. To minimize the modification of the original TALEN construct, the UTR effect on the translation effect of the mRNA has not been investigated in this work. Given the sequence is already settled, the

effect of the 5'cap analogue was investigated. The eukaryotic mRNA starts with a 7-methlyguanosine incorporated to the 5' end of the rest of the mRNA via a 5'-5'-triphosphate bridge (ppp) (m7GpppN) (153). The 5' cap structure helps the binding of the eukaryotic translation initiation factor 4E and protects the mRNA from the 5' to 3' degradation by the exonuclease Xrn1p(154). There are the conventional cap analogue and ARCA analogues that are easily commercially accessible. The ARCA cap can only be incorporated into the transcribed RNA sequence in the right orientation, whereas the conventional one is not, leaving it a 50/50 chance of reverse incorporation which renders the mRNA untranslatable. By comparing the translation efficiency of these two types of cap analogues, the result clearly showed that the ARCA analogues promote the transfection efficiency, consistent with the previous literature(155). Poly(A) tail is also an important part of the mRNA to protect the mRNA from 3'-5' degradation(156). It is located at the end of the mRNA and can be incorporated by Poly(A) Polymerase or directly transcribed from a poly(A) sequence downstream of the open reading frame on the template plasmid. At the beginning of this work, the adding of poly(A) tail was neglected and resulted in no GFP signal from the cell transfected with these mRNA without the tail (data not shown). Learning the fact, by adding the poly(A) tails using Poly(A) Polymerase, the result shown in this section showed GFP positive flow cytometry results. The positive result from the IVT mRNA eGFP has confirmed the kit I used was capable to transcribe the target sequence on the plasmids into functional mRNA, thus the mRNAs of both arms of TALEN were transcribed and electroporated to the GHOST-CXCR4-CCR5 cells line. After 3 days of incubation, a significant decrease of CCR5 expression by flow cytometry was observed, indicating that the

homemade TALEN mRNA was functional and then can be carried to the next step of CCR5 disruption on primary T cells.

Another modification of mRNA to enhance the transfection efficiency is to incorporate artificially modified nucleotides to replace the natural ones. Commonly used artificial nucleotides include 5-Methyl-CTPs and Pseudo-UTPs. The nature of the modified nucleotides changes the structure from the natural ones, resulting in the molecular groove for enzyme binding to change, thus masks these mRNA from the recognition and degradation of the innate immunity within the cells(157). This mechanism results in less innate immunity activation and attack against these foreign mRNAs introduced to the cell that leads to a higher translation activity(158, 159). Along with LNP-polymer-based chemical transfection methods, the nucleotides modified mRNAs together as a vaccine has for the first time been used *in vivo* against the SARS-CoV-2, commercially known as the Pfizer-BioNTech vaccine and Moderna vaccine, during the COVID-19 epidemic. Phase I-II trial reports, Phase III reports and real-world massive application reports have all confirmed the high efficiency of these vaccines comparing to traditional inactivated virus vaccines and viral-vector-based vaccines(160). However, using the modified nucleotides will further increase the cost of IVT, thus in my work, the final protocol for IVT was based on ARCA and natural nucleotides.

Finally, the gene-editing function of TALEN plasmids was firstly verified. Since the result showed an obvious downregulation of CCR5 on the surface of the GHOST-Hi5 cell line, the

TALEN mRNAs were tested for the function subsequently. The result of mRNA work not only confirmed the function of the TALEN mRNAs but also showed a stronger effect on the GHOST-Hi5 cell line, which was in agreement with the previous literature of using mRNA can result in a better gene-editing effect(161, 162). With the optimization of electroporation on lymphocytes to deliver mRNAs and the authentication of the function of TALEN, it was ready to be used o the activated primary T cells.

Chapter Four. CCR5 gene knockout on lymphocytes and its effects on HIV-1 resistance.

4.1 CCR5 gene knock-out mediated by TALEN mRNA

In this section, PBMCs from three blood cones of three different donors have been cultured and stimulated in R10 medium supplemented with soluble anti-CD3, soluble anti-CD28 and human IL-2 for three days. After washing the cells, an equal number of cells have been assigned to three groups (Table 4.1).

Table 4.1. Group design for the function test of TALEN mRNA

	Treatment
Untreated	No treatment
Single arm TALEN	Electroporation of GFP+ left arm TALEN mRNA
Double arms TALEN	Electroporation of GFP+ both arms TALEN mRNA

After treatment, each group of cells was cultured in R10 medium supplemented with IL-2 for a further 3 days for the TALEN complex to perform the gene editing. Then the cells were washed and went through the following tests.

4.1.1 Flow cytometry evaluation of CCR5 expression after TALEN treatment

The cells of each group have been stained with antibodies of CD4, CD8, and CCR5, then went through flow cytometry to compare the expression of CCR5 on CD4+ cells. The untreated group cells were not electroporated with GFP mRNA so the expression of CCR5 was tested among CD4+ cells. In the single arm and double arms TALEN group, the CCR5 expression was in CD4+GFP+ cells. As shown in Figure 4.1, the CCR5 expression in the single arm TALEN group was comparable or even higher than the untreated group, indicating that only one arm of TALEN mRNA is not able to perform the gene editing. Comparing to the single arm group, each sample in the double arms group showed downregulation of CCR5 expression (donor 1-3, single arm TALEN group, 54.3%, 31.5%, 23.4%, respectively; double arms group 40.2%, 20.4%, 15.1%, respectively). Comparing to the single arm group, the downregulation of CCR5 in each double arms group was 25.97%, 35.24%, 35.47%, respectively.

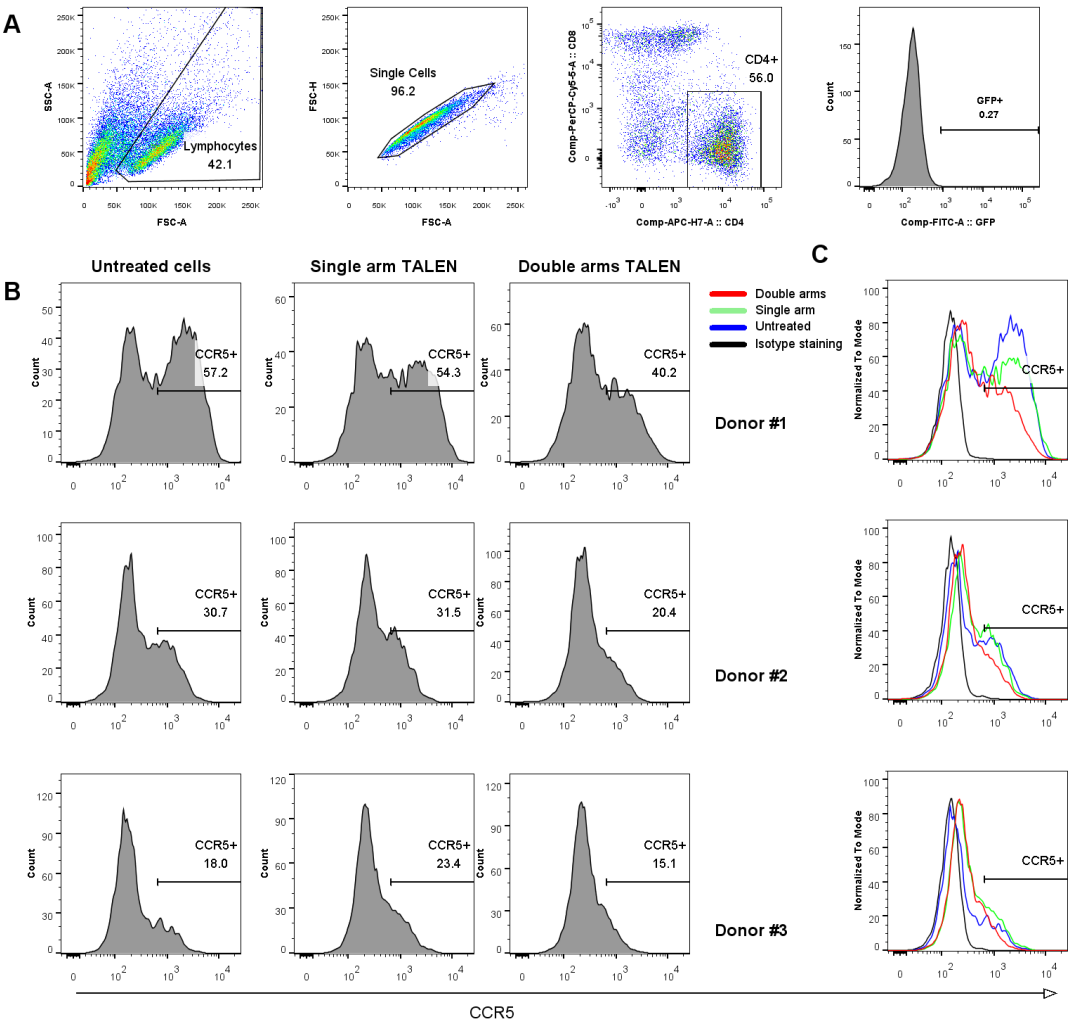

Figure 4.1. The CCR5 expression of all the samples three days after the electroporation of eGFP and TALEN mRNA. A) Gating strategy to scale CD4+GFP+ singular cells. B) the CCR5 expression among all CD4+GFP+ cells in each sample. C) Overlay histograms of the samples from each donor.

4.1.2 T7 endonuclease assay to detect CCR5 gene editing

The gDNA of each sample in the experiment above was extracted, then used to amplify the CCR5 gene fragment. Primers used in the PCR reaction scaled the base pairs that covered both the gene-editing site and CCR5-Δ32 site. The PCR product was purified then went through T7 endonuclease I digestion. This enzyme can cut any mismatches on the double strain DNA and the result can be shown by electrophresis. A positive result will show both

the original PCR amplicon and two additional bands shorter in DNA length, which indicates the occurrence of indels on part of the template gDNAs (Figure 4.2).

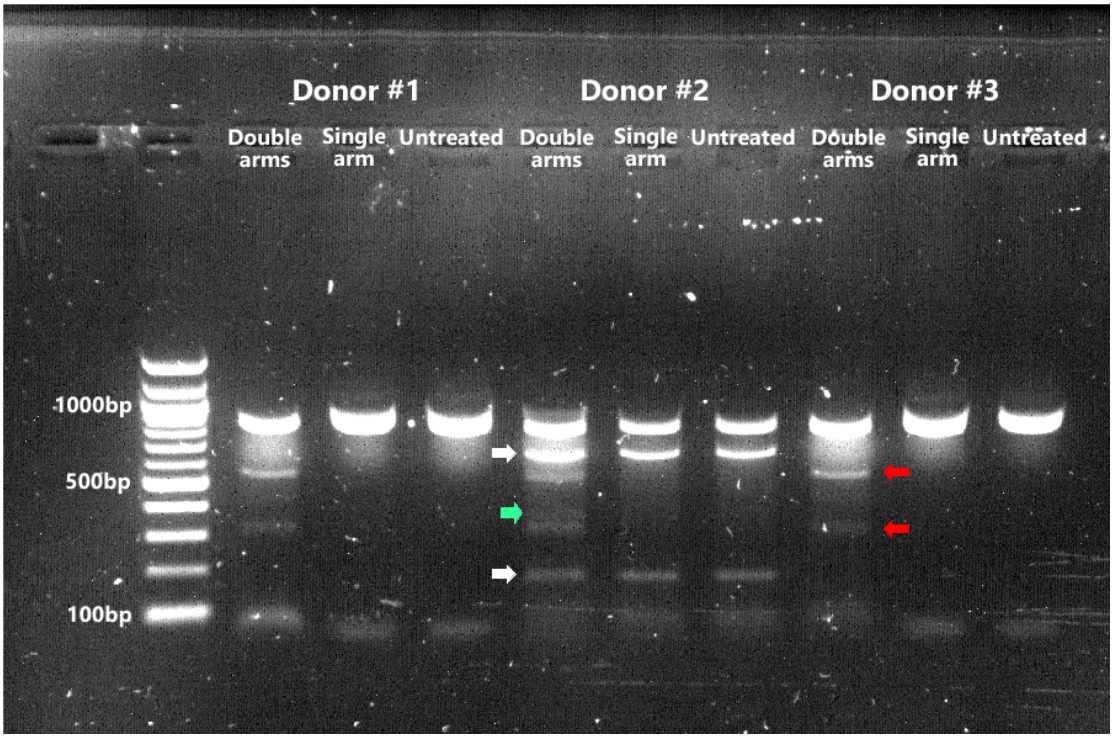

Figure 4.2. Gel image showing the result of the T7EI assay for all 9 samples from 3 donors. In the double TALEN arm group of each donor, two bands showed at the sites of the red arrows, indicating a mutation happened within a DNA sequence area narrower than the resolution of a gel run. Given the scale of sequence between two primers of PCR and the DNA cleavage site of TALEN, the cleavage separates the CCR5 PCR amplicon into two segments of the same length as the two bands with red arrows. Two additional bands indicated by the white arrows appeared across the three samples of donor #2, with the length indicating CCR5-Δ32. Finally, a very dim band appeared at the 400bp length position in the double arm group of donor #2, pointed at by a green arrow. This is an indication of two mutations co-existing in that sample.

After electrophoresis, the gel image (Figure 4.2) showed that two bands of shorter length comparing to the PCR amplicons, appeared at the same site in each double arms TALEN group, but not in single arm TALEN group and untreated group. Also, multiplying the length of the two shorter bands was the same as the length of the original PCR amplicon. This

result indicated that there were indels on part of the gDNA of the double arms TALEN group, whereas the gDNA from the single arm TALEN group and the untreated group remained intact.

It was worth noticing that in donor #2, two additional bands were also visible. The length of them corresponded to the cut site of CCR5-Δ32. In addition to that, there was a very dim band appeared close to the 400bp position, which indicates a double cut on the PCR amplicon that contained both TALEN gene editing and CCR5-Δ32. It led to the presumption that this was the result of heterozygous CCR5-Δ32. To further confirm that the two short bands that occurred in all double arms group were indeed the result of TALEN gene editing, along with confirming whether additional bands in donor #2 were indeed heterozygous CCR5-Δ32, the original PCR product was sent for Sanger sequencing service.

4.1.3 Sanger sequencing result of the CCR5 gene from the TALEN treated T cells

The PCR amplicons need to be cloned to a backbone plasmid vector for Sanger sequencing. To do that, the original PCR amplicons of each double arms TALEN group had been cloned to the pOPINJ plasmid backbone in the site of the lacZ gene. The plasmids of each sample have been transformed to *E. coli* and seeded on an X-gal LB agar plate. If insertion of the CCR5 amplicon was successful, it would have interrupted the lacZ gene thus the *E. coli* colony on the plate would appear white, otherwise blue due to the digestion of the X-gal by

the beta-galactosidase translated from the lacZ gene. Each sample had 30 white colonies picked out, cultured and then the plasmids extracted and sent for Sanger sequencing service (Figure 4.3).

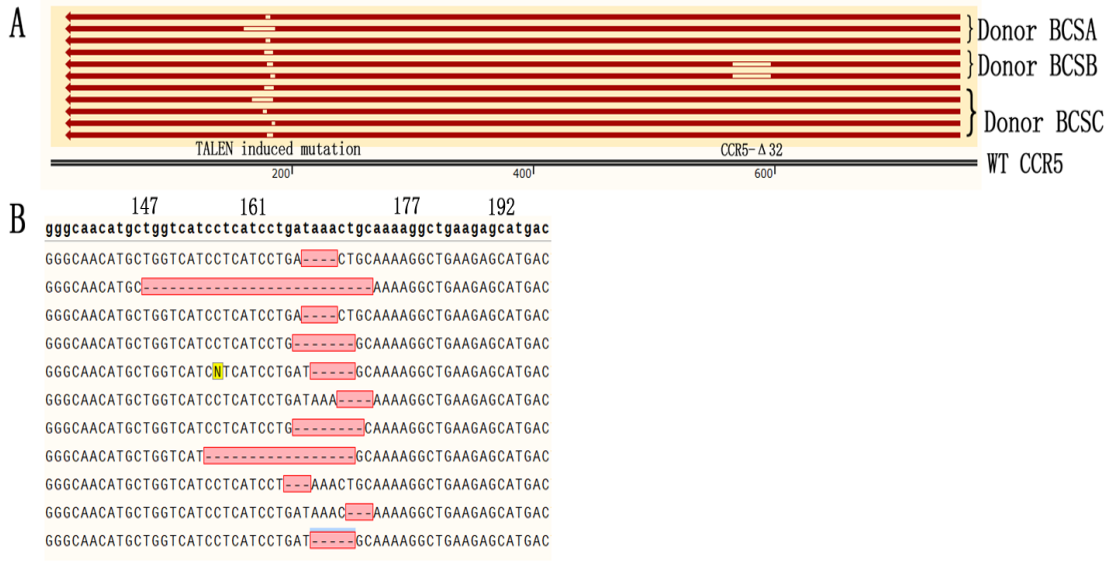

Figure 4.3. Sanger sequencing result of the CCR5 amplicons from the gDNA in the double arms TALEN group from each of the three donors. A) The overlap of all sequence maps that contained an indel at the site of the FokI cleavage site. The sequence map of wildtype CCR5 is included and marked with the site of FokI cleavage and CCR5-Δ32. Notably, samples of BCSB contained the CCR5-Δ32. B) the sequence data of all the results that carried a TALEN induced indel.

From the result of the Sanger sequencing (FIgure 4.3), all three samples contained amplicons that contained a deletion at the site, which is between the two DNA sequences that one or another TALEN arm recognizes, the site where the FolkI endonuclease forms a dimer and perform the DNA cut. The site is correspondent to the publication of the provider of the TALEN construct, where they described the CCR5 gene knockout mediated by the plasmids of this TALEN construct(135).

Moreover, 13 out of 28 successful sequencings from the donor #2 sample had CCR5-Δ32 detected, the rate was 40.7% (11 out 27 successful sequencings), close to 50% which confirmed that the extra bands that appeared on the T7EI assay were indeed the result of heterozygous CCR5-Δ32.

This result, along with the flow cytometry and T7EI assay results, confirms that the mRNA transcribed from the TALEN plasmid is able to perform gene disruption on CCR5, which leads to a change of cell phenotype of downregulation of the CCR5 molecule.

4.2 HIV-1 infection assay on TALEN treated T cells

To further investigate if the CCR5 disruption could result in increased resistance to HIV-1 entry to CD4+ lymphocytes, an HIV-1 infection assay was conducted on the double arms TALEN group, single arm TALEN group and untreated group. The design of the same three groups was to test not only the HIV-1 resistance of CCR5 downregulated cells comparing to untreated cells, but also rule out the effect of electroporation (single arm group vs. untreated group).

In addition, HIV-1 infection can kill the CD4+ lymphocytes, thus it was presumed that with the progression of HIV-1 infection, the CCR5 knocked-out cells would be enriched in its ratio to the whole set of cells. So this presumption was also tested in this section of work.

To do this, PBMCs from three different donors were used in this work. Before the TALEN treatment, CD8+ T cells were depleted from the stimulated lymphocytes by positive selection of human CD8 magnetic beads, in order to rule out the killing effect from the cytotoxic T lymphocytes. The CD8 depleted lymphocytes were then divided into three groups and received their designated treatment and then cultured for 3 days. Then a portion of each sample was taken for the flow cytometry test to confirm the expression of CCR5, another portion was taken to have the gDNA extracted for NGS sequencing to serve as the baseline data of the CCR5 knockout ratio.

Each sample was then spin-inoculated with the HIV-BaL strain and cultured in R10 medium supplemented with IL-2, the medium was changed every three days. On day 4 and day 12 after the spin-inoculation, a portion of the sample was taken and intra-cellularly stained for the p24 antigen and then tested by flow cytometry. Another portion was taken for gDNA extraction to test the CCR5 knockout ratio by NGS, to confirm the enrichment of CCR5 knockout.

4.2.1 Flow cytometry evaluation of CCR5 expression after TALEN treatment

Three days after treatment, a portion of each sample went through a flow cytometry test to assess the expression of CCR5, results shown in Figure 4.4 and Table 4.2.

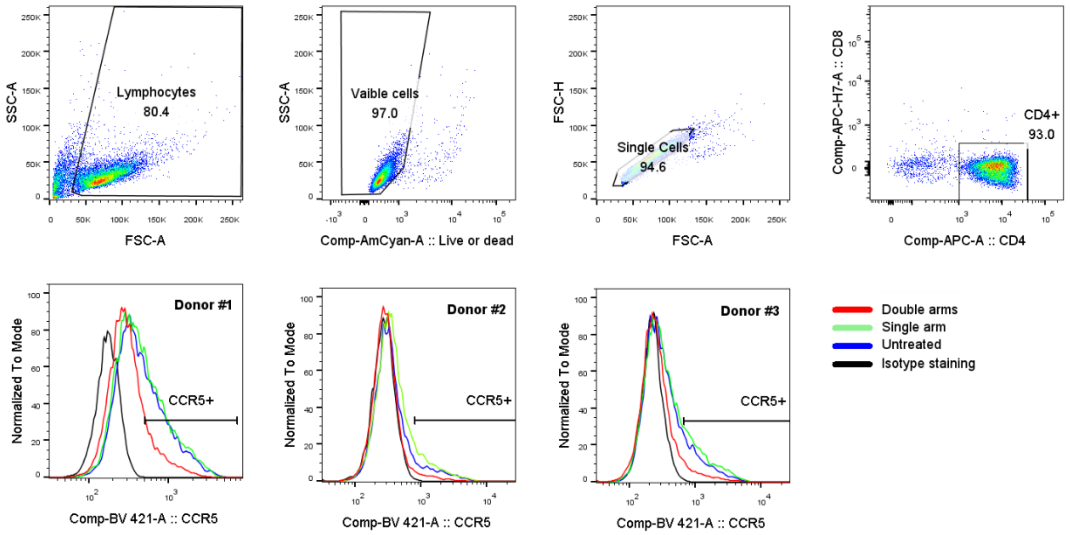

Figure 4.4. Gating strategy and the overlay of histograms of all the lymphocytes samples from three different donors before HIV-1 infection assay.

Table 4.2. The percentage of CD4+CCR5+ cells in each sample.

	Double arms	Single arms	Untreated
Donor#1	16.5%	37.4%	37.4%
Donor#2	3.34%	10.5%	9.17%
Donor#3	5.82%	17.3%	14.2%

All the samples in the double arms group showed a downregulation comparing to the single arm group (16.5%, 3.34%, 5.82% vs. 37.4%, 10.5%, 17.3%, respectively). A small aliquot of each sample was taken for gDNA extraction, the gDNA was kept at -20℃for CCR5 PCR-amplification and further NGS sequencing.

4.2.2 Flow cytometry evaluation of p24 expression after HIV-1 infection

Standard and prolonged HIV-1 infection assays have been conducted on each sample mentioned in the last section. The samples were spin-inoculated with HIV-BaL strain of HIV-1 at the MOI of 0.01, then cultured for 4 days. An aliquot of the sample was taken from each sample, part of it had the p24 intracellular staining to examine p24 expression and the rest was used for gDNA extraction. The rest of the sample was kept for culture till day 12 with regular change of medium, in order to test if the CCR5 disruption would be further enriched, because cells with intact CCR5 could be further killed by HIV-1. An aliquot at day 12 was also taken for gDNA extraction, to be prepared for NGS sequencing. The results are shown in Figure 4.5.1 and Figure 4.5.2 and summarized in Table 4.3.

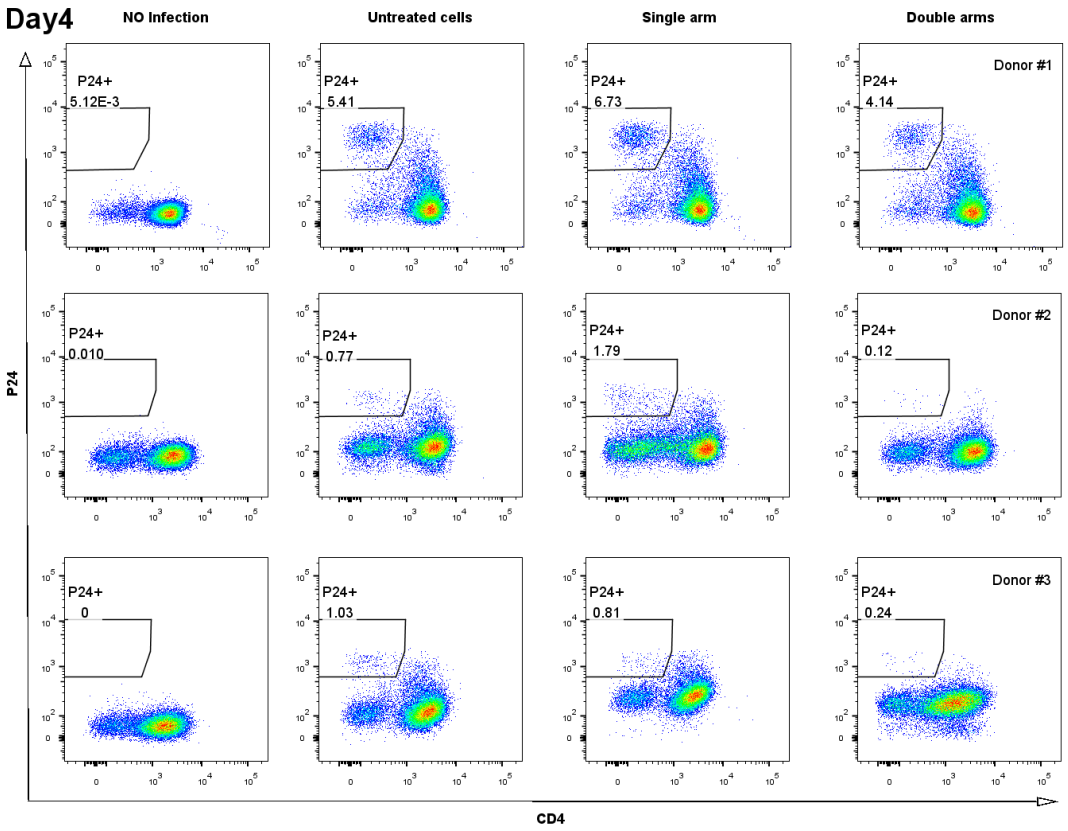

Figure 4.5.1. Result of HIV-1 infection assay on the lymphocytes from three donors with different TALEN treatments. All the samples have been spin-inoculated with HIV-BaL at the M.O.I of 0.01, 4 days after infection, an aliquot of each sample went through p24 intracellular staining and went through flow cytometry to examine the p24 expression. Donor #1 had an exceptionally high infection rate, presumably because of high CCR5 expression.

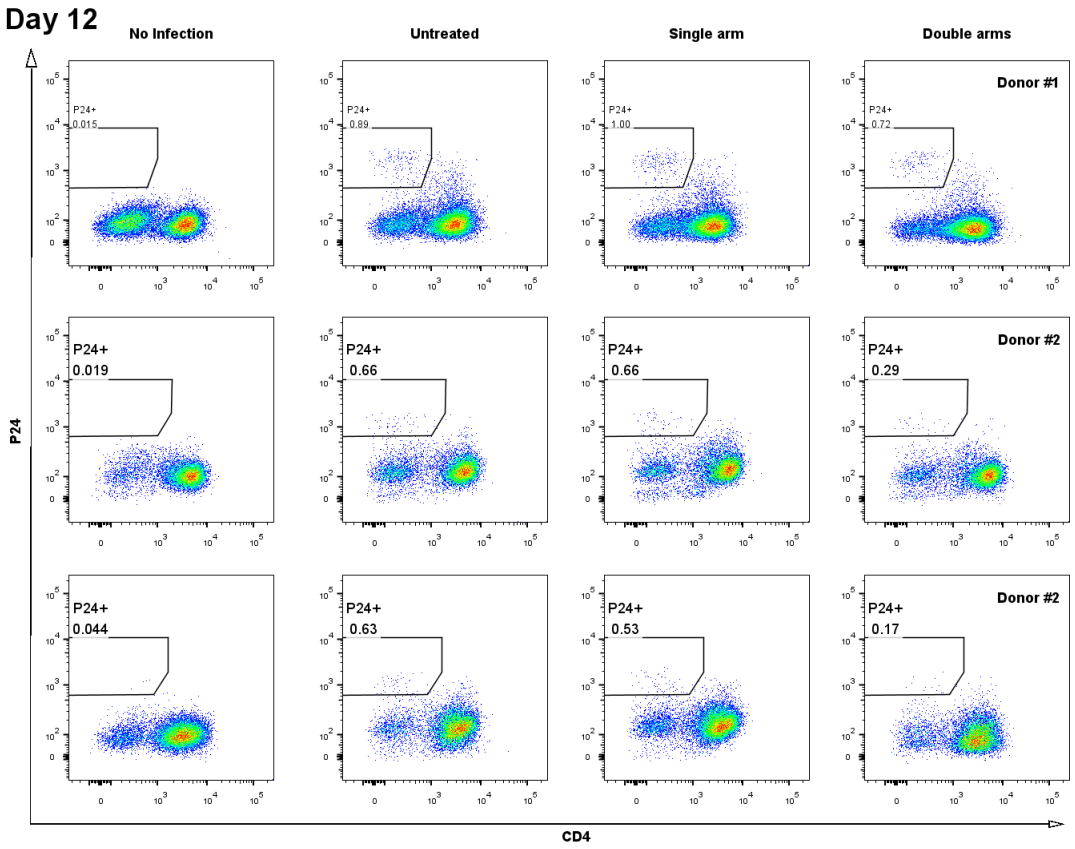

Figure 4.5.2. The p24 expression rate at day 12 of all samples. The double arms TALEN group still exhibited a stronger resistance than the other two groups.

Table 4.3. The p24+ rate tested on day 4 and day 14 post infection of HIV-BaL.

	%P24 day 4			%P24 day 12		
	Donor #1	Donor #2	Donor #3	Donor #1	Donor #2	Donor #3
Untreated	5.12	0.74	0.89	0.88	0.66	0.63
Single arm	6.38	1.65	0.66	0.99	0.66	0.53
Double arms	3.89	0.12	0.21	0.7	0.29	0.17

On day 4 and day 12, the double arms TALEN group showed a decline in the p24+ rate than that observed for the single arm TALEN group (51.44% and 46.78%, respectively; Figure 4.6), indicating an increased HIV-1 resistance from the cells treated with TALENs. Worth noticing was the cells from donor #1 had an exceptionally high infection rate. Since donor #1 had a much higher CCR5 expression than the other two donors, this might be the reason

for the high infection rate. On day 12, the infection rate of donor#1 was still higher but fell to a similar level to the other two donors. The difference in infection rate between the single arm group and the untreated group was much smaller than day 4 while the double arms TALEN group still exhibited an apparently lower infection rate.

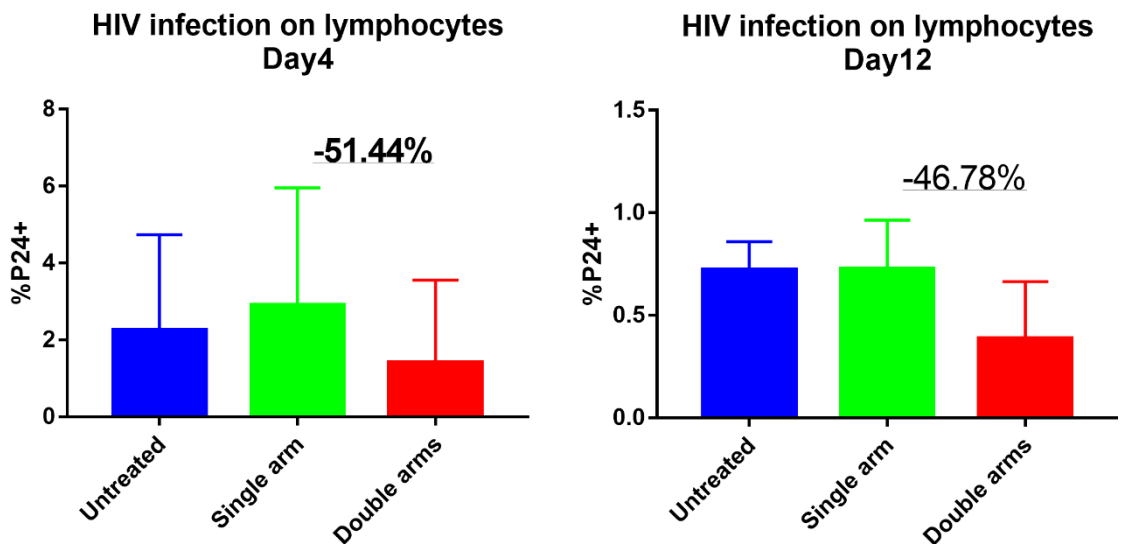

Figure 4.6 The comparison of %p24 in all three groups on day 4 and day 12 post-infection of HIV-BaL. The top of each bar indicates the mean value of HIV infection rate. The Error bar shows the mean value with standard deviation.

4.2.3 Next-generation sequencing to inspect the enrichment of edited CCR5 gene

The gDNA of the double arms TALEN group of every donor at day 0, day 4 and day 12 was extracted, and worked as the template for CCR5 gene PCR amplification. The amplicons of the CCR5 gene, which were designed 331bp long between primers and cover the TALEN cut site at the middle of it, were sent for NGS to see if there were enrichments of CCR5 editing through the duration of HIV-1 infection.

The NGS service provided every sequence read by Illumina technology in different formats. Among all the formats, there was a Microsoft Excel file showing all sequence readouts and the amount of each sequence readouts. The strategy used in this part of analysis was, the sequence between the two DNA recognition domains of TALEN arms (CAACATGCTGGTCATCCTCATCCTGATAAACTGCAA) was used as the reference sequence, the spreadsheet file of each sample was searched for every sequence that has this target sequence intact. All these readouts that have the intact reference sequence have been excluded from the calculation and all other readouts were regarded as the successful CCR5 editing. The number of all CCR5 editing was divided with the total readout number, the result was regarded as the CCR5 knockout ratio mediated by TALEN (Table 4.4). All three donors showed a continous enrichment of CCR5 editing through the process of HIV-1 infection.

Table 4.4. Percentages of the indel rate happened on the site of the FokI cleavage site of TALEN in every sample. All three donors had shown an increase of CCR5 disruption rate with time in the three time-points.

	Day 0	Day 4	Day12
Donor #1	12.85%	15.68%	19.49%
Donor #2	46.20%	63.50%	67.43%
Donor #3	26.64%	50.85%	62.32%

4.3 Discussion

As described in the introduction chapter, the CD4+ T cells are the most predominant target of HIV-1 infection and one major source of HIV-1 replication and spreading *in vivo*. Previous research using ZFNs to disrupt the CCR5 on CD4+ T lymphocytes and its effect against HIV-1 infection has been investigated *in vivo* (163). This study used the autologous T lymphocytes of HIV-1+ patients and transfected the cells with CCR5 targeting ZFNs, then transfused the cells back to the patients and interrupted the cART to test the safety and efficacy of this therapy. The result showed that this therapy was safe without significant adverse effects. However, classic viremia rebound and CD4+ T cell count decrease during the cART interruption were observed in all participants. But on the bright side of the result, most patients in the cohort showed a persistency of the CCR5 modified CD4+ T cells at a low level up to day 250 after the initiation of the therapy, indicating part of the modified cells can survival middle-termly. It is worth noticing that the reason for the lack of a significant therapeutic effect can be partly attributed to the low CCR5 disruption rate of the ZFN-cell product, with the highest rate among all 12 patients of just 27.7%. In that case, it leaves a question that if a higher percentage of CCR5 disruption in the cell product can bring some therapeutic effect.

It has been discussed in the introduction chapter that the provider of the TALEN constructs has used this TALEN design to knockout the CCR5 gene in primary CD4 cells but via delivery of the TALEN plasmids. The CCR5 disruption efficiency on the primary T cells was reported

at 13.1% from the cells transfected with the highest dose of TALEN-CCR5-515 plasmids, significantly lower than the rate in the ZFN research in the previous paragraph. Thus, it comes down to me to investigate if the mRNA form of TALEN from the same design will be able to achieve a better result.

Investigations on TALEN-mediated CCR5 disruption via mRNA delivery have been conducted by another group. Mock *et al* have designed their TALENs targeting another segment of the CCR5 gene sequence and conducted a series of work via mRNA delivery by electroporation(164). In their work, the CCR5 knockout rate has exceeded 50%, tested both by flow cytometry and digital droplet PCR. They further used modified HIV-1 strain with the *nef* gene substitute with eGFP gene or luciferase to see the change in HIV-1 resistance from the CCR5 modified cells. Not only did they find an increase of the resistance against HIV-1 entry at day 4 after infection, but also a near full suppression of the viral replication and enrichment of CCR5 knockout cells at day 12 after the initiation of infection. These data rise hope to the possibility of therapeutic effect once the CCR5 disruption reaches a significant level.

Other studies have also investigated CCR5 disruption using CRISPR/Cas9 against HIV-1 infection via different delivery methods, including electroporation and viral vector. The data from these works were consistent with previous studies by ZFNs and TALENs that the CCR5 disruption, once reaches a significant level, can increase the resistance of CD4+ cells to R5 strains of HIV-1(136, 165). Notably, some studies used CRISPR/Cas9 to knock both CCR5

and CXCR4 and confirmed resistance to a wide spectrum of HIV-1 strains, including the R5, X4 and R5X4 strains(166, 167).

With previous literature suggesting that the CCR5 gene disruption mediated with TALEN in the form of mRNA can be more efficient in knocking out the CCR5 gene and increase their resistance to HIV-1, it is to be answered that if the TALEN design in this work can achieve the same goal. Previous work has prepared the platform enough to carry the TALEN mRNAs to primary T cells. To evaluate the CCR5 disruption on CD4+ T cells and the effects it has against HIV-1 infection, two parts of the work have been done in this chapter similar to the work of Mock *et al*. The first part is electroporating T cells with both arms of TALEN mRNA and use the eGFP mRNA as a reporter to see if the mRNA of the TALENs used can disrupt the expression more efficiently than the TALENs plasmid. The data from all three different donors cells clearly showed a better efficiency of mRNAs than plasmids comparing to the plasmid works from Prof Linqi Zhang's team, the designer of this TALENs set. This CCR5 disruption was also validated on the genome level using the T7EI assay and Sanger sequencing, confirming the indels on the CCR5 gene sequence.

The second step of work was to evaluate the resistance to HIV-1 infection of the TALENs treated cells. Instead of using genetically modified HIV-1 strains, in this part of the work, the HIV-BaL strain, a laboratory-adapted but genetically unmodified strain was used. To mimic the natural infection of HIV-1 to the cells, it is better to use a genetically unmodified strain of HIV-1. However, it will increase the difficulty of testing the percentage of HIV-1-infected

cells. To test the percentage of HIV-1+ cells, an intracellular p24 staining is needed. The commonly used BD Cytofix/Cytoperm™ kit has failed the intracellular staining for p24, but fortunately, by using the protocol from Dr Hongbing Yang, the p24 staining was achieved. This protocol uses paraformaldehyde/50% methanol/Nonidet P-40 as the reagents for fixation and permeabilization of the cell membrane resulted in successful p24 staining on the HIV-1-infected cells. Furthermore, the gating strategy in Figure 4.5.1 and Figure 4.5.2 above showed only CD4-p24+ cells were gated, because of the CD4 downregulation effect from the *nef* gene. One function of the nef product is to rapidly downregulate the CD4 expression by triggering endocytosis of CD4 molecules and degrade them in the lysosome(168, 169), to prevent superinfection in case of the newly released HIV-1 virion binding to the CD4 molecule belonging to the cell that produces the virion(170). Since the HIV-BaL strain used to infect the cells are genetically unmodified virus and perform the full function of nef, only the CD4-p24+ population was gated which indicates that these cells are successfully infected, actively producing the new HIV-1 with all HIV-1 genes translated and every product doing its job.

The result of the second part of the work showed an elevated resistance to HIV-1 infection. The double arms TALENs treated cells of all three donors showed a decreased percentage of CD4-p24+ cells by flow cytometry, comparing to the single arm TALEN treated cells and untreated cells. Together with the first part of the work, it indicates that only treated with the intact structure of duo arms of TALEN can knock out the CCR5 gene and increase the HIV-1 resistance of these cells, not by a single arm of TALEN. Notably, donor #1 showed an

outstandingly low CCR5 disruption rate but a significantly higher susceptibility to HIV-1 infection. The reason for this incidence is not clear. Individual variation of genetic background can affect the susceptibility to HIV-1 infection, especially as this particular donor has a significantly higher CCR5 expression than the other two donors. Moreover, the other two donors showed a comparable HIV-1 infection rate compared to Mock *et al* who used a very similar infection assay protocol(164). This infection assay protocol is commonly used, but modifications like increasing the MOI value or treatment of polybrene can increase the infection rate. Moreover, the infection rate can be higher if tested earlier after infection, as replication and release of HIV can kill the cells, and cells become more resistant to the infection as the activation retrieves. In addition, the method and its strength in stimulating the T cells can impact the susceptibility of the cells(171)

Finally, in this part of work, prolonged duration of culture has been conducted to see if the CCR5 knocked out cells will enrich due to the death of the infected cells. The gDNA of each sample at three different time points was extracted and sent for NGS sequencing. The result sent back was further analyzed by adding up the indels that happened at the gap between the sequences recognized by each of the TALEN arms. Point mutation, insertion and deletion were all included. Among all three donors, the percentage of these kinds of mutation was indeed increased during the process, the most drastic increase happened between day 0 to day 4, indicating the acute infection has a stronger killing of the cells. From day 4 to day 12, the increase was not that drastic but still increased, suggesting that the infection has achieved a better balance between virion production and cells stability.

Chapter Five. CCR5 gene knockout on monocytes-derived macrophages and its effects on HIV-1 resistance

With the success of the CCR5 knockout on lymphocytes by TALEN mRNA and the consequential work that proved an increased resistance to HIV-1 infection, I looked forward to bringing TALEN to another linage of immune cells that is also the target of HIV-1, the mononuclear phagocytic system (MPS). Within the MPS, there are monocytes, macrophages and dendritic cells that are circulating in the blood. There is plenty of evidence that macrophages are an important reservoir of latent HIV-1 infection, so the goal of this work is to go through the same work on macrophages comparing to lymphocytes.

5.1 CCR5 gene knock-out mediated by TALEN mRNA on monocytes derived macrophages

5.1.1 Strategies to perform CCR5 genes knock-out

To acquire enough macrophages to conduct this section of work, the easiest way is to separate then differentiate the monocytes from blood into macrophages by cytokine stimulation. The differentiation process takes 7 days to complete, so the plan at the beginning was to electroporate the TALEN mRNA to the cells at day 3 and then let the

differentiation and gene knock out to be completed during the rest 4 days.

The first step was to detach the cells from the wall of the flask on day 3 of culture and use them to optimize the electroporation using GFP mRNA. Three settings have been tested, which were 300V250uF, 300V500uF, 300V950uF, respectively. All under exponential wave mode, in 4mm cuvette, 1ug mRNA/1*10^6 cells, 5*10^6 cells in 250uf OPTI-MEM medium as the buffer. After electroporation, the cells were immediately transferred to 5ml 37℃ R10 medium in a T25 flask, and then cultured overnight. Then the cells were examined under a fluorescence microscope and tested for GFP positive rate by flow cytometry. The result is shown in Figure 5.1, the average eGFP+ rate was 79.3% under the 300V250uF condition, 88.3% for the 300V500uF condition and 93.0% for the 300V950uF condition.

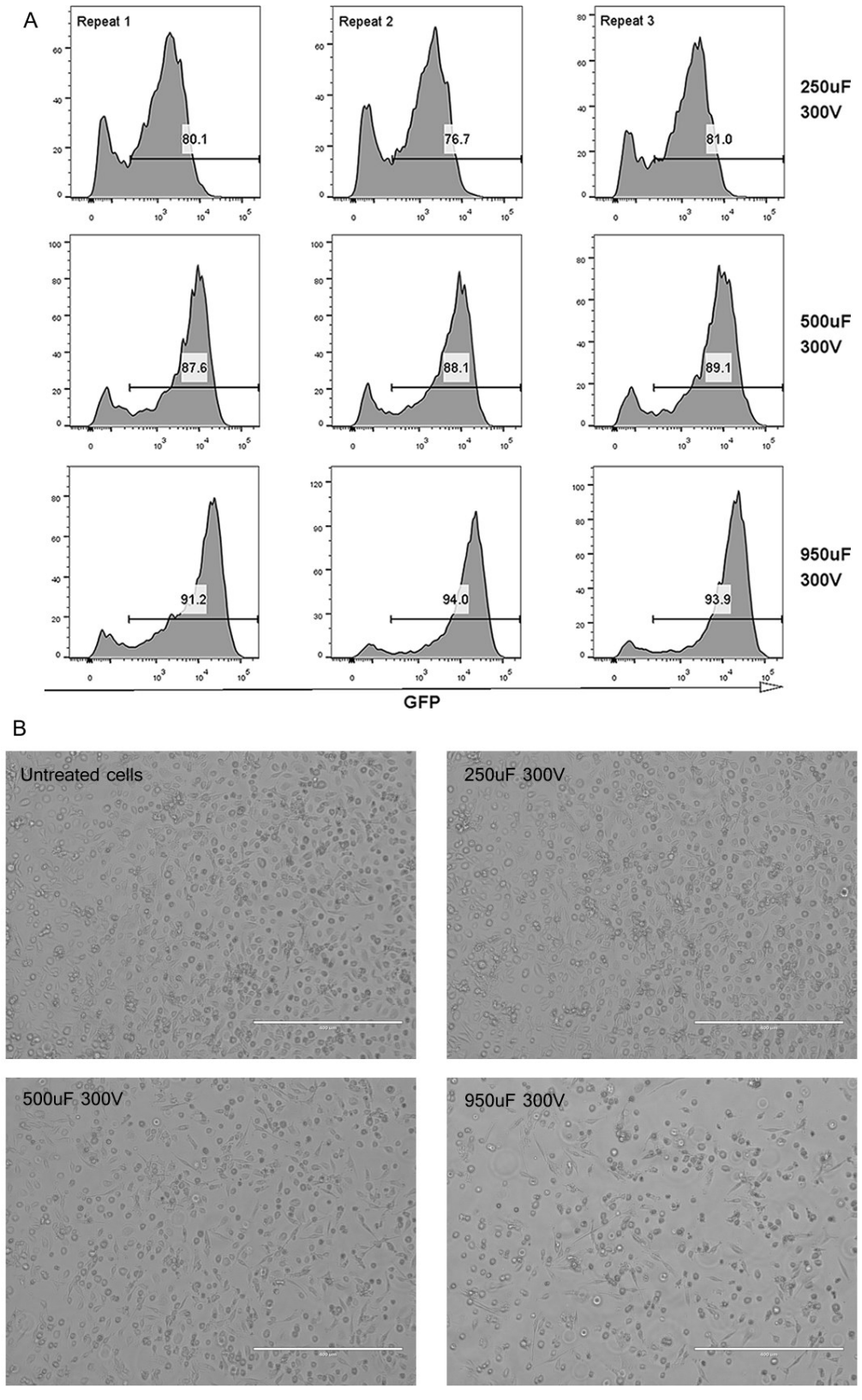

Figure 5.1 Result for electroporation optimization on monocytes cultured for 3 days. A) The result of GFP+ rate in the triplication of each parameter set after overnight culture. B) Cell confluency under a microscope after overnight culture. Each image was representative of its parameter condition.

The result showed that among all three settings, the 300V950uF resulted in excessive cell death that was unacceptable. Although the 300V500uf showed less cell confluency than the 300V250uF group, the decrease was acceptable and the GFP+ rate increased. Thus, the 300V500uf was taken as the set of parameters for the mRNA electroporation on monocytes after 3 days of cytokine stimulation.

With the electroporation parameters being settled, I moved to the CCR5 knockout stage. The cells were electroporated with 4ug of each arm of TALEN mRNA and 1ug GFP mRNA per $1x10^6$ cells. However, when I check with cells overnight, it appeared that there was hardly any cell in the right shape attached to the wall, in fact, it appeared to be only cell debris on the wall, and most cells floating (Figure 5.2). I left the cells to complete the three days of culture, then cell counting and flow cytometry only showed massive cell death. I tried again two more times, and the result all showed the same phenomenon.

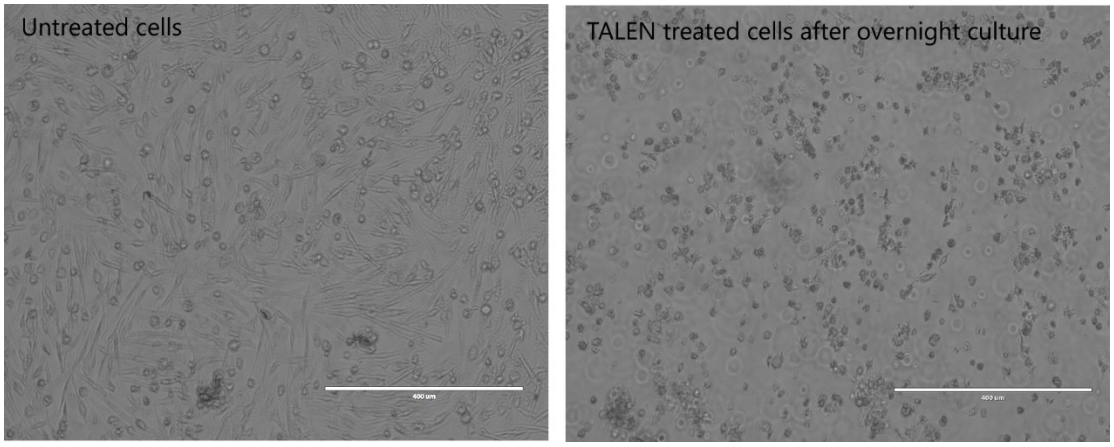

Figure 5.2. Images of overnight culture after transfecting TALEN to the monocytes from day 3 of culture. Almost all the cells attached to the flask wall were in the shape of cell debris, and most cells were floating, indicating the TALEN treatment caused excessive cell death.

To understand the reason for this, I did a literature review and found previous literature indicated that monocytes do not express the enzymes that repair the DNA damage, thus with TALEN cutting the DNA chain, it will leave significant genome damage that will not be repaired in time, which leads to apoptosis(172). Literature also indicated that the enzymes needed will resume being expressed when the monocytes have completed the differentiation into macrophages. Learning this mechanism, I had to change the protocol of this work, finish the whole differentiation period of 7 days, then treat the cells with TALEN.

To confirm a good set of parameters for the macrophages harvested at day 7 of cytokine-stimulation culture, I tested the 300V250uf and 300V500uf conditions, each set has been triplicated. The result indicated that the 300V500uf setting worked equally well on the macrophages, then I moved to TALEN work, using the same condition and dose of mRNA as in the monocytes work. However, overnight after the delivery of the TALEN and GFP mRNA to the 7 days cytokine stimulation differentiated macrophages, the same problem recurred. Almost none of the correctly shaped cells was attached to the wall of the flask, most attached structures seemed to be cell debris. At this stage, I assumed that this result showed that 7 days of differentiation is not enough for the DNA repair enzymes to be adequately expressed. To test this theory, I decided to prolong the culture duration to see if a longer time would make it work. However, the next batch of macrophages that went through 14 days of cytokine stimulation differentiation still showed the same result.

While the result showed no hope to perform the CCR5 gene knockout using the

conventional protocol to generate MDMs, the cells that have not been used but still under culture were left there in the incubator and the medium was not changed on time. After about 6 days without changing the medium, I found the medium was still not in the colour of yellow, which usually happens on day 3 after every change of medium. I assumed the withdrawn of cytokine has put the cell into a resting phase, although with the limitation of skills and equipment, I cannot confirm this assumption. Nevertheless, I decided to try for one last time to deliver the TALEN mRNA to this batch of macrophages, cryopreserve the rest of the cells. Much to my surprise, overnight after the electroporation, lots of correctly shaped cells were attached to the wall of the flask. This set of work was done without a control group, so I decided to alter the original protocol and repeat experiments.

5.1.2 Flow cytometry evaluation of CCR5 expression after TALEN treatment, T7EI assay and NGS to confirm CCR5 gene disruption

The new protocol was to first acquire monocytes from the blood cone and do the same cytokine stimulation differentiation for 7 days, then withdraw the cytokine to keep the cells cultured in R10 medium for another 6 days. The medium was changed every 3 days with and then without cytokine accordingly. After this step, the cells would be harvested and electroporated with TALEN and GFP mRNAs with the same dose as mentioned. Since the lymphocytes work already showed that only a single arm of TALEN is not capable of knocking out the CCR5 gene, thus an untreated group of macrophages will be used as the control. After electroporation, the two groups of cells would be cultured in R10 without

cytokine for 3 days then tested by flow cytometry to assess the expression of CCR5 (Figure 5.3). This work was triplicated.

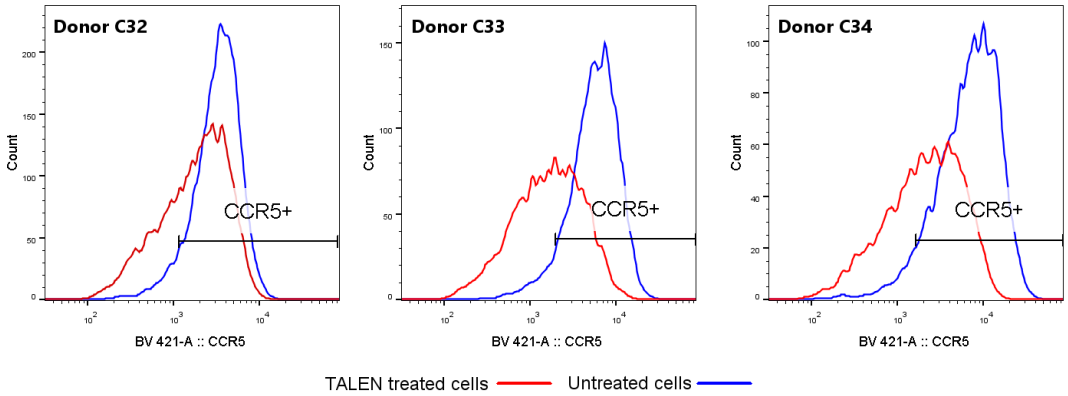

Figure 5.3. The CCR5 expression on the TALEN double arms treated cells and untreated cells among three different donors 3 days after electroporation.

As the results showed in Figure 5.3, each sample has shown a downregulation of the CCR5 molecule on the cell surface of the GFP+ macrophages. The knockoff rate was 26.2%, 51.9% and 34.7%, respectively. Then the genome DNA was extracted from each sample for PCR amplification of the CCR5 gene using primers modified for NGS as described for the lymphocyte work, then used for T7EI assay and NGS to confirm CCR5 disruption at the genomic level (Figure 5.4).

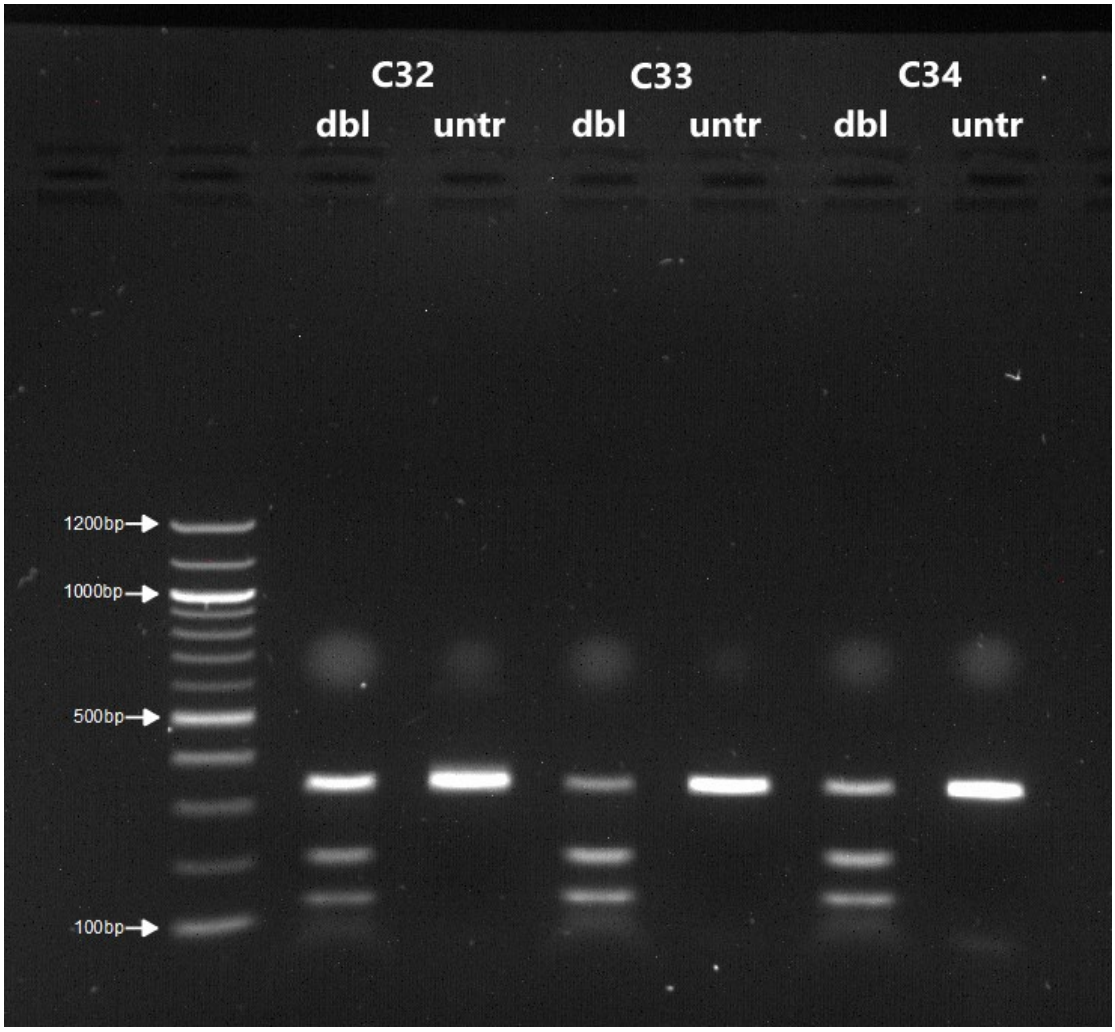

Figure 5.4. T7EI assay showing the successful introduction of indels on the CCR5 gene of every TALEN treated sample.

After the T7 endonuclease treatment, all TALEN treated samples showed the T7 endonuclease induced breakage, which resulted in three bars on the gel image, whereas there was one bar in each of the control samples, and the top bars of all six samples were at the sample site (Figure 5.4). As described in the lymphocytes work, this showed that in each sample of the TALEN treated group, at least one indel has occurred on the amplified DNA sequence. The rest of the CCR5 amplicon of each TALEN treated sample has been sent to the NGS service, the result showed various indels occurred on the CCR5 gene, at the site of the TALEN recognition area. The number of sequencing reads for each sample was 124377,

83413, 99902. The indel rate onsite of the FokI dimerizing zone for the three donors were 64.9%, 71.3% and 59.7%, respectively.

5.2 HIV-1 infection assay on TALEN treated macrophages.

As shown above, the TALEN mRNA can also disrupt the CCR5 on MDMs, then the question remains that is this disruption capable of enhancing resistance to HIV-1 infection. To investigate this issue, the plan was to perform the same process on the MDMs as described for the lymphocytes work.

As in this part of work, MDMs from any single donor were again divided into three groups, the undertreated group, the single TALEN arm group and the double TALEN arms group, to be in accordance with the lymphocytes work and to set appropriate controls. Three different blood cones were used in this part. So firstly, cells of three donors were used in this work in the same cycle of the experiment.

On day 3 of TALEN transfection, in all the three donors, the CCR5 expression of the single arm TALEN and the untreated group of TALEN showed no significant difference, whereas the double arms groups showed significant downregulation of CCR5 to the other two control groups (double arms group: 27.1%, 22.6% and 53.9%; single arm groups: 68.2%, 59.1% and 84.5%; untreated group: 64.4%, 61% and 81.1%, in respective, Figure 5.5, Table 5.1).

A small aliquot from the untreated group was taken and kept on culturing without infection. An equal number of cells ($1X10^6$) was taken from each sample and seeded to a 6-well plate and cultured overnight to allow full attachment to the bottom of each well. On the next day, these samples were infected with the HIV-ADA strain of HIV-1, then cultured for three days before intracellular staining for p24. The result of the p24 flow cytometry showed a very low infection rate, but still demonstrated a decrease of p24 percentage in all three double arms group compared with the other two control groups. (donor #1, #2 and #3, double arms group: 0.16%, 0.18%, 0.13%; single arm groups: 0.37%, 0.33%, 0.2%; untreated group: 0.35%, 0.29%, 0.22%, in respective, Figure 5.5, Table 5.1).

However, since the infection rate is too low to demonstrate a clear trend of elevated resistance to HIV-1, it is crucial to perform this on more donors to accumulate enough data for statistical analysis. Thus, two more sets of experiments were performed to have 5 sets of data for analysis. Three days after TALEN treatment on donors #4 and #5, the CCR5 expression rate in double arms group was 68.9% and 69.5%; single arm groups: 90.7% and 90.4%; untreated group: 90.5% and 91.5%, respectively (Figure 5.5, Table 5.1), after HIV-ADA infection, the p24 positive rate was 0.049% and 0.026% in double arms group, single arm groups: 0.093% and 0.088%; untreated group: 0.1% and 0.11%, respectively (Figure 5.5, Table 5.1). The infection efficacy was even lower on donors #4 and #5, but fortunately, the data of all five donors together in each group has passed the normal distribution test and thus can be analyzed by t-test (KS normality test, double arms group: p= 0.2148; single arm groups: p= 0.2504; untreated group: 0.2288. p>0.1 means the data set fits normal distribution).

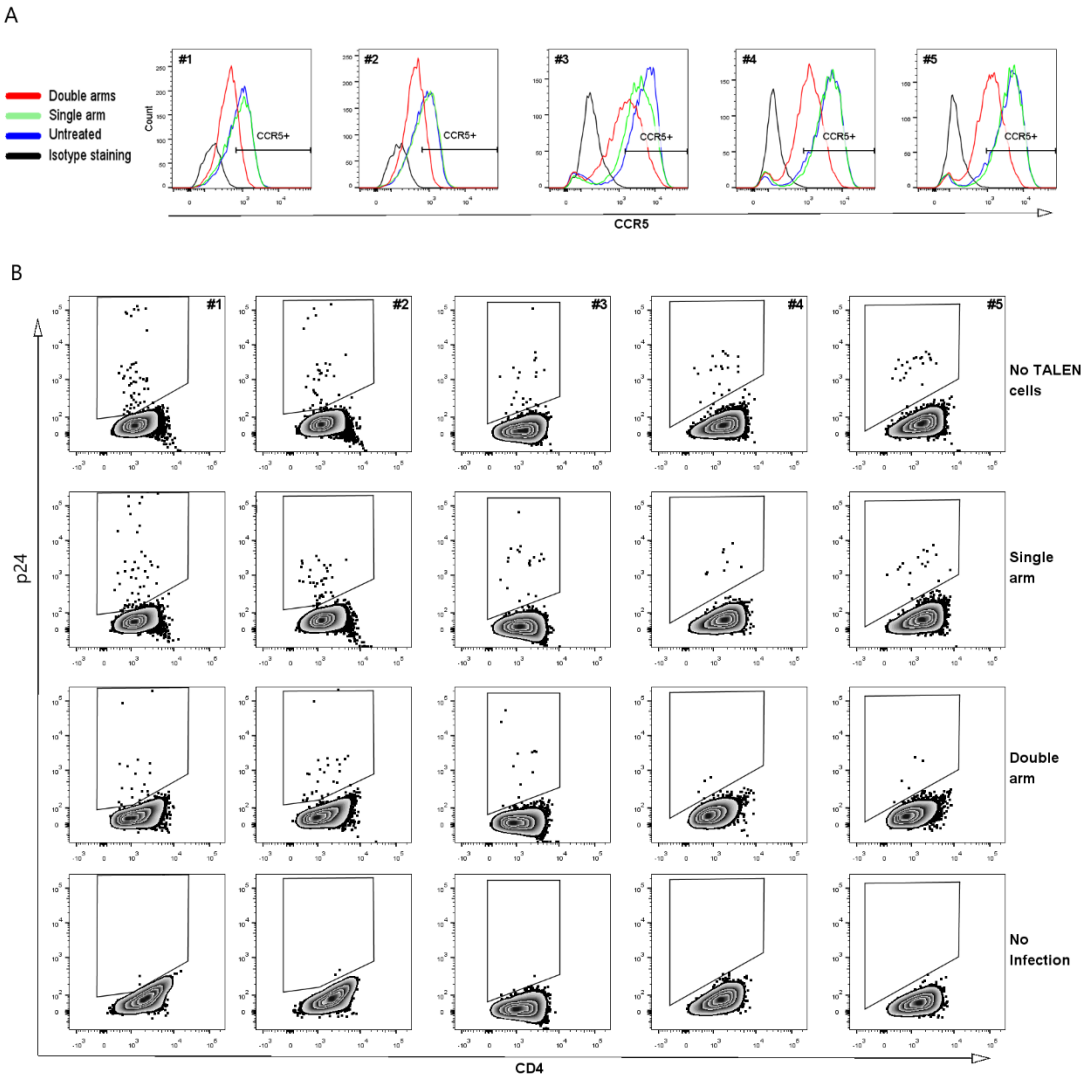

Figure 5.5. Result of the HIV-1 infection assay on macrophages. A) On the day of the HIV-1 infection assay, the CCR5 downregulation mediated by TALEN on all five different donors, three days after treatment. B) Three days after HIV-1 infection, the samples were intracellularly stained with p24 antibodies for flow cytometry, in order to compare the HIV-1 infection rate among different groups.

Table 5.1. List of the CCR5 and p24 expression of every sample from 5 different donors. 3 days after the TALEN treatment, all the samples had the CCR5 expression examined by flow cytometry. An aliquot of the untreated group was taken to culture without infection to serve as the negative control for the HIV-1 infection assay. Three days after HIV-1 infection, each sample was intracellularly stained with p24 antibody and went through flow cytometry to examine the p24 expression.

	%CCR5 day3 of TALEN treatment			**%p24 3 days after infection**		
	Double arms	Single arm	Untreated	Double arms	Single arm	No TALEN
Donor #1	27.1	68.2	64.4	0.16	0.37	0.35
Donor #2	22.6	59.1	61	0.18	0.33	0.29
Donor #3	53.9	84.5	81.1	0.13	0.2	0.22
Donor #4	68.9	90.7	90.5	0.049	0.093	0.1
Donor #5	69.5	90.4	91.5	0.026	0.088	0.11

After the paired t-test to compare the p24 expression, there was no statistical significance between the untreated group and the single arm group ($p=0.8641$), and the double arms group showed a statistically significant decrease of p24 level, comparing to the single arm group and untreated group ($p=0.02715$ and $p=0.0107$, respectively, Figure 5.6).

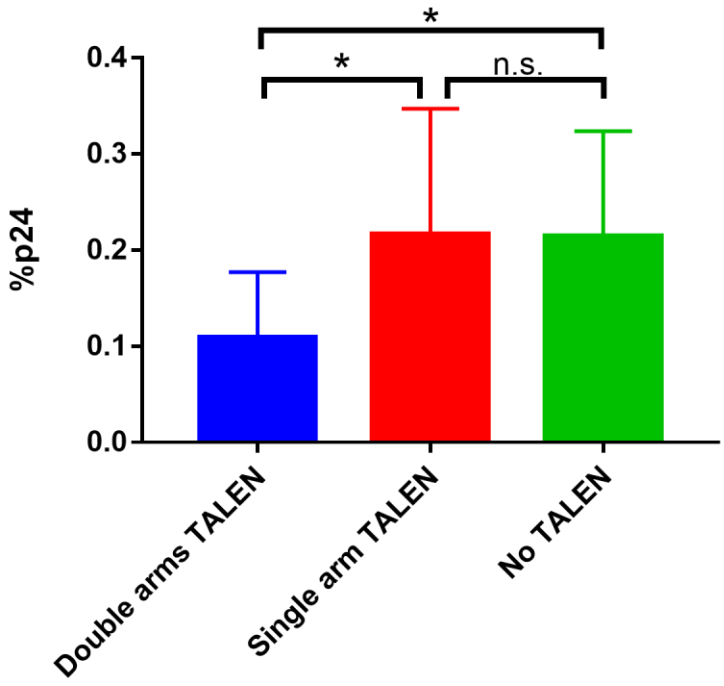

Figure 5.6 Statistical analysis of the HIV-1 infection rate between double arms TALEN treatment, single arm treatment and untreated cells from 5 different donors.

5.3 Discussion

Macrophage, abbreviated as Mφ, is a part of the mononuclear phagocyte system. The function of macrophages is to engulf and digest anything that macrophages are big enough to encapsulate when the object displays on its surface evidence that indicates this object is not a part of healthy tissue. This function makes the macrophages a very important category of immunity against cancer cells, microbes, cellular debris, foreign substances, etc. Macrophages can be found in almost all kinds of human tissues, the morphology and phenotype of it can vary depending on the tissue that the cells reside, thus are named histiocytes, Kupffer cells, alveolar macrophages, microglia, etc. Besides the phagocytic function, macrophages can also process the object it engulfs and deliver immunogenic antigen information to other effector immune cells, thus, serves as a kind of antigen-presenting cells.

Macrophages have two origins, they can either be differentiated from circulating monocytes, which serve as the main source of Mφs(173), or established before birth and sustained independent from circulating monocytes(174, 175). When a site in human tissue is injured, infected, or invaded by a non-infective but foreign substance, functionally tissue cells can produce and release stimuli as a part of inflammation reaction which leads to the secretion of some of the same cytokines by macrophages already on-site. These stimuli, particularly the chemokine C-C motif ligand 2 (CCL2), works as an attractant of monocytes to migrate to these sites, but also stimulates the differentiation, maturation of monocytes and macrophages(176). This effect is known as chemotaxis. During chemotaxis, the monocytes

migrate through layers of tissue by changing their morphology to squeeze through. During this process, the monocytes differentiate into macrophages and perform immune activities. In this way, tissue macrophages are replenished and participate in the anti-inflammation or tissue repair. Notably, macrophages are long-living cells, up to several months or even more.

Macrophages express CD4, CCR5 and CXCR4 which makes them susceptible to HIV-1 infection. Previous studies have shown that most X4 strains of HIV-1-1 cannot establish a productive infection in macrophages, thus Mφs are still considered a host for R5 strains(177). However, the biological patterns of HIV-1 infection in macrophages are quite different comparing to CD4+ lymphocytes. The major subset of CD4+ lymphocytes that are highly susceptible to HIV-1 infection is the activated T helper cells, they will be rapidly killed after infection and one round of new virion production that overload their hosting capacity(178). On contrary, the susceptibility of macrophages varies depending on the subtypes, but they are overall still far more resistant to HIV-1 infection comparing to the lymphocytes(177). After infection, the viral production in macrophages is increased in a linear manner, unlike in the lymphocytes which is robust and exponential and kills the cells within a week(179). Plus, because of the relatively mild production of HIV-1 virion and the long-living nature of macrophages, it constitutes a major source as a long-term active production of HIV-1 particles.

Another feature of macrophages in the spread of HIV-1 infection is its cell-to-cell

transmission, which is more effective to the virus-to-cell route(180). The newly produced virion in the macrophage can be accumulated in the surface-connected vesicular compartment during cycles of viral replication(181, 182), and the infective virion can be stored there long-termly(183), Although the same pattern can be found in T cells, the frequency of occurrence for cell-to-cell transmission from lymphocytes is significantly lower than macrophages and mainly causes non-productive infection and cell death(184). This pattern in lymphocyte cell-to-cell transmission leads to the question that if the transmission in the same route starting from macrophage and dendritic cells contributes the most to the spread of infection.

The reason for the lower susceptibility of the MDMs to HIV infection can be attributed to the sterile alpha motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1). SAMHD1 is a phosphohydrolase that regulates the pool size of intracellular dNTP, which depletes the pool of dNTPs available for the retro-transcription to produce HIV cDNA(185). SAMHD1 is expressed in monocytes, MDMs, DCs and resting T-cells, which accounts for the low susceptibility to HIV of these cell types(186, 187), because SAMHD1 deficient CD14+ cells have an enhanced HIV infection(188). SAMHD1 can be blocked by the vpx protein of SIV and HIV-2, but HIV-1 does not express vpx. Previous literature has indicated transfecting the monocytic linage of cells with SIV/HIV-2 vpx can greatly enhance the susceptibility to HIV-1(189). Unfortunately, I was not able to acquire the vpx DNA template for producing the vpx mRNA, moreover, to mimic the natural infection, I have not used SAMHD1 blockage in this work to enhance HIV infection to the MDMs.

As described above, the monocytes/macrophages have the ability to migrate to other compartments and reside there long-termly. However, given the long-lived nature and stable production of the virus of macrophages, the Mφs by themselves can be a courier of viral spread. For example, macrophages can infiltrate the blood-brain-barrier to enter the central nervous system where T cells are rarely presented. After residing there, by the cell-free and cell-to-cell transmission of the virus, HIV-1 can thus infect target cells in the central nervous system such as microglia and astrocytes(190). For other compartments that have easier access, the lymphoid tissue is also of importance. The lymphoid tissues are widely existing across the body and work as a major defense against infections. T cells are heavily accumulated there and so do macrophages. The HIV-1 provirus DNA has been detected in cART treated and untreated patients from nearly all lymphoid tissues(191-194). Moreover, the infected macrophages are more resistant to the attack from the cytotoxic lymphocytes, making them a major source of persistent viremia without cART and major latency site due to their long half-life under cART(195). With all these features, the monocytes/macrophages system plays a significant role in the spread of HIV-1 through the body and serves as a major reservoir of HIV-1.

Based on the mechanism of TALEN as a gene-editing tool, and given the result from my works along with previous literature on lymphocytes, TALEN is supposed to be able to knock out the CCR5 gene as in the lymphocytes. However, many predicaments have been encountered in the beginning in the early stage of this part of the work. The transfection of TALEN mRNAs to the monocytes differentiating to macrophages at day 3 of culture led to

massive cell death. This result corresponds to previous literature that monocytes lack the expression of key enzymes in repairing the damage of the genome(172, 196). These previous studies have indicated that the expressions of key proteins, such as XRCC1, ligase IIIα, poly(ADP-ribose) polymerase-1, and catalytic subunit of DNA-dependent protein kinase (DNA-PKcs), was absent in monocytes. These studies also indicated that during the maturation of monocytes into macrophages and dendritic cells, the expression of DNA repair enzymes will be upregulated and restored. However, the TALEN transfection at day 7 and even day 14 of culture still led to massive cell death. Fortunately, chance led me to try to treat macrophages that have been cultured without cytokine stimulus after the conventional 7 days maturation protocol, which surprisingly resulted in successful CCR5 disruption.

The reason for this is not clear. As described in the result section, the first batches of macrophages that had a successful CCR5 disruption were transfected with TALEN at day 14 of culture. The first variation that came to my mind was the second half duration of culture has the cytokines withdrawn. Given that 14 days of culture with the cytokines still led to massive cell death, it first made me think if the DNA repair function is still not restored, but this theory is directly contradicting previous studies mentioned above. To further challenge my hypothesis, very few recent studies have shown that CRISPR/Cas9 system can achieve a successful knockout of certain genes in macrophage or even freshly isolated monocytes(197, 198). These studies indicate that the failure in some of my protocols to knock out CCR5 might not root in the DNA repair process, since using CRISPR/Cas9 also needs to introduce

DSBs and induce the NHEJ function to conduct an unprecise repair of the DNA sequence, in order to disrupt the correct sequence of a gene. Notably, in both CRISPR/Cas9 studies cited, a Cas9 ribonucleoprotein mediated gene knock was performed, instead of introducing the mRNA of CRISPR/Cas9 to the cell, which leads to the next hypothesis that the cytokine stimulation related cellular activity played a part in this phenomenon. Many possible mechanisms can be the cause of this hypothesis: 1. The introduction of nucleotide unmodified mRNA can be immunogenic to the cells(199), but the cells transduced with eGFP mRNA survived well upon electroporation optimization, indicating that transfection with mRNA was not very likely to be the cause; and 2. Intracellular activities against TALEN proteins and/or get acerbated by TALEN protein. The previous two successful studies to knockout other genes in macrophages were conducted through transfecting the cells with Cas9 ribonucleoprotein (RNP) and did not result in acceptable cell death, together with my eGFP mRNA transfection, suggesting that foreign RNA and foreign protein from a different species and the subsequential DNA breakage might not, in general, be a problem for the macrophages to tolerate, and the TALEN's protein specifically might be the problem. There might be some innate immunity intra-macrophage against TALEN protein that induces apoptosis or the TALEN protein interacts with some biological process inside of the macrophages that induce cell death when the cells are still being stimulated by cytokines. Despite the assumption of causes for this phenomenon, the time limit of my PhD and my expertise prevented me to look further into this issue. At the same time, the absence of any study about TALEN mediated gene knockout on macrophage is an obstacle to verifying if it was just a problem that happened to me or the same to other researchers as well.

Nevertheless, I have built a successful protocol for the TALEN mRNA to disrupt the CCR5 gene in macrophages.

It is also worth noticing, the mutation rate onsite was significantly higher than the rate in the lymphocytes from the NGS data, with the lowest in macrophages (59.7%) still higher than the highest in lymphocytes (46.2%), this data suggests that it is possible that TALEN mediated CCR5 disruption is more efficient in macrophages conducted by this new protocol than in lymphocytes conducted by the protocol that was used in this work and the works from previous literature. Since the concentration of mRNA and cell density in the electroporation suspension were both the same between macrophages and lymphocytes electroporation, then the electroporation nature including the pulse wave mode might have an impact on the overall efficiency regarding the amount of mRNA that can be transduced into the cell. In addition, macrophages are non-dividing cells, especially withdrawn from cytokine stimulations for days, whereas the lymphocytes on the day of electroporation were at the dividing phase. It was reported that the DNA repair mechanism that the cell uses is cell cycle-dependent, the HDR is restricted to late S to G2 phases while NHEJ operates throughout most of the cell cycle(200, 201). This might contribute to the efficiency of gene disruption because the lymphocytes at day 3 post-activation stepped into the cell dividing phase, HDR can be utilized to precisely repair the gene, whereas the non-dividing macrophages can only use the NHEJ mechanism that usually leads to mutation.

Further investigation first confirmed that the macrophages are indeed resistant to HIV-1

infection as previous studies reported, and resulted in a significantly lower HIV-1 infection rate (highest in Mφ 0.37% vs lowest in lymphocytes 0.66%). However, the disruption of CCR5 still confirmed the resistance to HIV-1 infection, resulting in a statistically significant decrease in the HIV-1 infection rate. All the data from my work confirms that the TALEN technique is capable of knocking out the CCR5 gene in not only lymphocytes but also macrophages. The disruption of the CCR5 gene is capable to increase the resistance to HIV-1 infection of macrophages.

6. General Discussion

During the four decades since the discovery of the disease, tens of million people have died from AIDS. Until the end of 2020, it was estimated about 37.7 million people living with HIV-1 in the world. 680000 people died of AIDS-related diseases in 2020, and 1.5 million new cases were diagnosed in the same year(202). During the 40 years, numerous endeavors have been put into the understanding, management and curing this infectious disease. The finding of all the research not only helped us better understand this specific virus, but also expanded our understanding of viral immunology, viral genetics, human immunology activities, pharmaceutical mechanisms, and epidemiology models.

The research has led to successful controlling the disease by developing medications targeting different components crucial to the life cycle of HIV-1. Easier testing methods were also developed to allow faster and more accurate detection of the infection. The reports starting from 2014 have also confirmed that under successful cART with a fully suppressed viremia, HIV-1 can't be transmitted through the sexual route(203-205). With all the achievements in disease diagnosis, management and prevention, the UNAIDS has advocated the 90-90-90 initiative with the goal of eliminating HIV-1/AIDS, which is by 2020, 90% of all people living with HIV-1 will know their HIV-1 status, 90% of all people with diagnosed HIV-1 infection will receive sustained antiretroviral therapy, and 90% of all people receiving antiretroviral therapy will have viral suppression.

As described in the introduction chapter, the cART requires life-long adhesion, which means

taking the medication on time as instructed by the caring physician and it is usually daily. The financial burden of medication can be a problem for the universal health care system or the patients. Poor adhesion to the medication, especially taking the pills not on time can result in insufficient blood drug concentration which will rapidly cause the rise of drug-resistant viral strains (206). Although it can be salvaged by changing to medication based on different mechanisms, it is still possible to run out of options, or even worse, get contracted at the beginning with pan-drug-resistant strain. Thus, the eradication of HIV-1 infection is still needed and under constant investigation.

Based on the understanding of the virology of HIV-1, there are many targets of HIV-1 that can be the target of a strategy. Most of them fall into three categories: immunotherapy, gene engineering, and shock-and-kill. In the immunotherapy way, humoral immunity and cellular immunity are both pathways. The broad-spectrum antibody of HIV-1 can work as a prevention but also has therapeutic potentials(207). The cellular therapy uses re-programmed killer immune cells to specifically target and attack the HIV-1-infected cells. However, this would require a reversal of the HIV-1 latency for every infected cell to express HIV-1-specific antigens(208). The reversal of latency is not the core only for cellular immunotherapy, but also for the shock-and-kill strategy. The shock-and-kill strategy is based on using the HIV-1 latency reversal agents to fully induce the replication of all latent HIV-1, keep the patient on cART and let the already established HIV-1-specific immunity kill all the HIV-1 reservoirs while preventing consequential new infection to cells. However, the results till now are promising yet not satisfying. For example, the CTLs are found to not

necessarily have the ability to fully kill the latency reversed cells, meanwhile, have a pro-latency effect, thus need to be addressed for a better therapeutic effect of the shock-and-kill strategy(209-211).

The development of gene engineering technologies and gene therapy opened the window for genetically interfering with HIV-1 targeting different parts of its gene or life cycle. Furthermore, it facilitates the production of re-programmed killer cells with artificial components, for example, the chimeric antigen receptor T cells, that can be acquired in bulk as an effector tool and has far better efficiency than the traditional antigen co-cultured CTLs. Among the strategies based on gene engineering, the gene-editing tools have a promising potential on many members of the HIV-1 target cells. For example, certain parts of the integrated HIV-1 provirus DNA can be targeted and knockout from latently infected cells, resulting in dysfunctional provirus(212, 213). But to do this *in vivo*, many obstacles remain. Delivering the gene-editing complex to every cell that has the productive provirus is fundamentally challenging, the toxicity and accuracy of gene editing are also to be minded.

However, the “Berlin Patient”, “London Patient” and very likely the “Düsseldorf Patient” are the only cases of eradication of HIV-1 infection, in another word, CCR5-Δ32 HSCT is the only HIV-1 eradication strategy that has been proved a success and repeatable. Attention and resources have since been directed to investigate the interaction of CCR5 and HIV-1, with the aim of slowing the progress of HIV-1 infection and a cure. Due to the very low natural occurrence of homozygous CCR5-Δ32, the artificial disruption of CCR5 would

greatly expand the availability of CCR5 silencing therapy. Moreover, the bone marrow/hematopoietic cell transplantation would require myeloablation, that the peri-operation risk is very high and can cause death, let alone the GVHD that is from engraftment of allogenic HSCs which can be lethal. For the two reasons, the autologous cells with disrupted CCR5 would overcome the obstacles. Previous studies have studied CCR5 disruption on lymphocytes mediated by different gene-editing tools, with the effector complex in different forms, delivered by different methods. Clinical trials have been conducted with one trial already concluded and published(163). This particular trial using ZFNs as the gene-editing tool as described in the introduction section, did not achieve control of the HIV-1 viremia, due to the low CCR5 knockout efficiency over all of the cellular therapy products. Despite that, this trial has proved the safety of using ZFNs modified lymphocytes which gives hope to bring more artificial CCR5 disruptions cellular therapy products to clinical trials.

In my work, it started with building the platform in my laboratory for mRNA production, transfection by electroporation, and all the related protocols regarding the generation of CCR5 knocked out cells, testing system and further infection assay and its evaluation assay. During my PhD, the expertise of IVT mRNA and modification of the component has been built up and polished, lead to stable production of mRNA with a high level of translation efficiency under optimal electroporation. The eGFP mRNA produced by my final protocol has a potency that is very close to commercially available ones from companies that are highly regarded in the business, which laid the foundation for further investigation on the

CCR5 disruption through transfecting cells with TALEN's mRNA. The work on lymphocytes proves that the TALEN design provided by our collaborator is able to perform the CCR5 gene in its mRNA form, more importantly, it is more efficient in performing the function than its plasmid form counterpart. Further HIV-1 infection assay confirmed that with the TALEN treatment, the lymphocytes exhibit a stronger resistance to infection of genetically unmodified HIV-1 strains than the wild types.

Before my work, CCR5 gene disruption mediated by TALEN, in the form of mRNA and delivered by electroporation to lymphocytes has indeed been done by another group, using their own design of TALENs(164). Until this step, the novelty in this part of work is limited to verifying the function of the TALEN design in my work. But my further work on macrophages has stepped into a field that lacks previous literature. Serving as a major carrier of HIV-1 infection and HIV-1 reservoir, the macrophage is also a target of significant importance. The lack of report on CCR5 disruption can be due to the difficulties of genetically modifying the myelocyte linage, as the monocytes indeed display a higher sensitivity to gene damage. Although evidence has shown that the DNA repair function will be restored once the monocytes mature into macrophages and dendritic cells. This maturation process can be achieved using cytokine stimulation, but my data showed that the macrophages harvested when they are still exposed to the stimulation are still highly sensitive to the TALEN mediated gene editing. Fortunately, my further work has indicated that withdrawing the stimulation for a period of time is about to significantly decrease the sensitivity of the macrophages to TALENs and achieved in a very efficient CCR5 disruption.

Again, these TALEN-treated monocytes-derived macrophages exhibited a stronger resistance to their wild-type counterpart, confirming TALEN is also capable of disrupting the CCR5 gene in MDMs, resulting in elevated resistance to HIV-1 infection. There are some limitations of this protocol. Firstly, it is very time-consuming, the whole process of CCR5 knockout takes almost 16 days from monocyte isolation to completion of editing, Secondly, the mechanism of cytokine withdraws to result in successful gene editing by TALEN needs to be studied. It will not only be understanding the biological change of the macrophages after cytokine withdraws, but also the nature of the high sensitivity to TALEN while being stimulated by cytokines. To fully understand the mechanism might provide theories on modifying TALENs structure or design to shorten the time cost of the work.

CCR5 disruption on lymphocytes and macrophages is not the end of the investigation. There are still many limitations in this study. Firstly, the general efficiency in the CCR5 disruption rate. It is theoretically possible to knock out the CCR5 level to a certain level, so that the amount of cells without CCR5 is enough to main the immune function in vivo. However, due to that the reservoir of HIV contains some long-living cell type, to achieve the final eradication of HIV would cost decades. Meanwhile, the virus itself can mutate to X4 or dual tropism, which makes CCR5 disruption useless. Secondly, there are only two cell types investigated in this study, which cannot cover all target cell types. Moreover, the CD4 lymphocytes indeed have the ability to differentiate to memory T cells thus a part of them can remain long-termly existing and possibly replenish new cells. Whereas the MDMs as a terminally differentiated cell type, the CCR5 disrupted MDMs will inevitably extinct after a

period. Moreover, previous studies have indicated that many tissue residue subtype of macrophages are in fact, raised from embryonic development, phenotypically different from MDMs, behaves differently than MDMs and are independent of monocyte replenishment(174, 214). Thus, the study on MDMs is not enough to cover the tissue residue Mφs.

Along with the insufficient coverage of immune cell types, this study indicates only the *in vitro* effects. To achieve the goal of HIV cure, eventually, an *in vivo* therapy has to be developed. The short-lasting nature of T cells indicates that the CCR5 disrupted cell product needs to be administrated for rounds to see the maximum effect. When focusing on the T cells, it would be more convenient to develop an *in vivo* platform to disrupt the CCR5 gene. To this point, only viral vectors can be used to achieve massive transfection *in vivo.* However, the DNA delivery or genome integration nature of viral vector delivery would severally prolong the cellular exposure to TALEN or any other gene-editing complex, resulting in increased off-target effect as mentioned in the introduction chapter. Moreover, the majority of T cells in circulation are quiescent and none dividing, which makes them resistant to gene-editing(215).

To mimic the “Berlin” and “London Patient” and develop a therapy that can cover all sorts of immune cell types, eventually, the gene-editing needs to be conducted on hematopoietic cells. In fact, this kind of work has already been conducted. In 2019, Lei Xu *et al.* reported a case of an HIV-1+ patient who suffered from acute leukemia received an HSCT using

CRISPR/Cas9 mediated CCR5 knockout autologous HSCs(216). This therapy was proved safe but had no significant therapeutic effect on HIV-1 infection. The reason for this was mainly attributed to the low efficiency of CCR5 disruption (17.8%), which further decreased after engraftment since the transplantation was conducted with a mixture of treated and untreated HSCs resulting in a 4-fold dilution of the treated cells. After 19 months, a range of 5.2% to 8.28% CCR5 disruption was observed across different types of bone marrow karyocytes. This “mixture” transplantation was because of concerns that the CRISPR/Cas9 treatment might be able to impact the engraftment of the HSCs, to ensure the success of the engraftment regarding the safety and benefit of the patient. The HIV-1-related therapeutic failure not only indicates the impact of gene editing on the engraftment capacity of the HSCs, which needs to be further investigated, but also the efficiency of gene editing in HSCs needs to be enhanced.

Unfortunately, HSCs are not an easy target for gene editing. The same team in the clinical trial has published a previous proof-of-concept work using the same method and transplant the HSCs to mice, which indeed showed a better resistance to HIV-BaL strain infection reflected by the decrease of viremia starting from 4 weeks after viral challenge. However, the best efficiency of gene disruption they achieved was only 28.7%(217). This low efficiency was also observed in ZFNs mediated experiment, with the highest efficiency reported at 27%, 17% on average (n=21)(218). The low efficiency can be attributed to the response of HSCs to the double strain break of DNA. One study has provided evidence of activation of the p53 pathway triggered by DSB which will lead to apoptosis, and treatment

with p53 inhibitor results in reversal of p52 pathway activation(219). This study also showed that this p53 pathway activation is DSB-intensity dependent, which might give better cell viability to treat the HSCs with a smaller dose of CRISPR/Cas9. However, a smaller dose although also helps to minimize the off-target effect, usually means lower gene editing efficiency. This problem might be solved by multiple rounds of treatment. The HSCs can be expanded *in vitro*, thus multiple rounds of gene editing while expanding the amount of the HSCs in every interval might be a way to increase the efficiency, and possibly not necessarily with a low dose of effectors. However, one round of gene editing is already very time and cost consuming, the patient might very likely not have the time and/or financial ability for the treatment under this strategy.

Another obstacle for co-receptor knockout as a cure for HIV-1 infection is the CXCR4 receptor. CXCR4 is an important receptor participating in the chemotactic activity of lymphocytes. The knockout of CXCR4 might have a more severe impact on lymphocytic function than CCR5, but it does not appear to impact the survival of lymphocytes very much. Previous studies have shown that ablation of both CCR5 and CXCR4 confirmed resistance to multiple strains of HIV-1 of both R5 and X4 tropism(166, 167) without impact on the viability of the lymphocytes. But unfortunately, it is not the case for the HSCs. CXCR4's ligand SDF-1 plays a crucial part in the homing of hematopoietic stem cells to the bone marrow and hematopoietic stem cell quiescence(220, 221). Thus, the knockdown of CXCR4 is very likely to severely impair multiple functions of the HSCs, and lead to the failure of engraftment. This will limit the use of autologous co-receptor knockout HSCT to patients

that can be confirmed with only R5 strain infection.

Although many obstacles remain to be addressed, CCR5 knockout is still promising in the eradication of HIV-1 infection, although it might not be easier to be solved than other therapeutic strategies, the homologous CCR5-Δ32 carrying HSCT is by far the only way that has achieved a completed functional eradication of HIV-1 infection. With the difficulties mentioned above, there are still methods to enrich the CCR5 disrupted HSCs which can be the further follow-up of this study. Previous studies have described the method to fluorescence label mRNAs(222). The HSCs can be transfected with the TALEN mRNAs, each arm labeled with a different fluorescence. Then the HSCs can undergo FACS-based single-cell selection for the cells that emit high intensity of both fluorescences, which indicates the high level of mRNAs of both arms in the cells to ensure gene-editing. Since HSCs can be expanded, these fluorescence intense HSCs are expected to have very high or even complete CCR5 disruption and can be expanded to a level sufficient for the HSCT. Hopefully, I will have an opportunity to conduct this work in the future to test this hypothesis.

Furthermore, gene editing technologies have opened the window to correct gene deficits that cause different diseases. Additionally, modified TALEN and CRISPR have been used for non-gene-editing research, including genetic background screening, infection detection and so on. Thus, more work is needed, not just for the cure of HIV-1 infection, but also to expand the function of gene editing tools, facilitate research, expand our understanding of life science, and provide hope for many traditionally incurable diseases.

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