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Combating Human Immunodeficiency Virus Replication by Gene Therapy

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Abstract

A mutant HIV-1 Tat protein termed Nullbasic has a strong antiviral activity via three independent mechanisms that disrupts; 1) reverse transcription of the viral RNA genome into a DNA copy, 2) HIV-1 Rev protein function required for unspliced and singly spliced viral mRNA transport from the nucleus and 3) HIV-1 transcription by RNA Polymerase II.

The Nullbasic protein is derived from the HIV-1 subtype B strain BH10 and has only been tested against other HIV-1 subtype B strains. Using a gammaretroviral vector as a gene delivery system, fusion forms of Nullbasic, Nullbasic-mCherry and Nullbasic-ZSGreen1, were tested against representative HIV-1 strains from subtypes C, D and A/D recombinant to determine if it can inhibit their replication. The results show that Nullbasic-mCherry inhibits Tat-mediated transactivation and virus replication of all the HIV-1 strains tested in TZM-bl cells. Importantly, Nullbasic-ZSGreen1 inhibits replication of the HIV-1 strains in primary $CD4^+$ T cells without affecting cell proliferation, cytotoxicity or level of apoptotic cells. However, long-term expression of Nullbasic protein in the cells is required to provide a strong and durable antiviral activity.

Nullbasic antiviral activity *in vivo* has not been studied. Therefore, a humanized mouse model was established to test Nullbasic antiviral activity *in vivo*. This animal model harbors human $CD4^+$ T cells for a short period, and is therefore suitable for testing Nullbasic antiviral activity against an acute HIV-1 infection. The results also indicate that Nullbasic-ZSGreen1 inhibits HIV-1 replication *in vivo*.

Gene delivery to target cells using gammaretroviral system may raise safety concerns due to its oncogonic potential. Therefore, a safer and better vector, a lentiviral vector, was optimized to deliver Nullbasic to T cells. However, Nullbasic delivery to T cells using a lentiviral system was problematic because Nullbasic itself inhibits lentivirus replication. Therefore, we optimized the *nullbasic* delivery method by using viral and cellular proteins antagonistic to Nullbasic. Transduction efficiency of Jurkat T cells by Nullbasic lentiviral VLP was optimal when addition of Tat and DDX1 in combination with spinoculation method was used. Nevertheless, this method did not improve transduction of primary $CD4^+$ T cells by Nullbasic lentiviral VLP.

Overall, Nullbasic has antiviral activity against all strains from the HIV-1 tested *in vitro* and inhibits acute HIV-1 infection *in vivo*. Therefore, Nullbasic may have utility in a future gene therapy approach.

Declaration by author

This thesis *is composed of my original work, and contains* no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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2. Hongping Jin, Dongsheng Li, Haran Sivakumaran, Mary Lor, Lina Rustanti, Nicole Cloonan, Shivangi Wani and David Harrich. Shutdown of HIV-1 transcription in T cells by Nullbasic, a mutant Tat protein. *MBio.*2016;7(4). DOI: 10.1128/mBio.00518-16[2].
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3. Min Hsuan Lin, Haran Sivakumaran, Alun Jones, Dongsheng Li, Callista Harper, Ting Wei, Hongping Jin, Lina Rustanti, Frederic A Meunier, Kristen Spann and David Harrich. A HIV-1 Tat mutant protein disrupts HIV-1 Rev function by targeting the DEAD box RNA helicase DDX1. *Retrovirology.* 2014;11:121. DOI: 10.1186/s12977-014-0121-9[3].
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Contributor	Statement of contribution
Lina Rustanti	Designed experiments (30%) Significant technical procedures (85%) Statistical analysis (90%) Wrote the paper (60%)
Hongping Jin	Significant technical procedures (10%) Editorial advice (30%)
Mary Lor	Significant technical procedures (5%) Editorial advice (10%)
Min Hsuan Lin	Editorial advice (30%)
Daniel Rawle	Editorial advice (30%)
David Harrich	Designed experiments (70%) Statistical analysis (10%) Wrote the paper (40%)

Contributions by others to the thesis

Mary Lor has assisted the animal experiment using Nullbasic-ZSGreen. This work has been included in Chapter 4.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Keywords

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LIST OF ABBREVIATIONS

HIV	:	Human immunodeficiency virus
WHO	:	World Health Organization
cART	:	combined antiretroviral therapy
LTR	:	long terminal repeat
P-TEFb	:	Positive transcription elongation factor b
CDK9	:	Cyclin-dependent kinase 9
TAR	:	trans-activation response
DDX1	:	DEAD helicase box protein 1
VLP(s)	:	virus like particle(s)
NB-mCh	:	Nullbasic-mCherry
mCh	:	MCherry
NB-ZSG1	:	Nullbasic-ZSGreen1
ZSG1	:	ZSGreen1
FACS	:	fluorescence activated cell sorting
CDC	:	Center for Disease Control and Prevention
NRTIs	:	nucleoside reverse transcriptase inhibitor
NNRTIs	:	non-nucleoside reverse transcriptase inhibitor
RT	:	reverse transcriptase
IN	:	Integrase
PR	:	Protease
Gp	:	Glycoprotein
SU	:	Surface
TM	:	Transmembrane
MA	:	Matrix
Gag	:	group-specific antigen
Pol	:	Polymerase
Vpu	:	viral protein U
Vpr	:	viral protein R
Vif	:	viral infectivity factor
Nef	:	negative regulatory factor
Tat	:	trans-activator of transcription
Rev	:	regulator of expression of virion proteins
ORF	:	open reading frame
NELF	:	negative elongation factor

DSIF	:	DRB sensitivity inducing factor
AIDS	:	acquired immunodeficiency syndrome
HAND	:	HIV-associated neurocognitive disorder
LEDGF75	:	lens-epithelium-derived growth factor 75
NLS	:	nuclear localization signal
RRE	:	Rev response element
NES	:	nuclear export signal
CRM1	:	chromosome region maintenance 1
SHIV	:	simian-human immunodeficiency virus
siRNA	:	small interfering RNA
ZFN	:	zinc finger nuclease
PBMC(s)	:	peripheral blood mononuclear cell(s)
HSC	:	hematopoietic stem cells
SCID	:	severe combined immunodeficiency
PBL	:	peripheral blood lymphocytes
NK	:	natural killer
NOD-SCID	:	non-obese diabetic SCID
RAG	:	recombination-activating gene
IL	:	Interleukin
DMEM	:	Dubelcco’s modified eagle’s medium
FBS	:	fetal bovine serum
MFI	:	mean of fluorescent intensity
PBS	:	phosphate buffered saline
NT	:	non-transduced
SEC	:	super elongation complex
RLU	:	relative luminescence unit
SFFV	:	spleen forming focus virus
qRT-PCR	:	quantitative reverse transcription real time PCR
SD	:	standard deviation
RSV	:	Rous sarcoma virus
WPRE	:	Woodchuck hepatitis virus post transcription regulatory element
NB-EGFP	:	Nullbasic-EGFP
huTat2	:	humanized Tat2 intrabody
cPPT	:	central polypurine tract
SIN	:	self-inactivating

Retronectin	:	recombinant human fibronectin
PIC	:	pre-integration complex

CHAPTER 1

Introduction

This introductory chapter gives an overview of the main points of this thesis. This chapter is presented in five sections, firstly the background of the study, secondly rationale of investigations, thirdly hypothesis followed by aims and finally the study approaches.

1.1 Background

Human immunodeficiency virus type 1 (HIV-1) infection remains one of the most serious health problems. Globally, 36.7 million people were living with HIV-1 by the end of 2015 and 1.2 million people died per annum from acquired immunodeficiency syndrome (AIDS)-related causes[4]. Whilst current combined antiretroviral therapy (cART) as recommended by World Health Organization (WHO) is capable of controlling the disease by delaying the disease progression and spread, lack of a cure and drug adverse effects remain as problems. Antiretroviral therapy using multi-drug regimens is costly and has potential side effects with long term use. Adverse events following long term use of cART that have been reported include brain aging (50%), gastrointestinal effects including nausea, vomiting and diarrhea (28%), lipodystrophy, hyperlipidemia and hypercholesterolemia (3-5%), hyperglycemia (0.47%) and hepatotoxicity (25.5%)[5-10]. These adverse effects lead to low adherence to cART[6]. Also, the potency of cART can be hindered by viral mutations that lead to HIV-1 quasispecies diversity and result in different drug susceptibilities. The combined effects caused by poor adherence and viral mutations induce development of drug resistance[11]. Furthermore, although cART is able to suppress viral load, once the medication is discontinued, HIV reappears in plasma. HIV-1 vaccine developments are hindered by the huge genetic variation in HIV-1 strains[12]. Therefore, a novel approach to controlling this disease, such as gene therapy, is required. Unlike antiretroviral therapy, which requires repeated doses, gene therapy offers a one-shot-treatment over a lifetime. This thesis focuses on the development of a novel gene therapy approach targeting HIV-1 replication.

Because HIV-1 can develop drug resistance, understanding the virus and its life cycle is crucial to finding an agent that can target multiple steps of viral replication. The literature review in Chapter 2 discusses HIV-1 morphology and genomic structure as well as its life cycle. The spread of HIV-1 subtypes is also addressed. Current gene therapy approaches that have undergone pre-clinical or clinical trial in various stages are highlighted. The use of murine models to test the new potential gene therapies is also discussed.

A novel gene therapy agent called Nullbasic has shown strong antiviral activity against HIV-1 infection *in vitro.* Nullbasic is a transdominant negative mutant of Tat, a small viral regulatory protein that is critical for enhancing the HIV-1 transcription efficiency from the long terminal repeat (LTR) transcriptional promoter[13]. Detailed explanation about Tat roles are reviewed in Chapter 2. Nullbasic has the ability to inhibit HIV-1 at three steps during viral replication, unlike other molecular therapeutics, which inhibit a single viral target. Nullbasic's three antiviral mechanisms provide a strong incentive to investigate the development of Nullbasic as a potential future therapy for HIV-1. Because Nullbasic interferes with HIV-1 infection in multiple ways, Nullbasic therapy may reduce the risk of the virus developing resistance, thus maximizing the potential for treatment efficacy.

In earlier research, Nullbasic showed strong inhibition of HIV-1 subtype B strains replication *in vitro* in Jurkat cells and in primary $CD4^+$ T cells, indicating this protein may be useful as an antiviral agent in preventing HIV-1 infection[14,15]. Nullbasic interferes with HIV-1 replication by targeting three steps of HIV-1 life cycle, including Tat-mediated transactivation, reverse transcription and Rev-mediated transport HIV-1 mRNA[15,16]. Nullbasic inhibits HIV-1 transcription by obstructing Tat interaction with positive transcription elongation factor (P-TEFb). This transdominant negative protein acts as a competitive agent against wild type Tat by binding to Cyclin-dependent kinase 9 (CDK9), part of P-TEFb complex that plays a role in RNA polymerase II phosphorylation during early transcription[15,17]. In addition, Nullbasic can alter the HIV-1 LTR epigenetic state so that it inhibits the binding of TAR, located at the 5' end of HIV-1 LTR promoter, to RNA polymerase II[2]. Nullbasic also interferes with Rev nucleocytoplasmic transport activity and thereby disrupts the Rev nucleolar organisation and functions[16]. As a consequence, Nullbasic downregulates the expression of unspliced and singly-spliced HIV-1 mRNAs and therefore inhibits virus replication[15]. The other antiviral mechanism of Nullbasic is achieved by targeting the reverse transcription complex. Nullbasic binds to reverse transcriptase and

accelerates viral uncoating, leading to disruption of reverse transcriptase complex[18]. Details of Nullbasic antiviral mechanisms are reviewed in Chapter 2.

In this thesis, we observed that Nullbasic can inhibit different HIV-1 strains from different subtypes, as discussed in Chapter 3. Therefore, Nullbasic also has a potential to be developed as an antiviral agent against HIV-1 subtypes other than subtype B. Furthermore, in Chapter 4, an HIV-1 acute infection model development in BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice is explained. The human CD4^{+} T cells were engrafted in the mice and HIV-1 viremia was detected in a limited period. In Chapter 5, we reveal that addition and combination of Nullbasic antagonist proteins can overcome Nullbasic inhibition to a lentiviral vector used to deliver the *nullbasic* gene to Jurkat cells. However, lentiviral based *nullbasic* transduction on primary CD4^{+} T cells was not improved; thus, further study is required to optimize transduction efficiency of lentiviral vector to deliver *nullbasic*. These notable results and the combined conclusions from this thesis as well as the limitation of this study are discussed in Chapter 6. This includes the future development of Nullbasic for HIV-1 research and therapy.

1.2 Rationale of investigations

The Nullbasic protein is derived from HIV-1 subtype B Tat and has been extensively studied *in vitro* against HIV-1 subtype B, but the antiviral activity of Nullbasic against other HIV-1 subtypes has not been studied. The genome changes between different subtypes may result in amino acid substitutions in viral proteins, including Tat. The sequence variation of Tat between subtypes may influence its interaction with RNA and cellular proteins, which may affect Nullbasic antiviral activity. Hence, it is important to investigate Nullbasic antiviral activity against different subtypes of HIV-1 to determine if Nullbasic can also inhibit replication of diverse HIV-1 subtypes and provide a broad coverage therapy.

HIV-1 subtype B, commonly found in Europe and US, accounts for only ~10% of HIV infection worldwide, while other subtypes such as subtype C, mainly found in developing regions such as in Sub-Saharan Africa, India and South America, is responsible for ~50% of HIV infection worldwide[19]. Subtypes D and A/D are also spreading rapidly in Sub-Saharan Africa[20]. Details of these HIV-1 subtype variations are reviewed in Chapter 2. Chapter 3 elucidates Nullbasic antiviral activity *in vitro* against HIV-1 strains representative of subtype

B, C, D and AD. The investigation of Nullbasic antiviral activity against different strains representing diverse HIV-1 subtypes in primary $CD4^+$ T cells provides evidence that Nullbasic can be promising as an antiviral agent for different HIV-1 strains.

So far, Nullbasic efficacy and antiviral mechanism against HIV-1 subtype B strains has been extensively studied *in vitro*. However, the protection of Nullbasic against HIV-1 infection *in vivo* has not been studied. A preclinical study to observe the effect of Nullbasic against HIV-1 replication *in vivo* is required before it goes further into clinical trial. An animal model is useful in studying the HIV-1 pathology and reservoir. Therefore, an acute HIV-1 infected mouse model was established to study Nullbasic antiviral activity against acute HIV-1 infection *in vivo*, and this will be described in Chapter 4. The Nullbasic expressing cells are engrafted in the chosen mouse model in a short period and enables *in vivo* testing of Nullbasic activity against acute HIV-1 infection.

Nullbasic gene has been delivered to target cells using a gammaretroviral vector. However, there may be an oncogenic risk from retroviral vectors due to insertional mutagenesis. Lentiviral vectors are known to be safer than retroviral vectors and provides stable transgene expression in the target cells. Lentiviral vectors can transduce non-dividing cells and therefore are more promising for transgene delivery to primary cells.

Previous research studied the delivery of the *nullbasic* gene using a lentiviral vector, but the transduction efficiency of the target cells was low due to reverse transcriptase inhibition by Nullbasic proteins[14,21] and thus, transduction optimization is required. Nullbasic disrupts reverse transcription by direct binding to reverse transcriptase and leads to defective reverse transcription complex[18]. Rev, a viral protein that is required for transporting viral mRNA from nucleus to cytoplasm, is also inhibited by Nullbasic. The Nullbasic inhibition to Rev is indirect by binding to a cellular factor required for Rev activity named DDX1 (DEAD helicase box protein 1)[3]. The majority of lentiviral systems used to deliver genes to target cells are HIV-1 based vectors, therefore, Nullbasic can inhibit these vectors.

To improve the transduction efficiency of Nullbasic lentiviral system in T cells, we introduced an intrabody protein termed hu-Tat2, a synthetic intracellular antibody expressed within the cytoplasm[22]. The Nullbasic and Tat protein sequences are conserved except for the basic region. The hu-Tat2 intrabody protein can bind Tat amino acids 1 to 19, a proline rich region that is conserved in Nullbasic[23], and therefore it can prevent the Nullbasic effect on reverse

transcription. Furthermore, overexpression of Tat was found able to reverse the inhibitory activity of Nullbasic towards lentiviral vector. Chapter 5 in this thesis discusses the use of the use of Nullbasic antagonists to improve transduction efficiency of lentivirus in conveying Nullbasic to T cells. It was observed that the coexpression of cellular and viral proteins antagonists of Nullbasic in lentivirus producer cells could improve the efficiency of Nullbasic lentiviral virus like particle (VLP)-mediated transduction of Jurkat cells, but not primary cells.

1.3 Hypothesis

The hypothesis of this study is:

Long term expression of Nullbasic in human CD4+ T cells protects them from HIV-1 replication by a variety of strains both *in vitro* and *in vivo.*

1.4 Objectives

The hypothesis above is addressed by the specific objectives of this study, which are:

1. to determine if Nullbasic can inhibit replication of diverse HIV-1 strains representing different subtypes *in vitro;*
2. to establish an acute mouse model and investigate Nullbasic effects *in vivo* against acute HIV-1 infection.
3. to optimize the production and transduction efficiency of Nullbasic lentiviral VLP for T cells.

1.5 Study approaches

In order to prove the hypothesis, those three aims above were addressed by conducting the following study approaches:

Aim 1: to determine if Nullbasic can inhibit replication of diverse HIV-1 strains representing different subtypes *in vitro*

This aim is elucidated in chapter 3. We evaluated Nullbasic antiviral activity on TZM-bl cell lines and primary $CD4^+$ T cells. Four HIV-1 strains from different subtypes were chosen:

HIV-1_{NL4-3} (subtype B), HIV-1_{ZAC} (subtype C), HIV-1_{MAL} (subtype D) and HIV-1_{ELI} (subtype A/D recombinant). Firstly, we established TZM-bl cell lines expressing Nullbasic-mCherry (NB-mCh) and mCherry (mCh) by transduction and fluorescence activated cell sorting (FACS) selection. Then, by transfecting these cell lines with Tat expression vectors, we investigated if Nullbasic can inhibit transactivation by Tat from different HIV-1 strains. Using the same cell lines, we also performed infection assay to determine if Nullbasic can inhibit transactivation of the HIV-1 LTR from different HIV-1 strains.

Nullbasic inhibition on replication of the HIV-1 strains chosen was examined in the TZM-bl cell lines as well as in primary $CD4^+$ T cells using a different fluorescent fusion protein attached to Nullbasic called ZSGreen1 (NB-ZSG1). Using NB-ZSG1 fusion protein, Nullbasic antiviral activity against HIV-1 strains from different subtypes in primary $CD4^+$ T cells was revealed. We also monitor Nullbasic protein levels of expression by flow cytometry. Furthermore, to confirm that there is no toxicity effect of Nullbasic expression in primary $CD4^+$ T cells, we conducted an MTS, proliferation and apoptosis assays.

Aim 2: to establish an acute mouse model and investigate Nullbasic effects *in vivo* against acute HIV-1 infection

To achieve aim 2, a humanized mouse model was developed. BALB/c-$Rag2^{-/-}\gamma c^{-/-}$ mice were transplanted with NB-mCh or mCh or NB-ZSG1 or ZSG1 transduced primary $CD4^+$ T cells and then challenged with HIV-1_{AD8}. Engraftment of the human $CD4^+$ T cells in the mice was measured by flow cytometry. Viral load was measured by quantitative reverse transcription real time PCR (qRT-PCR) from blood, spleen and lung samples.

Aim 3: to optimize the production and transduction efficiency of Nullbasic lentiviral VLP on T cells

To achieve efficient transduction of Jurkat T cell line and primary $CD4^+$ T cells by NB-ZSG1 lentiviral VLP, we introduced Nullbasic antagonist cellular and viral proteins. A humanized anti-Tat intrabody (huTat2), HIV-1 Tat and Rev, as well as DDX1 proteins were added in the VLP producer cells. We also combined those methods with spinoculation. The transduction rate was measured by flow cytometry.

CHAPTER 2

Literature Review

2.1 HIV-1 epidemic and current therapy

HIV, a virus that impairs the human immune system and weakens the natural defense against infections, remains one of the world's biggest health challenges. Since the first pandemic identified in 1981 by the US Center for Disease Control and Prevention (CDC), HIV has infected more than 78 million people and caused more than 35 million deaths. The number of people living with HIV has been steadily increasing and reached ~38.8 million at the end of 2015[24]. More than 95% of HIV infections are in developing countries, with the highest prevalence (70%) is in sub-Saharan Africa[4,25]. Approximately 5700 people are infected every day, and 2.1 million newly infected cases were reported in 2015. Although the global number of AIDS related deaths has been declining steadily in the last decade, dropping from 1.8 million in 2005 to 1.1 million in 2015, death rates have increased in several developing countries such as Indonesia and the Philippines[25,26].

HIV not only makes people vulnerable to AIDS-related diseases including opportunistic infections and cancers, it also impacts negatively on their economic and social life. Stigma and discrimination towards people living with HIV threatens their access to sustainable employment and healthcare[4]. Even though cART coverage decreased AIDS-related mortality rate globally, the burden of disease remains high. Sustainable funding for HIV/AIDS interventions is required, especially in low income countries in where HIV prevalence is high and health resources are scarce[24].

First line cART recommended by WHO includes two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitors (NNRTIs). For example, the combination of efavirenz, tenofovir disoproxil fumarate and emtricitabine or lamivudine[27,28]. Second line cART consists of one or two NRTIs boosted with one protease inhibitors (PIs), such as lopinavir or Atazanavir[27,29]. Third line ART regimen includes integrase inhibitors such as raltegravir combined with second generations of NNRTIs such as etravirine and PIs such as Darunavir[27,29]. The most common reasons of therapy switching

from first line, second or third line ARTs are adverse event occurrence and virological treatment failure due to low adherence or drug resistance mutations[30]. A clinical study of 953 HIV-1 patients in Uganda showed that 67.3% of the patients were on first line cART, while the rest received third line cART. However, virological treatment failure was found in half of the treated patients, with one fifth of the cases caused by drug resistance mutations[29]. This increased the necessity of third line cART. Subsequent studies in the US and Thailand observed that third line cART was well tolerated and able to supress the viral load as few as 50 copies/ml[30,31].

Current HIV therapies using cART have reduced plasma viremia and slow down the disease progression, but virus eradication or a cure has not been achieved so far. Residual viremia persists and may arise from the infected cells that become latent viral reservoirs. Long-term medication helps reduce the viral load to an undetectable level, but soon after the therapy is discontinued, the viral load increases and accelerates disease progression[32]. Many adverse effects following cART administration have been reported, including gastrointestinal intolerance (nausea, vomiting and diarrhea), lipodystrophy, hyperlipidemia, hypercholesterolemia, hyperglycemia, neurotoxicity, hepatotoxicity, paresthesias, nephrolithiasis and skin rash[5-9,33]. These adverse effects reduce compliance. Furthermore, the high mutational rate of HIV-1 due to reverse transcription infidelity and recombination has created a number of HIV-1 strains that are not susceptible to cART. Non-compliance due to adverse effects of cART and the mutability of HIV-1 have increased drug resistance events. Therefore, an alternative therapy that can provide a combination of multiple antiviral activities without the need for lifelong medication, such as gene therapy, is required.

HIV-1 gene therapies aim to deliver transgenes to human target cells or delete unwanted genes to eliminate HIV-1 infection by inhibiting virus replication, rendering cells resistant to HIV-1 infection or providing immunity against HIV-1 antigens[34]. Therefore, understanding the virus structure and function, life cycle and host factors, pathogenesis, delivery vectors and animal models is important in developing gene therapy strategies.

2.2 HIV-1 – The virus

2.2.1 The basic structure of HIV-1

An HIV virion is a spherical particle with diameter 100 nm. This virion consists of a viral envelope, matrix proteins (MA) and a viral core. The envelope protein consists of an external part, composed of glycoprotein gp120, on the surface (SU) of the virion, and a transmembrane (TM) part, composed of gp41 (Fig. 2-1). Both proteins are formed by enzymatic cleavage of gp160[35,36]. During viral entry to the targeted cells, gp120 plays a role in attaching the virus to the host cell, while gp41 is required in the cell fusion process. The HIV structural matrix protein, which is made from the p17 protein, lies between the envelope and the core. The HIV-1 core contains a 24kD capsid (CA) protein, p24, and a nucleocapsid protein, p7 which enclose two single strands of viral mRNA and are involved in the reverse transcription process[36,37]. This p24 protein is abundantly present in the blood serum of newly infected people, and therefore p24 levels are used to diagnose acute HIV-1 infection[38]. Within the core, there are the essential viral enzymes reverse transcriptase (RT), integrase (IN) and protease (PR)[37].

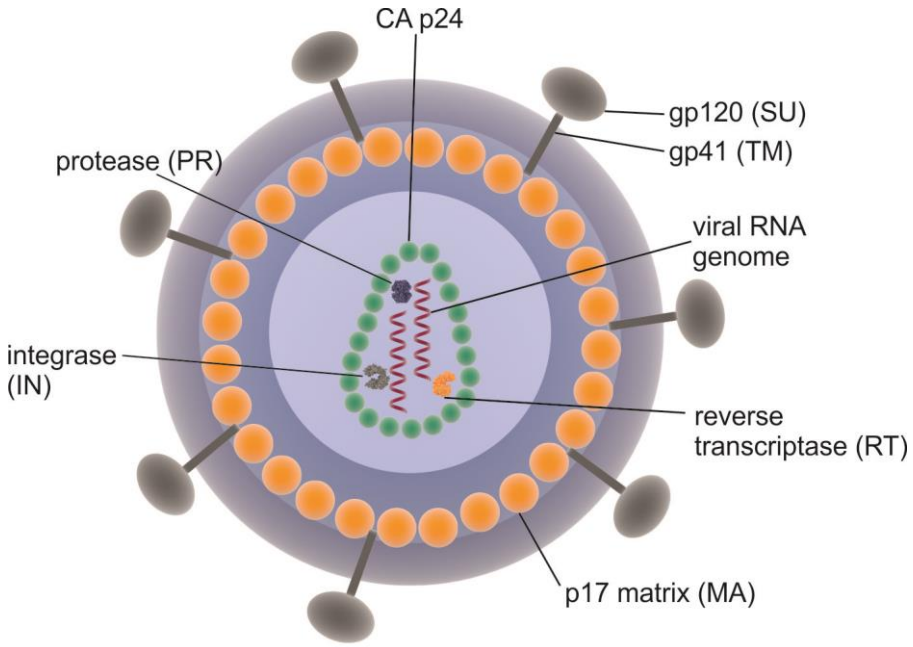

Figure 2-1. The basic structure of an HIV-1 virion
A HIV-1 virion has a viral envelope composed of glycoprotein gp120 on the surface and gp41 on the transmembrane. Other structural proteins such as matrix and capsid are shown. Inside the capsid, an HIV-1 has two single-stranded viral mRNA and the enzymatic proteins reverse transcriptase, integrase and protease.

2.2.2 The structure of HIV-1 genome

Each HIV-1 virion contains two copies of a single strand mRNA located in the viral core. These two mRNA copies serve as the HIV-1 viral genome. The full-length HIV-1 genome is approximately 9.7kb long. This genome contains nine genes coding for fifteen viral proteins. Figure 2-2 below shows the HIV-1 genomic structure. The proteins encoded by HIV-1 mRNA are classified into three groups: a) major structural proteins, b) regulatory proteins, and c) accessory proteins. The major structural proteins include a group-specific antigen (Gag), polymerase (Pol) and envelope (Env) proteins. Gag, Pol and Env are produced as polyproteins that are subsequently cleaved by protease to form individual proteins. Gag later becomes mature proteins, such as matrix (MA), capsid (CA) and nucleocapsid (NC). Gag expression alone is responsible for immature virus-like particles that bud from plasma membranes. Therefore, Gag proteins must interact with other components to form mature viruses. Together with *pol, gag-pol* encodes viral enzymes including reverse transcriptase (RT), integrase (IN), protease (PR) and DNA polymerase. The *env* gene encodes surface glycoprotein (SU) and transmembrane (TM) proteins of the virion. During early infection, these proteins form a complex that interacts specifically with chemokine receptor proteins on the targeted cell surface, such as CD4, CCR5 and CXCR4[39].

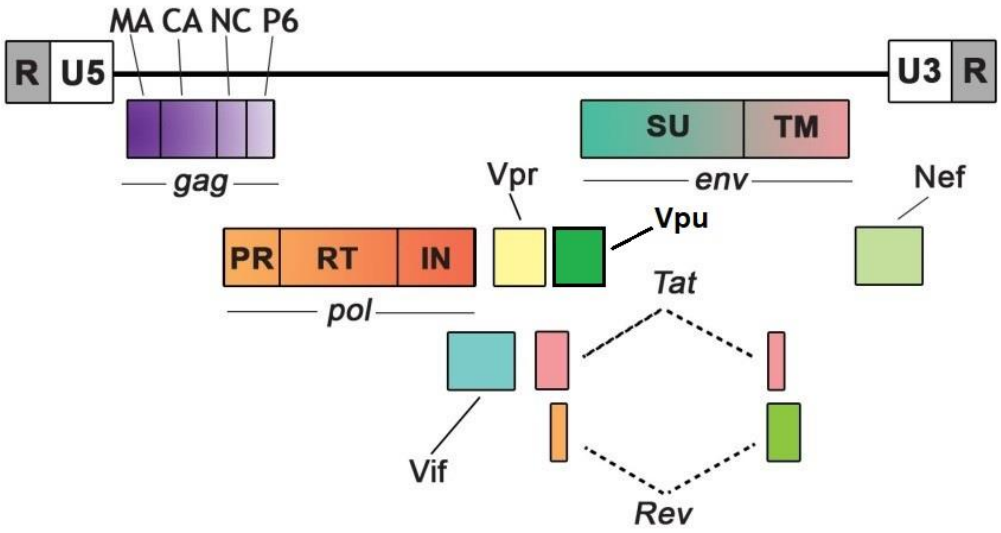

Figure 2-2. HIV-1 genomic structure
The HIV-1 genome composes of 3 major genes; *gag, pol* and *env*. *Gag* gene encodes matrix (MA), capsid (CA), nucleocapsid (NC) and p6 protein. Gag-pol genes encode protease (PR), reverse transcriptase (RT) and integrase (IN). *Env* gene encodes surface glycoprotein (SU) and transmembrane glycoprotein (TM). By splicing mechanisms, the HIV-1 genome also encodes viral accessory proteins such as Vif, Vpr, Vpu and Nef, as well as regulatory proteins such as Tat and Rev.

As a result of multiple mRNA splicing, HIV-1 genome produces accessory proteins such as viral protein unique (Vpu), viral protein R (Vpr), viral infectivity factor (Vif) and negative regulatory factor (Nef), also regulatory proteins include trans-activator of transcription (Tat) and regulator of expression of virion proteins (Rev)[40]. Tat protein is essential in regulating HIV-1 transcription and has diverse functions in HIV-1 pathogenesis, and therefore will be discussed further in this review.

2.3 Trans-activator of transcription (Tat)

Tat protein is expressed early after HIV-1 provirus is formed in the host cells. This protein is an important regulatory protein of HIV-1 as it has so many roles in HIV-1 replication cycle and pathogenesis. Tat open reading frame (ORF) has two exons separated by an intron. As a result of double mRNA splicing, the intron is removed, forming a Tat transcript that encodes 86-101 amino acids. The first exon of Tat consists of proline rich region, cysteine rich region, core region, arginine rich region (basic domain) and glutamine rich region[41].

Tat N-terminus domain plays a role in T cell immunosuppression by binding to dipeptidyl peptidase IV, a T cell activation marker (CD26) important in regulation of lymphocyte growth. This way, this proline rich region inhibits DNA synthesis in T cell lymphocytes[42]. Tat cysteine-rich and core regions induce migration and invasion of monocytes during HIV-1 expansion[43], while the glutamine rich region involved in T-cell apoptosis[44]. Tat basic domain, at position 49-57, plays an important role in enhancing HIV-1 transcription by RNA polymerase II. During early transcription, the basic domain of Tat binds to HIV-1 trans-activation response (TAR) RNA (Fig. 2-3). TAR RNA is a hairpin structured RNA located at the 5' HIV-1 LTR end serves as cis acting site for transactivation[45].

The bulge structure of TAR RNA plays a role as a Tat binding site. However, Tat binding to TAR RNA only is not enough to stimulate transactivation[46]. The stem-loop structure of TAR RNA supports transactivation by Tat as it binds to cyclin T1[47]. Tat also binds to cyclin T1 surface and interacts with CDK9. Both cyclin T1 and CDK9 are part of P-TEFb, a host protein that is responsible for controlling gene expression in eukaryote cells. A study on Tat-P-TEFb crystal structure shows Tat binds to both Cyclin T1 and CDK9[48]. CDK9 has a role in phosphorylating the C terminal domain of RNA polymerase II. It was observed that Tat protein alone has a flexible structure, but when it interacts with P-TEFb complex, Tat

acquires the stable conformation required for its transcriptional function. Tat changes its conformation by directly binding to both Cyclin T1 and CDK9. Tat cysteine-rich and core regions interact with two Zn ions to form a compact structure. One of these Zn ions forms a bridge with Cyclin T1. Mutational studies reported that His33 and all cysteine residues of Tat are important for this interaction, except Cys31, which often is replaced by serine in subtype C Tat, as this mutation does not change the protein function. The first 49 amino acids of Tat, ~37% of its folded surface, is complementary to the kinase. Consequently, Tat has a strong binding affinity with P-TEFb complex. This interaction changes CDK9 conformation and activates P-TEFb by altering its substrate binding surface[48]. Shortly after transcription initiation, negative elongation factor (NELF) interacts with DRB sensitivity inducing factor (DSIF), causing RNA polymerase II promoter-proximal pausing. Activated P-TEFb reverses this RNA polymerase II inhibition by phosphorylating NELF and DRB sensitivity inducing factor (DSIF)[48]. It also phosphorylates Ser2 and Ser5 in the carboxy terminal domain of RNA polymerase II, enhancing HIV-1 transcription processivity. Without Tat activation of P-TEFb, mRNA synthesis by RNA polymerase II is inefficient due to the promoter-proximal pausing, and results in short transcripts[41,49].

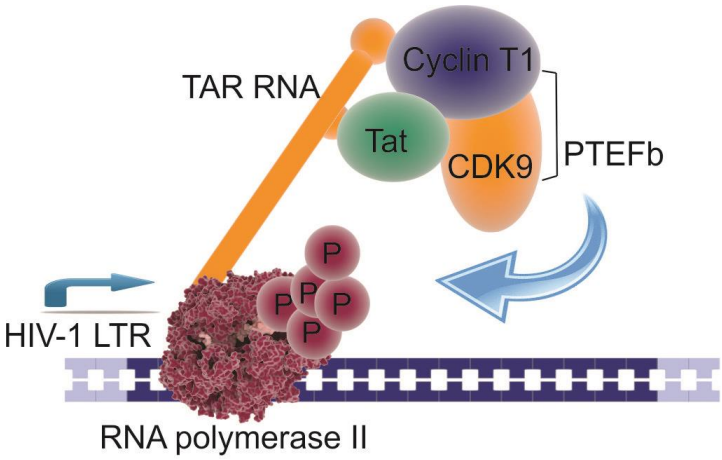

Figure 2-3. Diagram of HIV-1 Tat-mediated transactivation
Tat binds TAR RNA from the HIV-1 LTR then recruits pTEFb that is compose of Cyclin T1 and CDK9. This complex then hyperphosphorylates the carboxy terminal of RNA polymerase II, and in this way Tat enhances transcription efficiency.

Tat also has several functions in HIV-1 pathogenesis. Tat induces apoptosis in $CD4^+$ T cells, and this results in depletion of $CD4^+$ T cell lymphocyte numbers in HIV patients along with other mechanisms. Tat is expressed in the cytoplasm and can cross the membrane into the nucleus. Tat can also be released from infected cells and induces apoptosis in uninfected bystander cells. This mechanism is reported as the main cause of $CD4^+$ T cell lymphocyte

depletion in HIV patients[50]. However, Tat has been reported elsewhere to induce cell proliferation. A small concentration of Tat released from infected cells was able to induce proliferation of human epithelial cells[51]. This may explain incidents of AIDS-related cancer in HIV patients. Extracellular Tat was reported to induce Kaposi sarcoma[51]. Extracellular Tat can enter bystander cells and stimulates inflammation cytokines such as TNF-β, TGF-β and IL-6. It is also reported that Tat can inhibit immune response mediated by MHCII presentation and IL-2[41].

Tat is unique in having free cysteine residues in region II that support structure flexibility. The flexible structure of Tat protein is necessary for its diverse functions, from binding to RNA to crossing host cell membranes[48]. Tat can cross the blood brain barrier and causes HIV-associated neurocognitive disorder (HAND)[52]. Because of its abundant functional roles in HIV-1 replication and pathogenesis, Tat is a promising target for antiviral therapy.

2.4 HIV-1 life cycle

The HIV-1 life cycle starts from virus fusion, reverse transcription, integration, transcription and translation, followed by virus maturation and budding in the late phase of infection (Figure 2-4).

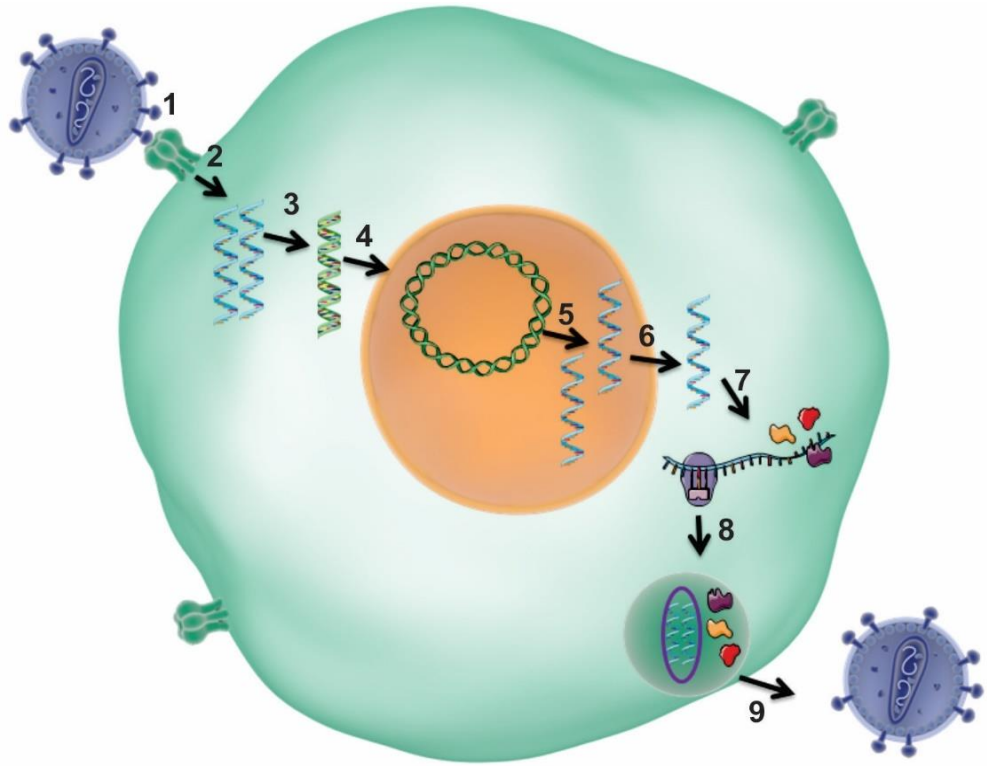

Figure 2-4. HIV-1 life cycle
The HIV-1 life cycle starts from; (1) virus fusion to the target cell, (2) uncoating and release the HIV-1 genetic material into the cell, (3) reverse transcription, (4) integration to the host chromosome, (5) transcription, (6) mRNA export from the nucleus to the cytoplasm, (7) translation, (8) assembly and (9) a new virus budding and maturation.

The HIV-1 life cycle can be divided into an early and a late phase of viral replication. The early phase includes the steps from viral fusion until integration, while the late phase starts with transcription and is finished by release of a mature new virion. Details of virus life cycle stages are described below.

2.4.1 *Virus fusion and entry*

HIV infects lymphocytes, monocytes, macrophages and dendritic cells. The HIV life cycle begins when the virus penetrates the host cells. The infection starts when the external part of the viral HIV envelope protein, gp120, attaches to a CD4 molecule on the target cell surface. This attachment changes the conformation of gp120 to enable binding to CCR5 or CXCR4 chemokine receptors in the host cell. This step is followed by a transmembrane protein (gp41) activity that allows cell fusion with the viral envelope[35]. Conformation change happens so that gp41 forms a coiled structure that exposes the hydrophobic region of gp41 and enables the virus to fuse with the lymphocyte cell membrane[53]. After that, the viral capsid releases the viral core, containing RNAs and proteins, into the host cell cytoplasm[35].

2.4.2 *Reverse transcription*

The viral RNA is then reverse transcribed into its complementary DNA (cDNA) by reverse transcriptase activity. This step results in viral genome that is transported into the nucleus. This transport is aided by nuclear signal transduction from the p17 matrix protein[53], Viral protein R (Vpr), Viral protein U (Vpu) and transportin-3[35]. Subsequently, the viral RNA template is degraded by ribonuclease activity.

2.4.3 Integration

The next step of HIV-1 life cycle is DNA integration, which serves to stabilize the viral cDNA in the host chromosome[54]. A cofactor named human lens epithelium-derived growth factor 75 (LEDGF75) interacts with integrase and enhances strand transfer activity during the viral genome integration. Once the viral genome is inserted into the host chromatin, a provirus is formed[55].

2.4.4 Transcription, nuclear transport and translation

RNA polymerase II in the cytoplasm initiates transcription of the integrated DNA into mRNA. Due to the splicing process, transcription results in short mRNA transcripts during the early stage of infection. Small and doubled spliced mRNAs encode Tat, Rev and Nef. Singly spliced mRNAs encode Env, Vif, Vpr and Vpu, while unspliced full length mRNAs encode Gag and Pol. Next, translation occurs in the cytoplasm forming proteins that are transported further into the nucleus through the nuclear import mechanism[11]. The first protein that is produced from this process is Tat. As described in section 1.4, Tat is important for HIV-1 transcription as it enhances the transcription processivity.

Rev prevents full length RNA splicing and increases the nucleocytoplasmic transport of the viral mRNAs[3,56]. Rev is transported from the cytoplasm to the nucleus through the interaction of its arginine rich nuclear localization signal (NLS) with transportin or importin β. Mediated by nucleophosmin, Rev is then transported into the nucleolus. In the nucleolus, Rev forms a complex with unspliced or singly spliced HIV-1 RNA containing Rev response element (RRE). RRE is a ~350 nucleotide recognition sequence located at the envelope region of unspliced or singly spliced HIV-1 RNA. Rev multimerization on the RRE masks Rev's NLS and exposes its nuclear export signal (NES). Rev then interacts with nuclear export factors and chromosome region maintenance 1 (CRM1), which mediates transport of unspliced and singly spliced RNAs into the cytoplasm[57].

2.4.5 Budding and maturation

The full length mRNAs are translated into Gag and Gag-Pol, while singly spliced mRNAs go on to make Env, Vif, Vpr and Vpu[53,54]. Gag assists the virus assembly by binding to the plasma membrane, mediating protein-protein interaction to create a spherical particle and interacting with RNA packaging sequence to package the HIV-1 genomic RNA. An immature virus is then ready to bud through the plasma membrane. During budding, the amino-terminal Gag domain binds to the plasma membrane and recruits envelope glycoprotein to encapsulate the virion. Protease is activated as the immature virion buds. Gag is then undergone proteolysis into viral structural proteins, such as matrix, capsid and nucleocapsid. Gag-Pol , a protein precursor generated from ribosomal frame shift, is cleaved by protease and forms reverse transcriptase, integrase and protease[35]. At the end of this stage, a mature virion is formed and ready to be released from the cell and infect another target cell[58].

2.5 HIV-1 pathogenesis and disease progression

HIV-1 disease progression includes acute infection stage, clinical latency infection and AIDS. Figure 2-5 explains how untreated HIV-1 infection progressing by time indicated by depletion of CD4+ lymphocytes in relation with viral load levels[59,60]. These three stages are discussed below.

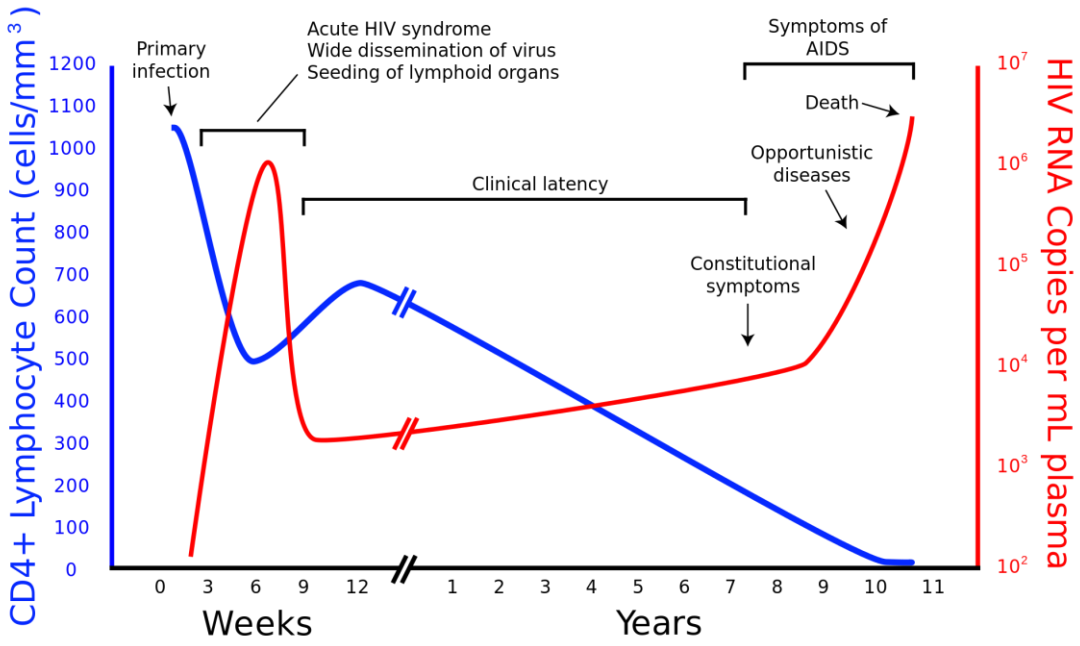

Figure 2-5. Time course of disease progression in untreated HIV-1 infection
Viral load levels are shown in correlation with depletion of $CD4^+$ lymphocyte count at time indicated.

2.5.1 Acute infection stage

Acute infection stage occurs during HIV-1 exposure and completion of initial immune responses. This stage, also called seroconversion, may last for few weeks to 3 months. Seroconversion is the period between initial infection and development of detectable levels of antibodies. During the first 2 to 6 weeks after HIV-1 infection, HIV-1 infected patients produce virus in large amounts as well as mounting a robust immune response. High rate of viral replication and the immune response generation during seroconversion cause severe flu-like symptoms, the acute retroviral syndrome, which may include fever, swollen glands, rash, headache, myalgia, arthralgia, lethargy/malaise, pharyngitis and fatigue[61]. Lymphadenopathy, anorexia, nausea, mucus membrane ulcers, night sweats, diarrhea and weight loss have also been reported[61,62]. A recent case report from 10 acute HIV infected patients indicated hemophagocytic lymphohistiocytosis, a systemic inflammation condition[63].

HIV-1 replicates in $CD4^+$ T cell lymphocytes and causes cell death. Therefore, $CD4^+$ T cell count falls dramatically and the viral load increases during the acute infection stage[64]. A cohort study of 284 HIV infected individuals in Thailand showed that administration of cART in this stage suppressed the viral load up to ~2.5-fold by day 12, ~135-fold by day 30 and ~1148-fold by day 120[65]. Taking antiretroviral during this stage helps to decrease the viral

load as well as increase the $CD4^+$ T cell count and also reduce the duration of acute retroviral syndrome[66].

2.5.2 Clinical latency stage

After the acute infection stage, the disease moves to clinical latency stage. This phase is also called chronic HIV infection or asymptomatic HIV infection. During this period $CD4^+$ T cell count decreases steadily. This phase is asymptomatic and may last for about eight years. The latently infected cells create a barrier for HIV-1 eradication [67].

HIV latency occurs when provirus is formed in $CD4^+$ T cells but translation machinery is shutdown[68]. The HIV latency reservoir is generated when infected $CD4^+$ T cell lymphocytes survive the HIV cytopathic effect and the increased immune response. This leads to the development of cell memory in resting CD4+ T cells carrying integrated provirus, but cannot facilitate viral gene expression[69]. In the resting T cells, Cyclin T1 is degraded and Tat production declines[68]. The lack of these host and viral factors cause limited activity of the HIV-1 long terminal repeat during transcription[70], and therefore very low levels of HIV-1 plasma viremia are present in this latency stage. However, these resting $CD4^+$ T cells may become reactive when the cART treatment is discontinued, or fails due to drug resistance, and lead to a rebound of viremia[69,71]. Furthermore, induction of Tat production can reactivate HIV transcription, replication and spread. Latency reactivation can also occur as a result of cell stimulation by cytokines or T cell receptor activation[68].

2.5.3 AIDS

In the late stage of infection, the capability of the immune system decreases significantly and the patient becomes vulnerable to cancer and opportunistic infections. The $CD4^+$ T cell count falls below 200 cells/mm^3 and clinical manifestations including pulmonary, gastrointestinal, neurologic and cutaneous diseases follow. Without appropriate treatment, the life expectancy for patients in this stage is about three years and may decrease to about one year with opportunistic infections[72-74].

2.6 HIV distribution

HIV is part of *Retroviridae* family and a member of *Lentivirus* genus. Based on the sequence differences of the viral genomes, HIV is classified into HIV-1 and HIV-2. Based on

evolutionary comparisons, both HIV-1 and HIV-2 arose from multiple cross-species transmissions of Simian Immunodeficiency Virus (SIV). The origin for HIV-1 was SIV in the chimpanzee, while for HIV-2, it was the sooty mangabey SIV[75]. HIV-1 is most prevalent worldwide and will therefore be the focus of this section.

HIV-1 genetic diversity remains a major obstacle for the virus eradication. Extensive genetic diversity of HIV-1 has been characterized. The viral genetic diversity emerged due to the rapid rate of viral mutation, replication and recombination[76]. Among the multiple factors that contribute to genetic variation, errors during virus replication are the most likely causes of the mutations[77]. HIV-1 replication involves three different polymerases. The enzyme best known to trigger mutations is reverse transcriptase, a viral enzyme that converts HIV-1 RNA to cDNA. Reverse transcriptase has low fidelity when transcribing RNA into DNA because it lacks proofreading activity[76,78,79]. DNA polymerase can also contribute to mutations during replication of the integrated provirus. However, this is thought to be a less important source of error because cellular DNA replication is known to be less error-prone[76,80]. Mutations can also occur during transcription by RNA polymerase II[79], but this polymerase possesses transcriptional proofreading[81-83], and therefore mutations caused by this enzyme are less frequent relative to those caused by reverse transcriptase. Genetic variation in HIV-1 can also be caused by environmental and host factors[84]. In addition, recombination can also cause HIV-1 variations, known as Circulating Recombinant Forms (CRF). The HIV-1 virion contains two strands of viral genomic RNA. Recombination can occur when a person is coinfected with more than one virus from related or different strains of HIV-1. CRFs are formed when single cell harbors two or more different proviruses, and one RNA transcript from each provirus is encapsidated form a new virion. When this new virion infects another cell, reverse transcription happens between the two RNA synthesizing a recombinant form of cDNA[12]. Currently, over 90 CRFs have been assigned and these CFRs account for ~20% HIV-1 infection worldwide[85].

Based on envelope nucleotide sequence differences, HIV-1 has four distinct lineages: groups M, N, O and P[75]. Worldwide, group M is the most prevalent type of HIV-1, and is classified into nine major subtypes or clades (A-D, F-H, J-K) and a small number of recombinant forms of those subtypes[36,53,75]. The geographical distribution of HIV-1 variants is summarized in Table 2-1 below.

Table 2-1. Distribution of HIV variants[75,84]

Group	Subtype /Clade	Distribution
M	A	East and central Africa, Central Asia, Europe
	B	America, Western Europe, East Asia, Oceania
	C	India, South and East Africa, Brazil
	D	East Africa
	F	Central Africa, Romania, Latin America
	G	Eastern and Western Africa, Taiwan, Russia
	H	Central Africa, Belgium
	J	Central America
	K	Cameroon
	A/E	South East Asia, China, Central Africa
	A/D	Central Africa
	A/G	Eastern and Western Africa, Central Europe
N		West Africa, Cameroon
O		Cameroon, Gabon, Equatorial Guinea
P		Cameroon

HIV-1 is divided into two classes based on the use of chemokine receptors: R5 infecting macrophages and T lymphocytes by binding to CCR5 co-receptor, and; X4, infecting T lymphocytes by binding to CXCR4 co-receptor[86]. The subtype or clade variation of HIV-1 correlates with the use of different co-receptors and different disease progressions. Subtype A and C viruses, for example, are associated with exclusive use of CCR5 co-receptor even in the late stage of disease progression, whereas subtype D viruses tend to use both CCR5 and CXCR4 co-receptors (R5X4). CXCR4 virus is correlated with faster disease progression[87]. It has been reported that subtype C causes the fastest disease progression, while subtype A and G have the slowest disease progression[88]. Subsequent studies found that subtype D and its recombinant forms, such as subtype AD, caused faster disease progression compared to subtype A[87,89,90].

The HIV-1 diversity also impacts on drug susceptibility. For example, non-B subtypes develop drug resistance more rapidly than B subtype viruses[84]. This is a challenge for HIV-1 eradication and hence a broad spectrum of HIV therapeutic strategy is required to overcome this problem.

2.7 A Resurgence in Gene Therapy as an Approach to Curing HIV/AIDS

HIV therapy research has included novel HIV therapeutic interventions such as gene therapy. Gene therapy is the use of genes to prevent or treat diseases by replacing mutated genes that cause diseases, knocking out target genes or introducing a new gene to fight diseases. Gene therapy for HIV-1 is mainly aimed at making cells resistant to HIV-1. Conventional therapy using cART is a long-life treatment that requires multiple dosages of medication. Compared to that conventional therapy, gene therapy may provide an advantage as it offers an ongoing manner therapy with a single shot treatment. Several gene therapy approaches to combatting HIV-1 that have been through preclinical and clinical trials are discussed below.

2.7.1 Anti-Tat single chain intrabody

An intrabody is an intracellular antibody that is designed to specifically bind to a protein target in cells. Anti-Tat intrabody is an intracellular antibody that binds Tat protein. The Anti-Tat intrabody protein binds to the proline rich region in Tat, located across amino acids +1 to +19 from the N terminal of Tat[23]. This region is an important epitope of Tat for transactivation function.

An intrabody variant called anti-Tat sFvs1 inhibits Tat-mediated transactivation as well as syncytia formation and HIV-1 replication. This single chain antibody is abundantly expressed in the cytoplasm and nuclear compartment of transduced primary $CD4^+$ T cells without any detectable cytotoxicity[91]. This variant has been developed into a humanized antibody named sFvhuTat2 that has undergone a pre-clinical study in mice and a clinical trial on HIV patients[23]. Using an MLV based vector, transduction efficiency of $CD4^+$ T cells by sFvhuTat2 was 28%. These transduced cells were able to expand up to 30 days. Viral load was suppressed up to ~80pg/ml CAp24[92]. These studies indicate that, delivered by a lentiviral system, sFvhuTat2 may prevent HIV-associated neurotoxicity in mouse neuron cells and suppress HIV replication *in vitro*[93]. Furthermore, the action of sFvhuTat2 against Simian HIV

(SHIV) in rhesus macaques has been studied using a retroviral delivery system, and this intrabody has been found to cause a decrease in viremia by 3-fold, as well as prolonged $CD4^+$ T cell survival post infection[94].

Extensive research has been performed in order to optimize the use of this intrabody for HIV gene therapy. However, transduction efficiency of huTat2 delivered by a lentiviral vector in primary cells was low. Furthermore, the *in vivo* model developed to examine huTat2 only represents acute but not chronic HIV-1 infection[93]. Another limitation of the use of intrabodies for HIV treatment is that a single chain antibody usually has low affinity to the target protein partly because of incorrect folding in the reducing environment of the cytoplasm[95]. More extensive clinical trials are required to improve this gene therapy efficacy.

2.7.2 *Tat/vpr* anti_HIV-1 specific ribozyme

Phase I and II clinical trials were conducted to examine safety and efficacy of gene therapy using a *tat/vpr* anti HIV-1 specific ribozyme (OZ1)[96,97]. Ribozymes are small RNA composite molecules that catalyse cleavage of phosphodiester link of complementary RNA fragments[98]. OZ1 contains a ribozyme that targets the overlapping *vpr* and *tat* RNA sequences. Using an MLV based vector, OZ1 was delivered to $CD34^+$ hematopoietic progenitor cells and transplanted to 38 HIV-1 infected patients[97]. The result showed no serious adverse effect up to 5 years post transplantation. Slightly decreased viral load (up to 10.000 copies/ml) was observed in OZ1 treated patients at week 40-48. However, low engraftment levels of OZ1 transduced $CD34^+$ cells (0.01%-0.38%) were detected in the peripheral blood [97]. Further studies are required to improve the efficacy of OZ1, for example by increasing the number of transduced $CD34^+$ cells to obtain better engraftment levels.

2.7.3 RevM10

Clinical studies using a Rev mutant protein as a therapeutic approach found that Rev M10 can inhibit HIV replication. RevM10 is a Rev mutant protein that acts as a transdominant negative protein against wild type Rev activity during HIV replication. RevM10 was created by substitution of two amino acids in the NES region of wild type Rev that interact with CRM1. Rev is an HIV regulatory protein that plays a role in nuclear localization, RNA binding and nuclear export[99]. During the HIV replication process, Rev binds to RRE, a stem loop RNA element located in the envelope coding region of the viral genome. The Rev-RRE complex mediates the transport of unspliced and singly spliced viral mRNAs from the

nucleus to the cytoplasm[100]. RevM10 multimerizes by forming a non-functional heteromeric complex with Rev and thus prevents wild type Rev from interacting with CRM1[101]. Introduction of RevM10 to human T cells inhibits Rev activity, preventing export of Rev-dependent viral mRNAs from the nucleus by disrupting CRM1 binding sites and consequently inhibiting HIV-1 replication[102].

Subsequent clinical trials of RevM10 in HIV-1 patients using non-viral and retroviral vectors showed the survival rate of $CD4^+$ T cells expressing RevM10 was higher than that of untreated cells[102,103]. This suggests RevM10 protects cells from HIV-1. However, a subsequent study found this therapy cannot eliminate viral loads in blood plasma because HIV-1 may develop mutations in a RRE region that alter the structure of RRE and thus causes resistance to RevM10[104]. Another clinical trial using $CD34^+$ cells transduced by RevM10 transplanted in HIV-1 patients showed the transplanted cells survived for four months but gradually diminished over two years[105]. Further study should focus on anticipating resistance to RevM10 as well as improving cell survival.

2.7.4 siRNA

Another gene therapy approach is downregulation of CCR5 expression using small interfering RNAs (siRNA). siRNA targeted gene silencing is achieved through the RNA interference (RNAi) pathway[106]. siRNA can be introduced into mammalian cells to induce degradation of specific intracellular RNA[40]. One form of siRNA that stably inhibits HIV-1 is short hairpin RNA (shRNA)[107]. A highly efficient shRNA called shRNA1005 has been found to downregulate CCR5 expression in systemic lymphoid organs in a mouse model without showing toxicity[107]. The gene knock-down was stably maintained long term as indicated by sustained downregulation of CCR5 expression in $CD34^+$ transplanted animals. *Ex vivo*, the downregulation of CCR5 by shRNA1005 was shown to be able to inhibit CCR5 HIV-1 infection[107]. Furthermore, a clinical study of downregulation of CCR5 by siRNA was carried out in patients undergoing transplantation for AIDS-related lymphomas. The expression of siRNA and ribozyme in peripheral blood samples was maintained up to 24 months after stem cell infusion and the viral loads were undetectable for patients maintained on cART treatment[108].

Recent studies utilized shRNA named shPromA to inhibit HIV-1 transcription was conducted by Kazuo and colleagues. It was reported that shPromA induced transcriptional gene

silencing (TGS) of HIV-1 by targeting conserved region in the tandem repeat NFκB sequence in the U3 region of HIV-1 5'LTR in MOLT-4 and Hela cells[109]. This shPromA is sequence specific to HIV-1 NFκB and therefore the possibility of off-target effects is minimum[110]. An *in vivo* study using a (NOD)/SCID/Janus kinase 3 (NOJ) knockout humanized mouse model showed that shPromA can prevent $CD4^+$ T cell depletion and lower the plasma viral load[111].

NFκB binding region is conserved in most HIV-1 subtypes, except for subtype C that can have three possible NFκB binding regions. Therefore, a double-stranded RNA (ds RNA) termed S4-siRNA was created subsequently to target subtype C HIV-1 promoter[109,112]. S4-siRNA induces TGS by histone methylation mechanism that forms heterochromatin of the HIV-1 LTR. This ds RNA showed long term suppression of HIV-1 subtype C in TZM-bl and peripheral blood mononuclear cells (PBMCs)[112].

These siRNA approaches can be promising to block HIV-1 transcription and enforce long term TGS suppression. However, HIV-1 may develop mutations by nucleotide substitution or deletion in or near the siRNA target sequence. Such mutations may cause mismatches with the siRNA or alter the RNA folding and prevent binding to siRNA so that HIV-1 can escape from siRNA mediated gene silencing[113].

2.7.5 Zinc Finger Nuclease

Zinc Finger Nuclease (ZFN) technology is useful for genome engineering of various organisms including human cells. ZFN induces a double strand break (DSB) in a specific target sequence that can be followed by desired gene modifications. ZFN has a region containing a Cys2-His2 zinc finger that can recognize three base pairs of DNA as a binding site, fused with a nuclease enzyme that can cut DNA at that binding site. Consequently, ZFN can be used to mutate a targeted gene in order to knockout the gene[114,115].

It has been reported that ZFN therapies can produce HIV-1-resistant $CD4^+$ T cells. A clinical study on 12 patients using ZFNs to knock out the CCR5 gene in $CD4^+$ T cells showed a significant increase in the $CD4^+$ T cell count of all the patients one week after modified $CD4^+$ T cell infusion[116]. The modified $CD4^+$ T cell half-life was approximately 48 weeks. Modified $CD4^+$ T cells were found to survive longer than unmodified $CD4^+$ T cells and the viral load in the blood decreased after the treatment. This research also found that T cells were mostly

found in mucosal tissues, as indicated in rectal biopsy specimens. These findings suggest that the modified cells were engrafted and that the CCR5 gene deletion provides protection to $CD4^+$ T cells against HIV infection. However, adverse events following this treatment were also found. These included fever, chills, joint and back pain[116].

2.7.6 Nullbasic

A novel transdominant negative HIV-1 Tat protein termed Nullbasic has been shown to have a strong antiviral activity against HIV-1 infection *in vitro*. Nullbasic is considered to be a Tat transdominant negative protein because it can block the activity of wild type Tat. Nullbasic has a unique ability to potently inhibit multiple steps of HIV-1 replication. Nullbasic is a mutant of Tat, a small viral regulatory protein critical for enhancing the HIV-1 transcription efficiency from the LTR transcriptional promoter[13]. Wild type Tat has an arginine-rich basic domain with an important role in mediating TAR RNA binding. In Nullbasic, this domain contains glycine and alanine residue substitutions (Figure 2-6).

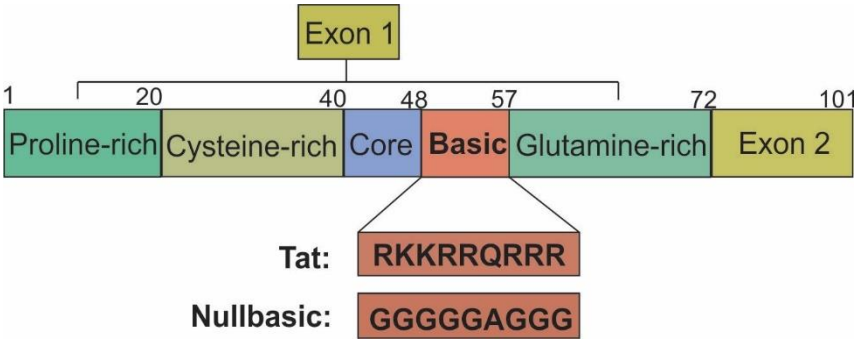

Figure 2-6. Schematic of Tat and Nullbasic domains
Tat protein is encoded by two exons. The first exon encodes five domains, as shown, including a basic domain rich in arginine. In Nullbasic, the basic domain is mutated to be glycine rich with an alanine residue.

Nullbasic has potential as a therapy to prevent the spread of HIV infection, showing strong inhibition of HIV-1 replication *in vitro* in primary $CD4^+$ T lymphocytes[15]. Nullbasic interferes with HIV-1 replication by targeting three steps in the HIV-1 life cycle, including Tat-mediated transactivation, Rev-mediated transport of HIV-1 mRNA[16], and reverse transcription[15]. Nullbasic obstructs Tat interaction with P-TEFb during early transcription because Nullbasic can compete with Tat for binding to P-TEFb, and therefore it is considered as a transdominant negative inhibitor of wild type Tat. Unlike Tat, Nullbasic does not activate HIV-1 transcription[15,17]. Furthermore, Nullbasic has been shown to inhibit HIV-1 reverse transcription, resulting in the suppression of virion infectivity. Nullbasic binds directly to RT

subunit p51 as well as p66 and disrupts the reverse transcription complex, thereby causing premature viral uncoating[15]. Nullbasic has also been shown to interfere with Rev nucleocytoplasmic transport activity and thereby disrupt the Rev nucleolar organisation and functions. Recently, it was shown that Nullbasic specifically targeted the cellular RNA helicase DDX1, which is a Rev binding protein that regulates its function in mRNA transport. DDX1 directly interacts with the multimerization domain of HIV-1 Rev protein to promote Rev oligomerization on the RRE[16]. As a consequence, Nullbasic downregulates the expression of unspliced and singly-spliced HIV-1 mRNAs and therefore inhibits virus replication[15].

However, the antiviral activity of Nullbasic against HIV-1 has only been tested on a laboratory clade B virus strain[12]. Genome variations between different clades may result in amino acid substitutions in viral proteins that can influence susceptibility of the virus to antivirals[84]. Hence, more research into Nullbasic antiviral activity against different clades of HIV-1 is necessary.

2.8 Humanized Mouse Models for HIV Research

In order to generate preclinical data about promising HIV gene therapy approaches, appropriate animal models are required. Nonhuman primates such as chimpanzees are genetically and physiologically similar to humans. In term of disease susceptibility, a chimpanzee is the only non-human animal that is naturally susceptible to infection with HIV and therefore is considered as the gold standard of animal model for HIV. However, the use of this primate for biomedical research is prohibited in many countries due to ethical issues. This leads to generation of small animal models to represent the human biological system and disease susceptibility, such as humanized mouse models. The humanized mouse model is less laborious and less costly than the primate model.

Humanized mouse models are immunodeficient mice engrafted with functional or modified human cells or tissues or human transgenes[117]. Based on the aim of experiments, different kinds of humanized mouse models are utilized. For example, mice transplanted with PBMC obtained from human blood allow investigation of human $CD4^+$ T cell depletion, viral load levels and the effect of therapeutic interventions against HIV infection. This model provides rapid and reproducible data in HIV therapy research[118]. The mice can also be transplanted

with hematopoietic stem cells (HSC) isolated from human cord blood or fetal liver to study a completely naïve immune system during HIV infection. This model sustains human hematopoietic cell engraftment over a longer time frame[118].

The discovery of humanized mouse models in HIV-1 research was summarized in Table 2-2. Severe combined immunodeficiency (SCID) mutation in mice has led to the development of a humanized mouse model (hu-PBL-SCID) through grafting of human peripheral blood lymphocytes (PBL)[119]. Because of the SCID mutation, these mice lack functional T cells and B cells and hence are unable to mount immune responses to reject xenogeneic transplants[119]. The potential of this model to support a pathogen that replicates in human cells such as HIV was investigated, but studies indicate this model only shows a low level of human cell engraftment (0.5-5%) and is unable to mimic human adaptive immune responses against HIV-1 infection[120]. In addition, at 10-14 weeks old, SCID mice become leaky and generate unwanted functional T and B cells as well as natural killer (NK) activity[120]. In order to eliminate NK activity, a non-obese diabetic SCID (NOD-SCID) mouse model was developed. Unfortunately, the NOD mutation often causes thymic lymphomas that shorten the life span of the mice[121].

As an alternative, a new generation of humanized mouse models was developed through mutation of recombination-activating gene (RAG). RAG1 and RAG2 proteins play an important role in forming an enzyme complex that joins T and B cell receptor genes. The RAG deficient mice were not leaky in T and B cell production and less radiosensitive compared to the SCID mice. Yet, RAG1 and RAG2 mice generate unwanted NK cells so mutations of common gamma chain receptor (γ_c) to prevent NK cell maturation were also required. The gamma chain is an important subunit protein of interleukin receptors, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. The lack of IL-2 and IL-7 signaling prevents T and B cell development as well as NK cell maturation[122]. This resulted in BALB/c-Rag1$^{-/-}\gamma c^{-/-}$ and BALB/c-Rag2$^{-/-}\gamma c^{-/-}$ that are susceptible to PBL or hematopoietic stem cell (HSC) transplantation and HIV infection.

The BALB/c-Rag2$^{-/-}\gamma c^{-/-}$ mouse is susceptible to transplantation of human PBL or HSC. The level of human cell engraftment ranged from 5-85%. Humanized mice grafted with HSC are receptive to infection of R5 and X4 tropic HIV-1, and exhibit prolonged viremia (up to 30 weeks) and $CD4^+$ T cell depletion as in humans. Also, BALB/c-Rag2$^{-/-}\gamma c^{-/-}$ mice develop a

functional human immune system[123,124]. Thus, this model is useful for investigating gene therapy against HIV-1.

Table 2-2. Humanized mouse models for HIV infection studies

Mouse Model	Advantages	Limitations
SCID	Lacking B cells and T cells	Low levels of engraftment Unable to mimic human adaptive immune responses against HIV-1 After 10-14 weeks can generate B cells, T cells and NK Radiosensitive
NOD-SCID	Lacking NK	Develop thymic lymphoma Radiosensitive
RAG1 and RAG2	Lacking B cells and T cells Non-radiosensitive	Generate NK

Gene therapy may be the answer for a functional cure of patients with HIV-1/AIDS. Have been extensively studied, Nullbasic, with its combined antiviral mechanisms, can be a promising gene therapy agent against HIV-1. In the next chapter, Nullbasic antiviral activity against diverse HIV-1 strains from different subtypes will be elucidated.

CHAPTER 3

Nullbasic Inhibits Replication of Different HIV-1 Strains from Diverse Subtypes in Human Cells

3.1 Introduction

The HIV-1 pandemic remains a huge social and economic burden. By 2014, 36.9 million people were living with HIV and 1.2 million AIDS related death cases were reported[4]. One of the major obstacles in treating this disease is the high genetic diversity of HIV-1 that leads to different rates of disease progression and drug resistance to antiviral drugs[11,125]. We have investigated an anti-HIV-1 agent that targets three different steps of virus replication by targeting viral and cellular proteins, and therefore may have efficacy against HIV-1 with diverse genetic backgrounds.

The agent is a Tat mutant protein derived from subtype B HIV-1_{BH10} that strongly inhibits HIV-1 replication in human cells[15], and is referred to as Nullbasic. Wild type Tat is an essential HIV-1 protein required for transactivation of the HIV-1 LTR promoter, resulting in high levels of viral mRNA transcription by RNA polymerase II[126]. It also plays a role in HIV-1 reverse transcription[127,128]. HIV-1 virions lacking *tat* gene were unable to initiate reverse transcription[128]. Furthermore, recombinant Tat proteins were able to enhance reverse transcription in a RNA independent manner, through Tat-RT interaction[127]. Tat is also involved in other cellular processes, such as immune suppression and induction of inflammatory cytokines[41,129,130]. Tat causes immune suppression by binding to CD26, a T cell activation marker that plays a role in lymphocyte growth, as well as inhibiting dipeptidyl peptidase IV, an exopeptidase important for lymphocyte proliferation and activation[42]. Induction of inflammation by Tat occurs via by stimulating production of IL-6 and IL-8 cytokine production in dendritic cells and monocytes via the NF-kB activation pathway[131]. Tat can also induce cell apoptosis by hyperactivation of $CD8^+$ T cells[122,132].

Nullbasic, which has been described previously[2,15,127], has a substitution mutation spanning the entire basic domain; amino acids 49 to 57, RKKRRQRRR, are replaced with

GGGGGAGGG. Studies show that Nullbasic is expressed in cells and located in the nucleus and cytoplasm[16] , and inhibits HIV-1 replication by: 1) inhibiting HIV-1 transcription by RNA polymerase II through interaction with p-TEFb and causing epigenetic silencing of the HIV-1 LTR [2,14,15]; 2) inhibiting Rev-dependent viral mRNA transport from the nucleus by binding to DDX1[3,16], and; 3) inhibiting reverse transcription by directly interacting with reverse transcriptase, leading to accelerated uncoating kinetics post-infection and defective viral DNA synthesis[18].

HIV-1 sequence diversity is categorized by HIV-1 subtypes that are defined by comparisons of *env* genes. These subtype variations can also be observed as differences in viral proteins, such as Tat, Rev and RT. Amino acid sequence variation in the viral proteins of various HIV-1 subtypes can affect virus replication and virulence[133]. For example, the RT from subtype C isolates differs from subtype B by ~7–10%, which can affect drug susceptibility and cause drug resistance[133]. Tat proteins from different subtypes can vary up to 40% without significantly affecting Tat transactivation ability[134], but the effects on alternative functions of Tat[135] have not been studied in detail.

To date, Nullbasic antiviral activity has only been tested against HIV-1 subtype B strains such as HIV-1_{NL43}[14,15]. However, subtype B strains only accounts for ~10% of HIV-1 infections globally and HIV-1 Tat sequences vary between subtypes, especially for subtype C, which is responsible for ~50% HIV-1 infection worldwide[136,137]. Subtype C is predominant in sub-Saharan Africa, India and South America[75], while subtype D and recombinant A/D are increasing in sub-Saharan Africa[20,138]. Whether sequence variations in different HIV-1 subtypes alter the antiviral effect of Nullbasic has not been examined. These differences could influence the ability of Tat to interact with RNA and cellular proteins and thus could affect the antiviral activity of Nullbasic. Therefore, in this study, the ability of Nullbasic to inhibit replication of HIV-1 strains from different subtypes, including subtypes C, D and A/D recombinant, was evaluated.

In this study, we examined Nullbasic antiviral activity against those HIV-1 strains in TZM-bl cell line as well as in primary $CD4^+$ T lymphocytes. To enable protein expression detection in targeted cells, Nullbasic was tested in the form of fusion proteins as Nullbasic-mCherry (NB-mCh) and Nullbasic-ZSGreen1 (NB-ZSG1). The NB-mCH and NB-ZSG1 genes were delivered to TZM-bl cell lines and primary $CD4^+$ T cells by a gammaretroviral vector. Our

results show that Nullbasic inhibited replication of the HIV-1 strains tested in both cell lines and primary cells.

3.2 Materials and Methods

3.2.1 Cell lines and cultures

HEK 293T(ATCC), TZM-bl[139,140] and Phoenix-Ampho[141] cell lines were grown in Dubelcco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% ($^{v}/_{v}$) fetal bovine serum (FBS), penicillin (100 IU/ml) and streptomycin (100 µg/ml) (referred to as DF10 medium). TZM-bl expressing NB-mCh and mCh cell lines were established by transduction of NB-mCh and mCh gammaretroviral virus like particles (VLPs) respectively and then selected by FACS for the top 10% of mCherry positive cells by mean of fluorescent intensity (MFI).

3.2.2 Plasmids constructs

pSRS11-SF-γC-EGFP was obtained from Axel Schambach and Christopher Baum[142]. pSRS11 -SF-γC-NB-mCh, pSRS11-SF- γC-mCh, pSRS11-SF-γC-NB-ZSG1 and pSRS11-SF-γC-ZSG1 constructs were made by replacing EGFP in pSRS11-SF-γC-EGFP with NB-mCh, mCh, NB-ZSG1 and ZSG1 respectively. A proviral plasmid pGCH (GenBank accession number AF324493) was previously described to make HIV-1_{NL43}[16]. The proviral plasmid pZAC (GenBank accession number JN188292.1) was obtained from Jochen Bodem[143]. The proviral plasmids pELI and pMAL (Los Alamos accession number A07108 and A07116 respectively) were provided by Damian Purcell[144]. The exon *tat* genes with HA epitope were synthesized by GenScript and ligated into pcDNA3.1^{+} plasmid (Thermofisher Scientific).

3.2.3 HIV-1 and VLP production

HIV-1 subtype B, C, D and A/D recombinants were produced from pGCH, pZAC, pELI and pMAL proviral plasmids respectively. HEK 293T cells were grown on a 10 cm plate at ~80% confluency and transfected with 10 µg of each proviral plasmid, then incubated for 24h at 37 °C. On the next day, the transfected cells were washed with 1 x phosphate buffered saline (PBS) and the DF10 media was replaced. The supernatant containing HIV-1 VLPs

was collected 48 and 72 hours post transfection and the amount of CA in each supernatant was measured by ELISA (Zeptometrix), as recommended by the manufacturer.

NB-mCh or mCh or NB-ZSG1 or ZSG1 VLPs were produced in Phoenix-amphotropic retroviral packaging producer cell line by cotransfection of 7.5 µg of SRS11-SF-γC vector expressing NB-mCh or mCh or NB-ZSG1 or ZSG1 and 1.5 µg of Gag-Pol expressing plasmid using 9µl X-tremeGENE^{TM} DNA transfection reagent (Roche) in a 10 cm plate. Six hours post transfection, the cells were gently washed with PBS and the media was replaced. The VLPs were collected 48 and 72 hours post transfection and filtered through a 0.45 µm filter.

3.2.4 Western blot analysis

Cell lysates were made from 5×10^6 NB-mCh or mCh-TZM-bl cell lines in cell lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% ($^{v}/_{v}$) Triton X-100). The total protein concentration was measured by a Bradford assay using Bio Rad protein assay (Bio Rad) and equivalent amounts of protein were used for analysis. The blot was stained with anti *α*-mCherry rabbit antibody (BioVision) followed by anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology) and β-tubulin antibody (Sigma Aldrich) followed by anti-mouse IgG HRP-linked antibodies (Cell Signaling Technology).

3.2.5 Transactivation assay

Tissue culture dishes (6 cm) were seeded with 5×10^5 TZM-bl cells expressing NB-mCh or mCh and then co-transfected with 1 µg of each subtype Tat plasmid and 150 ng of Gaussia luciferase expression plasmid. After 48 hours, the cells were washed with PBS and cell lysates were made using Glo Lysis buffer (Promega). Luciferase assays were performed in 96-well white polystyrene microplates, as per the manufacturer's instructions, using 10 µl of the cell lysates and Dual-Glo® luciferase substrate (Promega). Luciferase activity in each sample was measured within 20 minutes by using a luminescence microplate reader and relative values were normalized to Gaussia luminescence in the sample.

Next, 3×10^5 of non-transduced (NT) TZM-bl cells or TZM-bl cells expressing NB-mCh or mCh were seeded in 6-well plates. The next day, the cells were infected with HIV-1_{NL4-3} (subtype B) or HIV-1_{ZAC} (subtype C)[143] orHIV-1_{ELI} (subtype D) or HIV-1_{MAL} (recombinant A/D subtype) virus supernatant containing 20 ng CA for 48 hours. The cells were washed with

PBS and then cell lysates were made using Glo Lysis buffer (Promega). Luciferase activity was measured as described above.

3.2.6 Transduction of NB-ZSG1 or ZSG1 VLPs in CD4$^+$ T cells

Primary CD4$^+$ T cells were purified from PBMCs that were isolated from healthy donor's buffy coat. The CD4$^+$ cells purity was determined by flow cytometry using anti-human CD4 antibody. The purified CD4+ T cells were then stimulated using human CD3 and CD28 antibodies for 48 hours. NB-ZSG1 or ZSG1 VLPs were concentrated using the precipitation method, through the addition of 20% ($^v/_v$) of 34% polyethylene glycol 8000 (Sigma Aldrich) and 10% ($^v/_v$) of 0.3M sodium chloride solution. The solution mixture was incubated at 4 °C for 1.5 hours, mixed every 30 minutes and then centrifuged at 1500 $\times$ g for 1 hour at 10 °C. The supernatant was discarded and the precipitate was resuspended in 600 µl RF20 IL-2 medium. The concentrated VLPs (150 µl) were added to Retronectin (Takara) coated 24-well plate and incubated at 37 °C for 30 minutes. 5×10^5 stimulated CD4$^+$ T cells were added to each well and incubated for 3 days. Transduced cells were processed by FACS to collect ZSG1 positive cells, which were grown for 3 more days. The RF20-IL2 media was replaced every day.

3.2.7 Infection of HIV-1$_{NL4-3}$ (subtype B), HIV-1$_{ZAC}$ (subtype C), HIV-1$_{ELI}$ (subtype D) and HIV-1$_{MAL}$ (A/D recombinant subtype) in TZM-bl cell lines and primary CD4$^+$ T cells

NT TZM-bl cells or TZM-bl cells expressing NB-mCh or mCh (3×10^5 cells/well) cultured in 6-well culture dishes were infected with a virus stock containing 20 ng CA of HIV-1$_{NL4-3}$, HIV-1$_{ELI}$ and HIV-1$_{MAL}$ or 40 ng CA of HIV-1$_{ZAC}$ for 2 hours at 37 °C. A larger amount of HIV-1$_{ZAC}$ was required to yield measurable infections. The virus was then removed by washing 3 times with PBS and the infected cells were cultured at 37 °C with 5% of CO_2. The culture supernatants were sampled on day 3 and 5 post infection. The amount of HIV CA present was measured using a CAp24 ELISA kit (Zeptometrix) according to the manufacturer's instruction.

Stimulated primary CD4$^+$ T cells (5×10^5 NB-ZSG1 or ZSG1 or NT) were infected with virus stocks containing 2 ng CA of each HIV-1 subtype for 2 hours at 37 °C. After infection, the cells were washed with PBS and cultured in RF20 IL-2 medium. Cell and supernatant samples were collected on days 0, 3, 7, 10 and 14 by centrifugation at 500 $\times$ g for 5 minutes. The amount of viral CA in the supernatant was measured by ELISA. The cells were fixed

with 1% paraformaldehyde in PBS solution and NB-ZSG1 or ZSG1 expression was measured by flow cytometry.

3.2.8 Cytotoxicity assays

Cell metabolic activity was measured by MTS using a CellTiter 96® aqueous one solution cell proliferation reagent (Promega) according to the manufacturer's instructions. Cell proliferation was quantified using a Violet Proliferation Dye 450 (BD Horizon™) assay in accordance with the manufacturer's instructions, and violet fluorescence was measured using a violet laser-equipped BD LSRFortessa™ IV flow cytometer. Apoptosis events were quantified using a PE Annexin V apoptosis detection kit (BD Pharmingen™), as per the manufacturer's instructions. Camptothecin (Sigma Aldrich), which strongly induces apoptosis in $CD4^+$ T cells, was used as a positive control.

3.2.9 Statistical analysis

Mean values of percentage of transactivation inhibition between strains were compared using Kruskal-Wallis one-way analysis of variance. A 95% confidence interval was used, therefore a p value less than 0.05 was considered to be significant.

3.3 Results

3.3.1 Inhibition of transactivation and replication of diverse HIV-1 subtypes in TZM-bl cells by Nullbasic-mCherry (NB-mCh) fusion protein

A schematic diagram of Nullbasic protein and the basic domain mutations (amino acids 49-57) to glycine and an alanine are shown in Figure 3-1 (a). A NB-mCh fusion protein, used previously to investigate Nullbasic inhibition on HIV-1 Rev activity[3], was inserted into a SIN gammaretroviral vector SRS11-SF-γC (Fig. 3-1b)[142]. VLPs produced in Phoenix amphotropic packaging cells[145] were used to transduce TZM-bl cells and those stably expressing NB-mCh or mCh, respectively, were collected by FACS. Expression of NB-mCh and mCh was confirmed by flow cytometry analysis of the purified cells (Fig. 3-1c) and Western blot analysis using an anti-mCherry antibody (Fig. 3-1d).

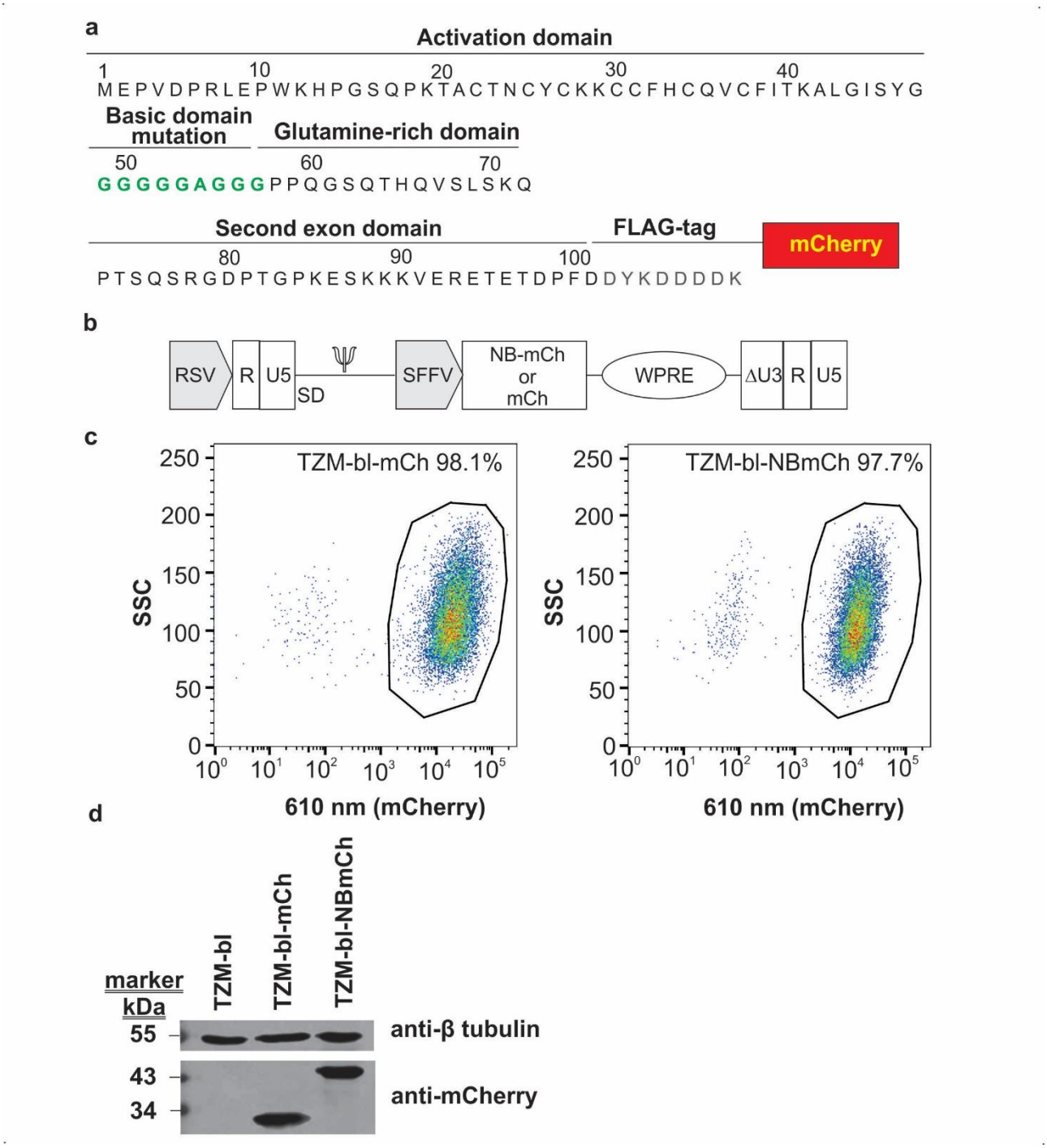

Figure 3-1. Expression of NB-mCh in TZMBL cells
(a) A schematic of the NB-mCh amino acid sequence. The basic domain of wild type Tat was mutated to glycine or alanine residues as shown (green) and other domains are labeled. (b) A schematic of SIN gammaretroviral SRS11-SF-γC vector showing NB-mCh or mCh expressed by a Spleen forming focus virus (SFFV) internal promoter. Rous Sarcoma Virus (RSV) promoter is used to transcribe mRNA for packaging in VLPs. The woodchuck hepatitis virus post transcription regulatory element (WPRE) was inserted adjacent to the NB-mCh or mCh to enhance the gene expression. (c) Purity of selected TZM-bl. TZM-bl cells expressing these proteins were collected by FACS, as shown. d. NB-mCh and mCh expression in TZM-bl cells expressing NB-mCh or mCh analyzed by flow cytometry. (d). NB-mCh and mCh expression in TZM-bl cells was detected by Western blot using anti-mCherry antibody. The blot was also stained using an anti-β tubulin antibody.

It is worth reviewing the amino acid sequence heterogeneity of Tat by comparing the alignment of Tat proteins from HIV-1_{NL4-3} (subtype B), HIV-1_{ZAC} (subtype C)[143], HIV-1_{ELI} (subtype D) and HIV-1_{MAL} (a recombinant A/D subtype). Amino acid substitutions occur in all domains except for amino acids 43 to 56 (Fig. 3-2, boxed), which includes a completely conserved basic domain. The first 20 residues of each Tat protein include 17 positions that are conserved or functionally similar. The carboxyl terminal amino acids 90 to 100 of subtype C and D Tat proteins have conserved sequences KKKVE and ETDP (Fig. 3-2, underlined), which are also present in Nullbasic (derived from HIV-1_{BH10}). All four Tat proteins maintained the cysteine residues in the cysteine-rich domain with one exception; HIV-1_{ZAC} (subtype C) has a C31S substitution (Fig. 3-2, blue circled).

Tat mediates HIV-1 transactivation by binding to TAR RNA in the R region of HIV-1 LTR and recruiting P-TEFb[49], which in turn binds a super elongation complex (SEC)[146,147]. P-TEFb consists of Cyclin T1 and CDK9[49]. In Tat, K41 is important for intramolecular hydrogen bonding and structural integrity of the Tat core[48] and is present in all Tat proteins shown except in HIV-1_{ZAC} which has T41 (Fig. 3-2, blue circled). A crystal structure of the Tat:P-TEFb complex showed that the surface of 37% amino acids 1–49 are complementary to the kinase complex, and this model indicates that the interactions between Tat and P-TEFb can accommodate substitutions commonly present in different Tat genes[48]. However, Tat interacts with other cellular proteins, many of which are important for HIV transcription, for example cyclin-T1, a regulatory subunit of pTEF-b CTD kinase complex[148]. Therefore, it is possible that these subtle differences could affect the ability of Nullbasic to inhibit the transactivation by Tat from other HIV-1 strains shown.

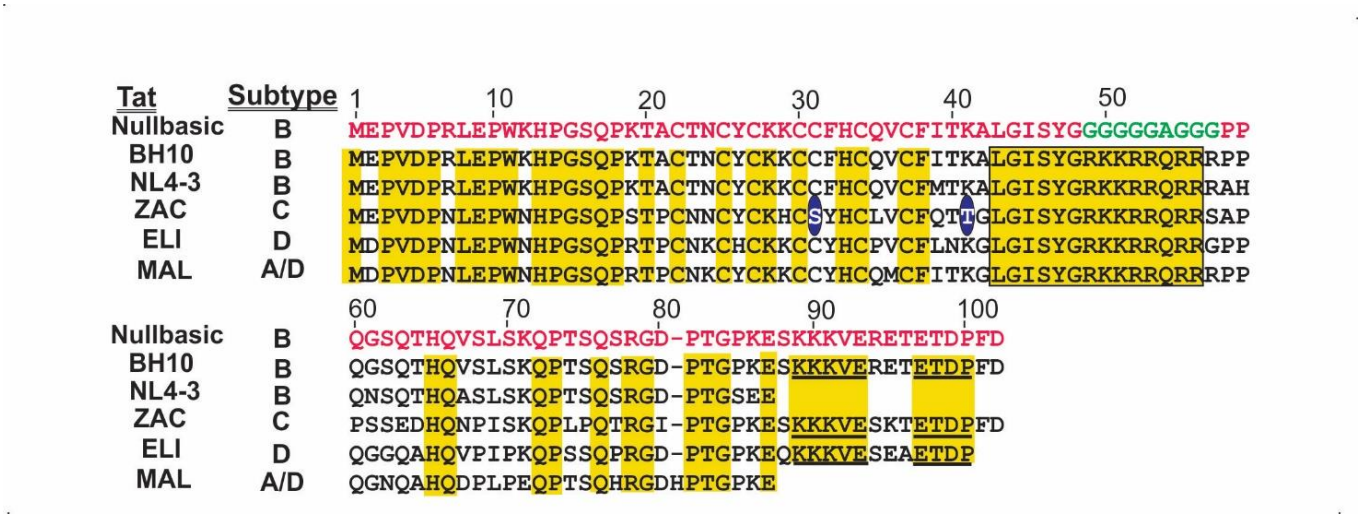

Figure 3-2. A comparison of the NB amino acid sequence against an amino acid sequence alignment for Tat_{BH10}, Tat_{NL43}, Tat_{ZAC}, Tat_{ELI} and Tat_{MAL}
Amino acids shown in yellow are conserved for the Tat variants. Tat_{ZAC} S31 and T41 residues are circled (blue). The basic domain is indicated as a black rectangle.

To test if transcription by different Tat varieties can be inhibited by Nullbasic, TZM-bl-NB-mCh and TZM-bl-mCh cell lines stably carrying a HIV-1-LTR firefly luciferase reporter were co-transfected with eukaryotic expression plasmids that express either Tat_{BH10}, Tat_{ZAC}, Tat_{ELI}, Tat_{MAL} or an empty expression plasmid and a Gaussia luciferase expression plasmid Gaussia luciferase is used to control for transfection efficiency. Tat can activate the HIV-1-LTR firefly luciferase reporter in TZM-bl cell lines, while Gaussia luciferase reporter enables linear quantification of relative transfection efficiencies between samples[149]. The amount of firefly luciferase activity present in lysates from the transfected TZM-bl cells was measured and the relative luminescence unit (RLU) values were normalized to Gaussia luciferase RLUs measured in the culture supernatant. The results show that expression of NB-mCh in TZM-bl-NB-mCh cells reduced transactivation of the HIV-1 LTR luciferase reporter in all subtypes tested from ~70 to ~90% (Fig. 3-3). The data shown is a representative from two independent experiments with similar results, each experiment was carried out in triplicate. In future, at least one more set of experiments should be done to confirm the results. Although Tat_{ZAC} showed a trend towards less inhibition than others, this difference did not achieve statistical significance. Overall, the level of inhibition observed is similar to that of Nullbasic in a previous report[15].

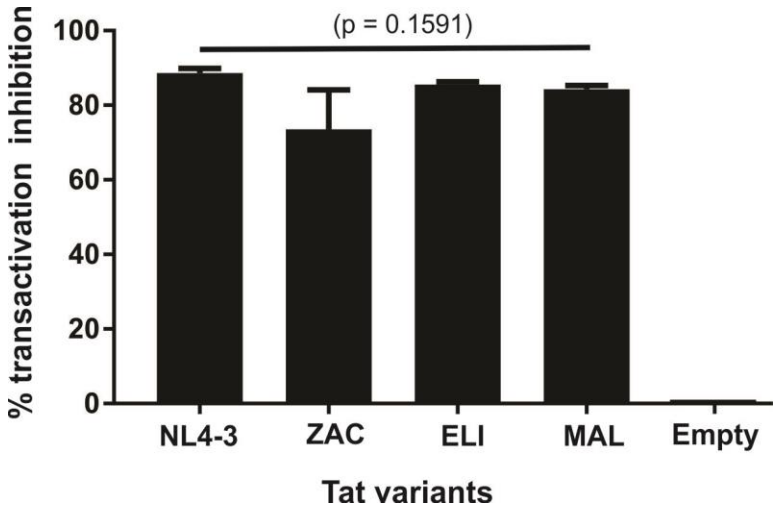

Figure 3-3. NB-mCh inhibits Tat-mediated transactivation after transfection of TZM-bl by Tat variants from different strains
TZM-bl cell lines expressing NB-mCh or mCh were transfected with pCDNA3.1+ containing each Tat variant or with empty Pcdna3.1+. A Gaussia luciferase expression plasmid was co-transfected as a control for transfections efficiency. Luciferase activity was measured 48 hours post transfection. Bars indicate mean percentage of transactivation inhibition by NB-mCh compared to that of mCh. The p value of the data set is shown. Data shown is representative from two independent experiments each carried out in triplicate.

The HIV-1 LTR reporter in TZM-bl cells is based on a subtype B LTR promoter. Sequence alignment of TAR shows variations among the strains, as seen in Figure. 3-4(a), especially for MAL TAR, where two deletions occurred and changed the bulge structure. Predicted structures of bulge and stem loop of the TAR RNA shown in Figure 3-4 (b) indicate variations of TAR RNA bulge and stem loop structures between different HIV-1 strains. These variations could affect TAR RNA function during transactivation by Tat or inhibition of transcription by Nullbasic. Consequently, we tested if each subtype-specific proviral encoded Tat protein could transactivate the associated proviral HIV-1 LTR promoter in the presence or absence of NB-mCh.

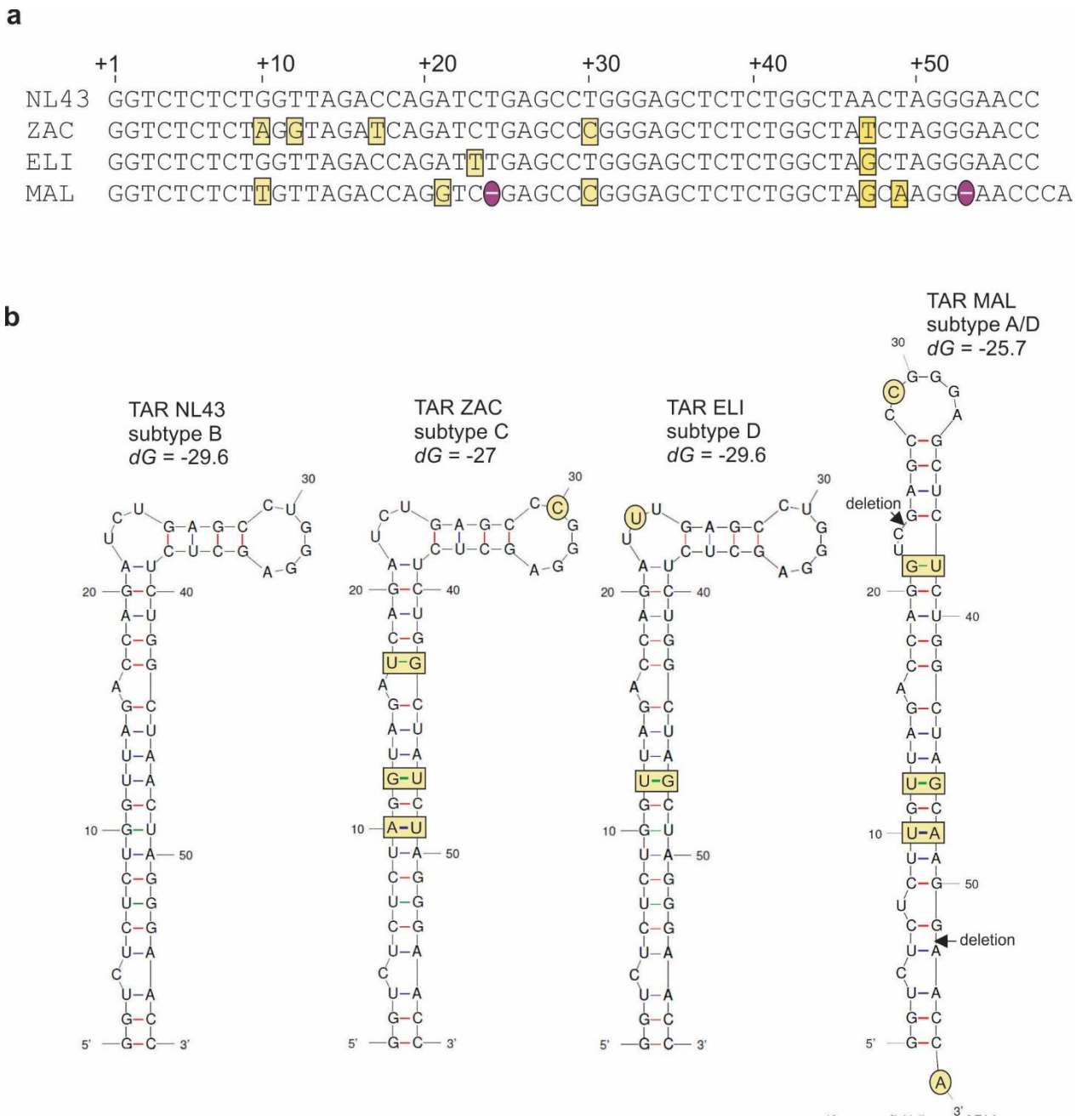

Figure 3-4. TAR RNA sequence and secondary structure.
(a) DNA sequences of TAR_{NL43}, TAR_{ZAC}, TAR_{ELI} and $TAR_{MAL.}$ TAR_{MAL}: TAR_{MAL} has deletions at nucleotides 24 and 53, relative to TAR $_{NL43}$ (circled in purple). Boxes indicate nucleotide polymorphisms in each TAR sequence that affect RNA structure stability relative to TAR NL43. Similarly, nucleotide changes in the bulge or loop region are indicated by yellow circles. (b) The predicted secondary structure determined by Mfold of TAR_{NL43}, TAR_{ZAC}, TAR_{ELI} and TAR_{MAL}: the relative free energy (dG) calculated by Mfold for each structure is shown. Tat interacting residues are located on the bulge structure, nucleotides 22-24.

Post infection, the integrated provirus produced Tat that can activate the HIV-1-LTR luciferase reporter in TZM-bl cells, which is an indicator of virus infection and replication. To evaluate Nullbasic inhibition on Tat-mediated transactivation of different HIV-1 strains, we infected TZM-bl-NB-mCh and TZM-bl-mCh cells with HIV-1_{NL4-3} (subtype B), HIV-1_{ZAC}

(subtype C), HIV-1_{ELI} (subtype D) and HIV-1_{MAL} (recombinant A/D subtype) produced in HEK293T cells or with mock supernatant. Lysates prepared from HIV-1 infected TZM-bl cells 48 hours post infection were assayed for firefly luciferase activity and the results were normalized to total protein concentration in the cell lysates. NB-mCh significantly reduced the amount of RLUs produced compared to control lysates made from infected TZM-bl-mCh cells. HIV-1_{NL4-3} viral Tat transactivation of the LTR-luciferase reporter was inhibited by ~97%; slightly lower inhibition of transactivation was measured for all other HIV-1 strains: HIV-1_{ZAC} by ~90%, HIV-1_{ELI} by ~89% and HIV-1_{MAL} by 91% (Fig. 3-5). However, difference between transactivation inhibition in HIV-1_{NL43} and other strains did not achieve statistical significance. The combined above experiments demonstrate that NB-mCh can inhibit transactivation of the TZM-bl LTR reporter by each Tat tested, albeit at a slightly reduced level compared to HIV-1_{NL-43}.

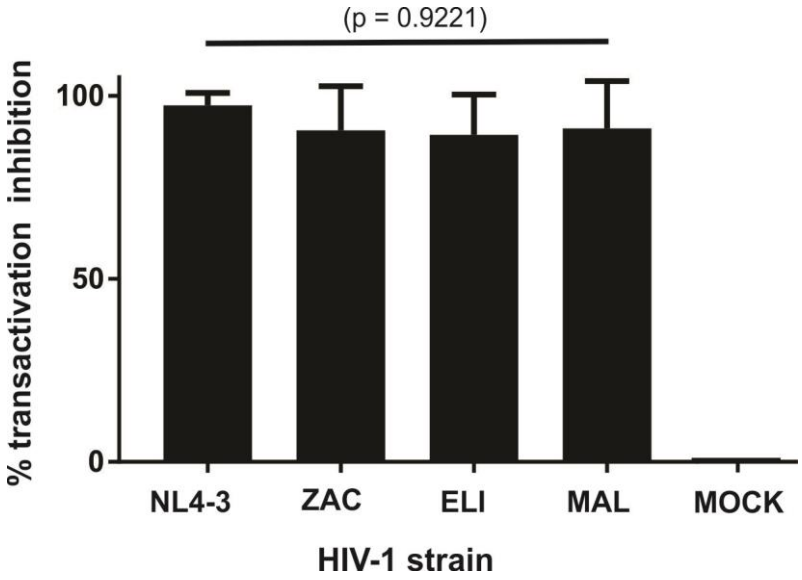

Figure 3-5. NB-mCh inhibits Tat-mediated transactivation after infection of TZM-bl by different HIV-1 strains from different subtypes
TZM-bl cell lines expressing NB-mCh or mCh were infected with each HIV-1 strain. Luciferase acivity was measured from the cell lysates 48 h post infection and normalized by total protein concentration in the samples. Bars indicate mean percentage of transactivation inhibition by NB-mCh compared to mCh. Error bars indicate standard deviation of three independent experiments carried out in triplicate. The p value of the data set is shown.

Finally, we tested if the presence of NB-mCh could inhibit viral transactivation and replication (rather than assessing effects on the integrated HIV-1 LTR-luciferase reporter in the cell lines) of each HIV-1 strain in the TZM-bl cell lines. In this 5-day experiment, detection of CA by ELISA requires virus replication. Therefore, TZM-bl-NB-mCh and TZM-bl-mCh cells were infected and supernatants were collected on day 3 and 5 post infection, and the amount of

CA in each sample was by measured by ELISA. As shown in Figure 3-7 a-d, the data shows that each HIV-1 strain replicated in NT TZM-bl and TZM-bl-mCh cells, with an increasing level of CA from day 3 to 5. However, HIV-1 replication of all strains was inhibited in TZM-bl-NBmCh cells. Comparing day 5 CA levels by TZM-bl-NB-mCh to TZM-bl-mCh cells show the production of HIV-1 CA levels dropped in TZM-bl-NB-mCh by >99% for HIV-1_{NL43}, ~97% for HIV-1_{ZAC}, ~98% for HIV-1_{ELI}, and ~97% for HIV-1_{MAL}. The combined results support the hypothesis that Nullbasic can inhibit virus replication of all the HIV-1 strains tested in TZM-bl cells.

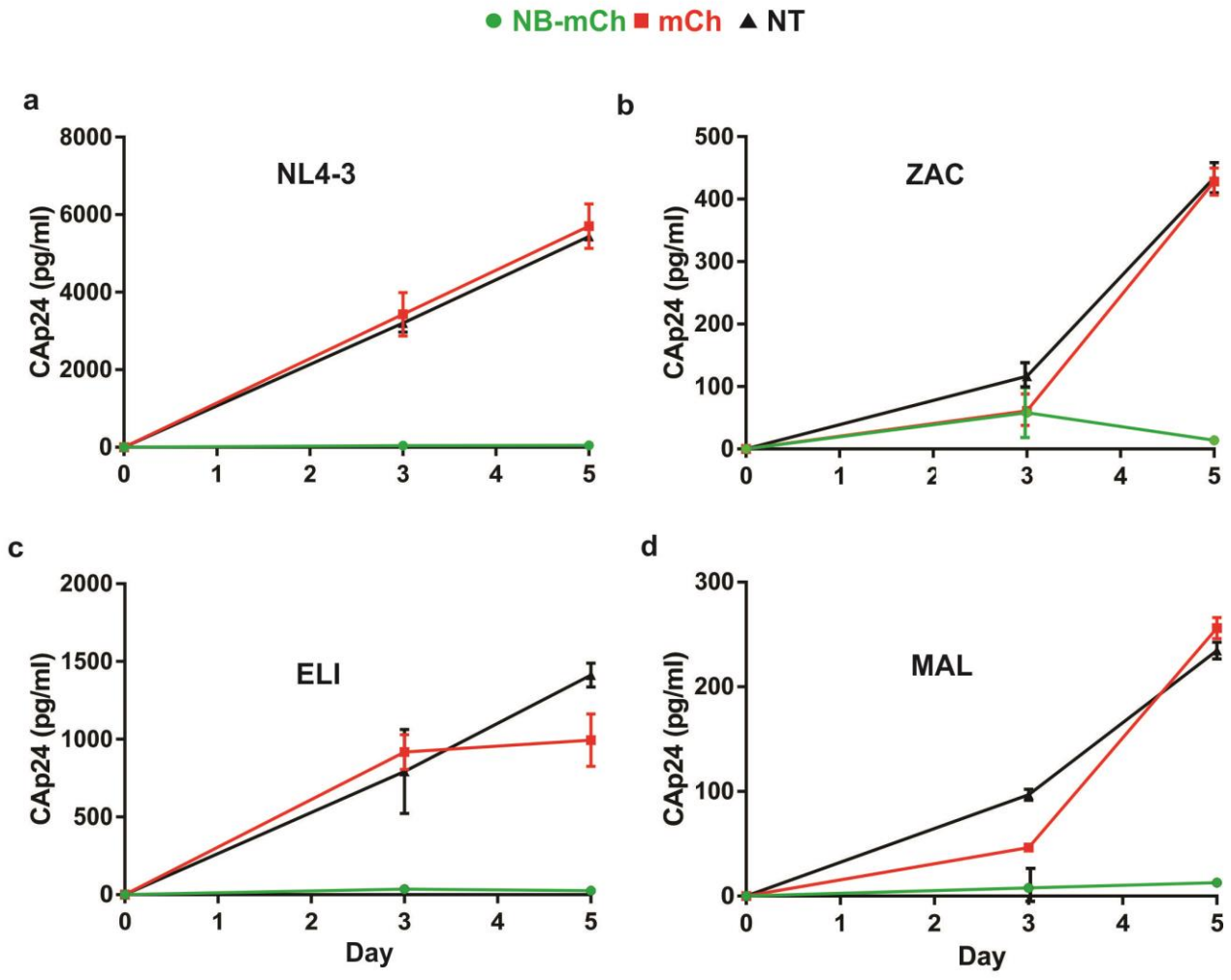

Figure 3-6. NB-mCh inhibits virus replication of all the strains tested in TZM-bl cells
TZM-bl cell lines expressing NB-mCh or mCh or NT were infected with each HIV-1 strain. (a) HIV-1_{NL4-3} (subtype B), (b) HIV-1_{ZAC} (subtype C), (c) HIV-1_{ELI} (subtype D) and (d) HIV-1_{MAL} (recombinant A/D subtype). On day 3 and 5 post infection, culture supernatant was assayed for HIV-1 CA by

ELISA. The experiment was performed in triplicate and mean values and standard deviations are shown from two independent experiments.

3.3.2 Antiviral activity of NB-mCh and Nullbasic-ZsGreen1 (NB-ZSG1) in primary CD4$^+$ T cells against 4 HIV-1 subtypes

To evaluate Nullbasic antiviral activity on primary CD4$^+$ T cells, the cells were transduced with SIN-based γ-retroviral vectors encoding NB-mCh or mCh and sorted by FACS for high level expression of the fluorescent proteins. As previous studies using NB-ZSG1 fusion protein showed strong antiviral activity against HIV-1_{NL4-3} on Jurkat cells[2,150], here we also used ZSGreen1 fluorescent protein fused to Nullbasic. The primary CD4$^+$ T cells were transduced with SIN-based γ-retroviral vectors encoding NB-ZSG1 or ZSG1 and selected by FACS at the same level as CD4-NB-mCh or CD4-mCh (Fig. 3-7). However, it is known that mCh is expressed as a monomer while ZSG1 is expressed as a tetramer[151]. This difference could influence the Nullbasic fusion proteins antiviral activity. Thus, we compared the antiviral activity of NB-mCh and NB-ZSG1 in primary CD4$^+$ T cells.

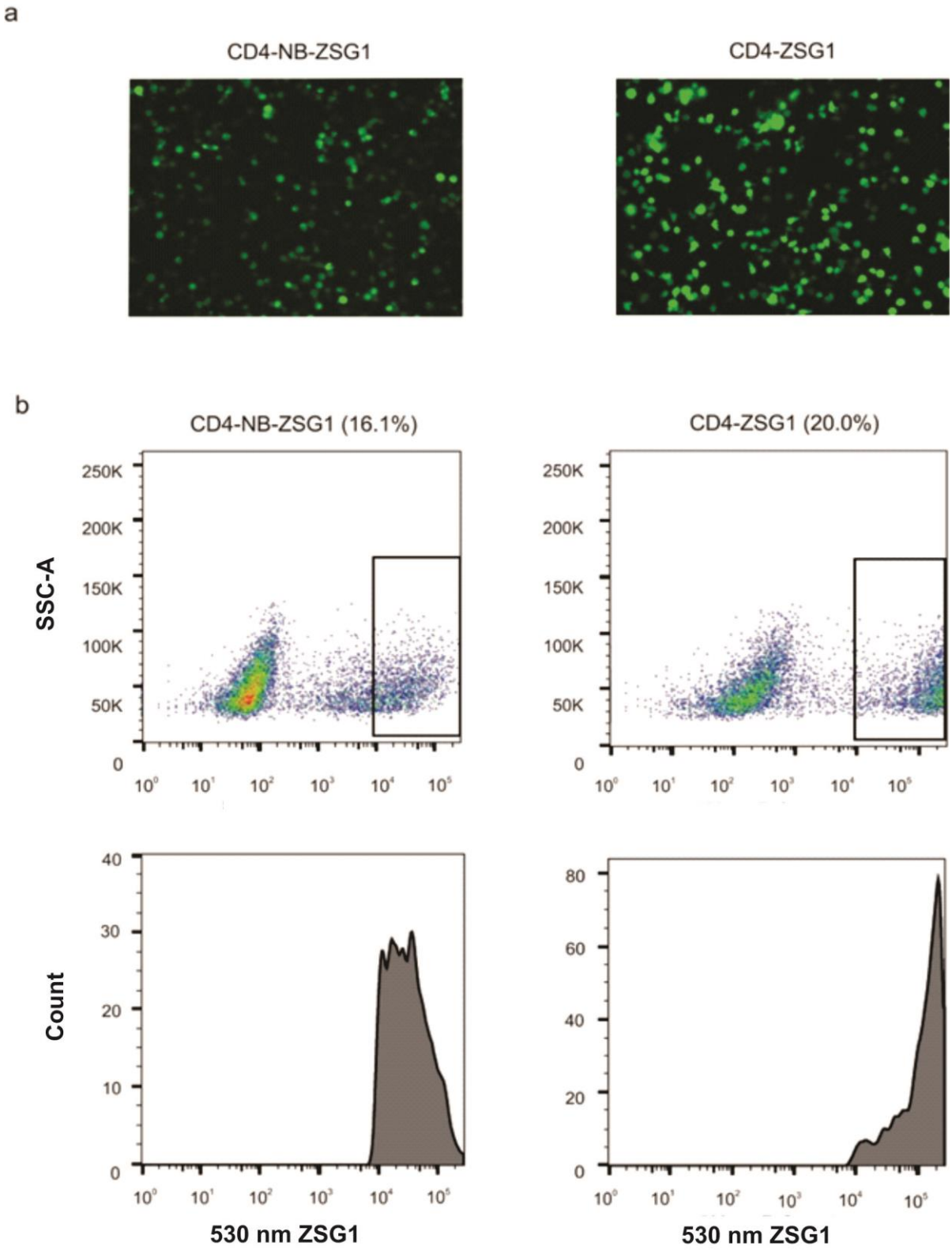

Figure 3-7. Primary CD4⁺ T cells transduced by NB-ZSG1 or ZSG1
(a) Expression of ZSGreen1 in NB-ZSG1 or ZSG1 transduced activated CD4⁺ T cells under EVOS™ cell imaging system before cell sorting. (b) Expression of ZSGreen1 in NB-ZSG1 or ZSG1 transduced CD4⁺ T cells by FACS. The cells were selected based on the rectangle gate shown. The histograms show the sorted cell population. A representative of at least 6 independent experiments is shown.

CD4-NB-mCh, CD4-mCh, CD4-NB-ZSG1, CD4-ZSG1 and NT CD4+ T cells were infected with HIV-1_{NL4-3}. The cell supernatants were collected on day 3, 7 and 10, then assayed for HIV-1 CA concentration by ELISA. The result suggests that both NB-mCh and NB-ZSG1 inhibited the virus replication, however, NB-ZSG1 showed ~6 times stronger antiviral activity compared to NB-mCh (Fig. 3-8). Therefore, we used ZSG1 fusion proteins to evaluate other HIV-1 strains in primary $CD4^+$ T cells.

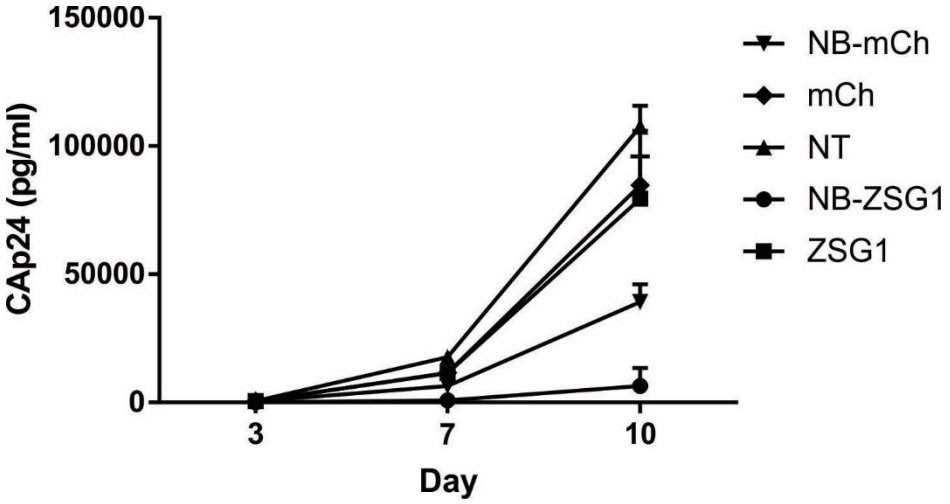

Figure 3-8. NB-ZSG1 inhibits HIV-1_{NL4-3} replication stronger than NB-mCh
Primary CD4+ T cells expressing NB-mCh, NB-ZSG1, mCh, ZSG1 and non-transduced cells were infected by HIV-1_{NL4-3} and HIV-1 CA concentrations were measured from the supernatants on each time points shown. The experiment was performed two times in duplicate and mean values and standard deviations are shown.

Next, CD4-NB-ZSG1, CD4-ZSG1 and non-transduced cells NT were infected with each HIV-1 strain and virus replication was monitored for 14 days (Fig. 3-9). All HIV-1 strains were inhibited, but some strain specific differences were noted. For example, at day 14 post infection, CA levels reduced by 90% for HIV-1_{NL43} and 88% for HIV-1_{ELI} and HIV-1_{MAL} in CD4-ZSG1 to CD4-NB-ZSG1 cells, whereas no CA was detected after infection with HIV-1_{ZAC}. It is worth noting that HIV-1_{ZAC} replicated poorly in $CD4^+$ T cells (Fig. 3-9b). Compared to HIV-$1_{NL43,}$ HIV-1 ZAC replication in CD4+ T cells on day 14 post infection was ~100-fold lower. A reduced level of HIV-1_{ZAC} replication compared to HIV-1_{NL4-3} was reported previously[143]. Therefore, this may account for a lack of detectable CA by HIV-1_{ZAC} in CD4-ZSG1 cells.

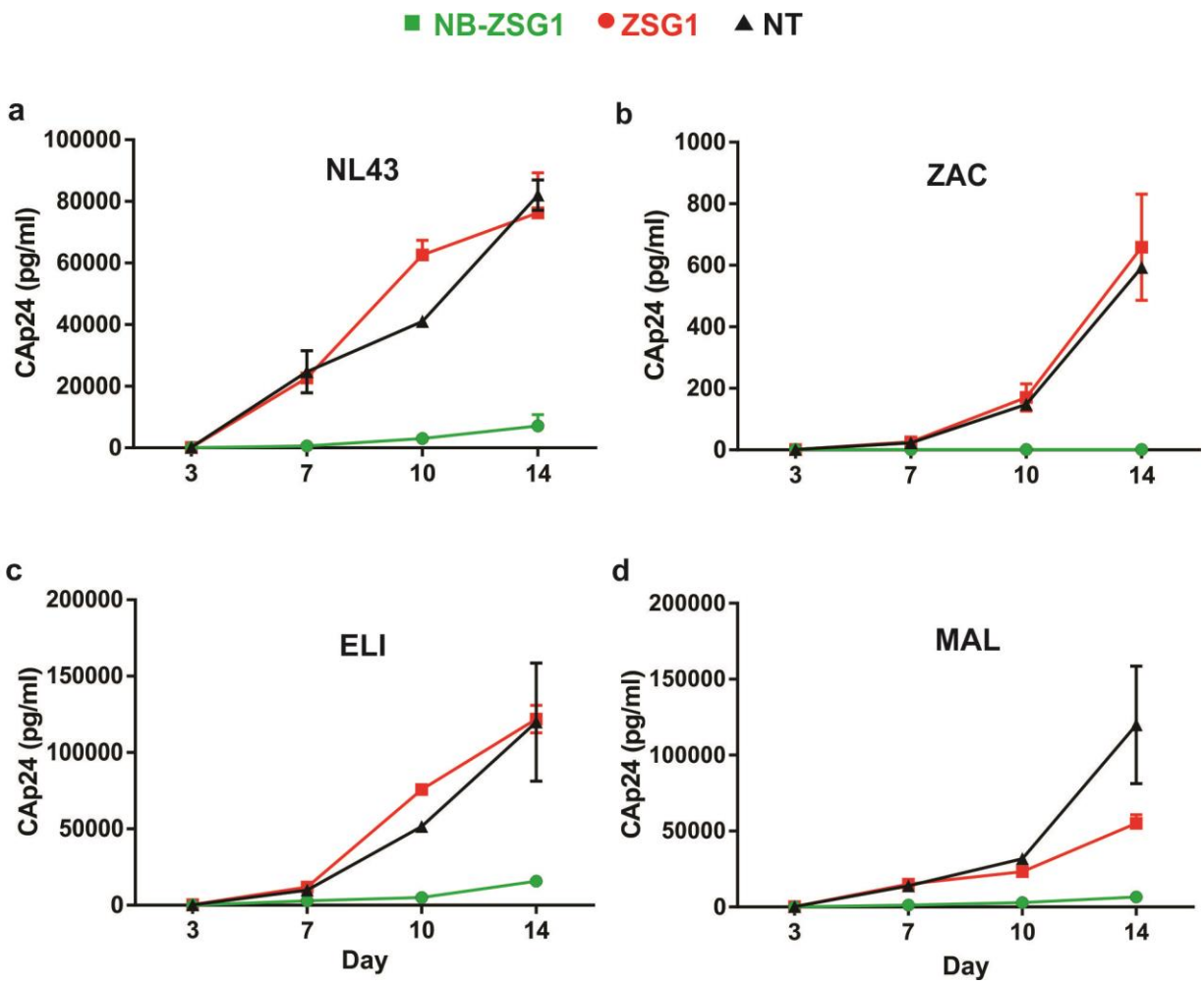

Figure 3-9. NB-ZSG1 inhibits virus replication of all the strains tested in primary CD4⁺ T cells

Primary CD4⁺ T cells expressing NB-ZSG1 or ZSG1 or NT were infected with each HIV-1 strain: (a) HIV-1NL4-3 (subtype B); (b) HIV-1ZAC (subtype C); (c) HIV-1ELI (subtype D), and; (d) HIV-1MAL (recombinant A/D subtype). The culture supernatant was assayed for CA at 3, 7, 10 and 14 days post infection. The experiment was performed in triplicate and culture supernatant from each replicate was assayed by ELISA for CA at 3, 7, 10 and 14 days post infection. The mean value and standard deviation for each time point are shown. A representative of two identical experiments with similar results is shown.

NB-ZSG1 and ZSG1 expression was monitored in uninfected and HIV-1-infected CD4⁺ T cells by flow cytometry. CD4⁺ T cell populations were initially collected by FACS so that ZSG1 positive cell population was >97%. The percentage of ZSG1 positive cells in the population at day 14 ranged from 90% (for HIV-1_{ELI}) to 99% (uninfected CD4⁺ T cells) in all experiments, whereas NB-ZSG1 levels were approximately 82% in uninfected and infected CD4+ T cells (Fig. 3-10). NB-ZSG1 expression reported here was significantly higher than in previous studies using pGCsamEN vectors, where the percentage of CD4⁺ T cells expressing NB-ZSG1 ranged between 40 and 50% of cell populations[14].

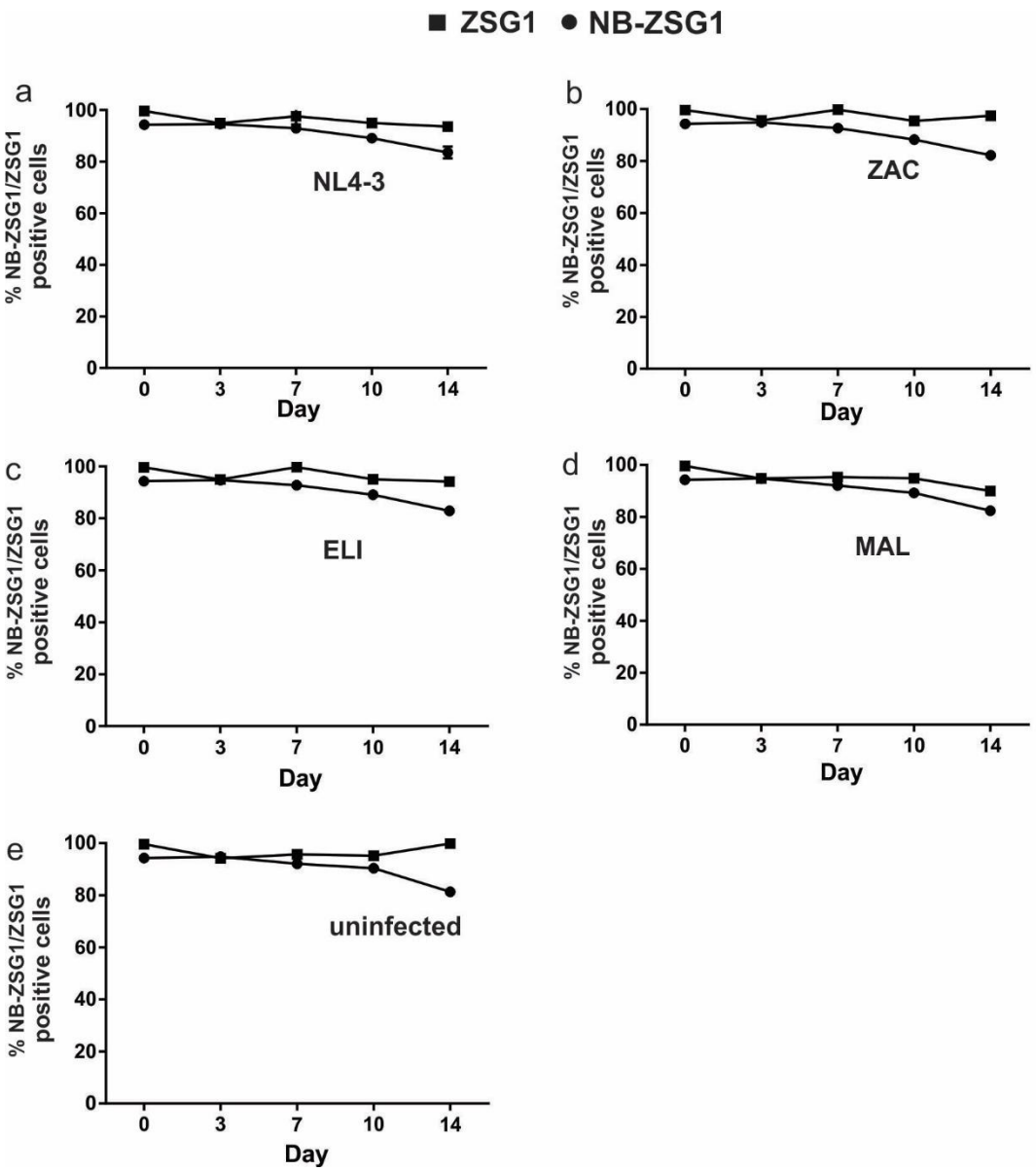

Figure 3-10. A temporal analysis of NB-ZSG1 or ZSG1 expressed in CD4+ T cells
A temporal analysis of NB-ZSG1 or ZSG1 expressed in CD4+ T cells. Primary CD4+ T cells expressing NB-ZSG1 or ZSG1 or NT were infected with each HIV-1 strain: (a) HIV-1NL4-3 (subtype B), (b) HIV-1ZAC (subtype C), (c) HIV-1ELI (subtype D), (d) HIV-1MAL (recombinant A/D subtype), and (e) uninfected. The level of NB-ZSG1 or ZSG1 positive cells in the population was monitored by flow cytometry on day 3, 7, 10 and 14 post-infection. The experiment was repeated three times with similar results and a representative experiment is shown.

We evaluated MFI at each time point; the results suggest that the fluorescent intensity of CD4-NB-ZSG1 cells was lower compared to that of CD4-ZSG1 cells. We also observed that the CD4-NB-ZSG1 MFI decreased at a higher rate than CD4-ZSG1 MFI (Fig. 3-12). This occurred in all infected or uninfected CD4-NB-ZSG1.

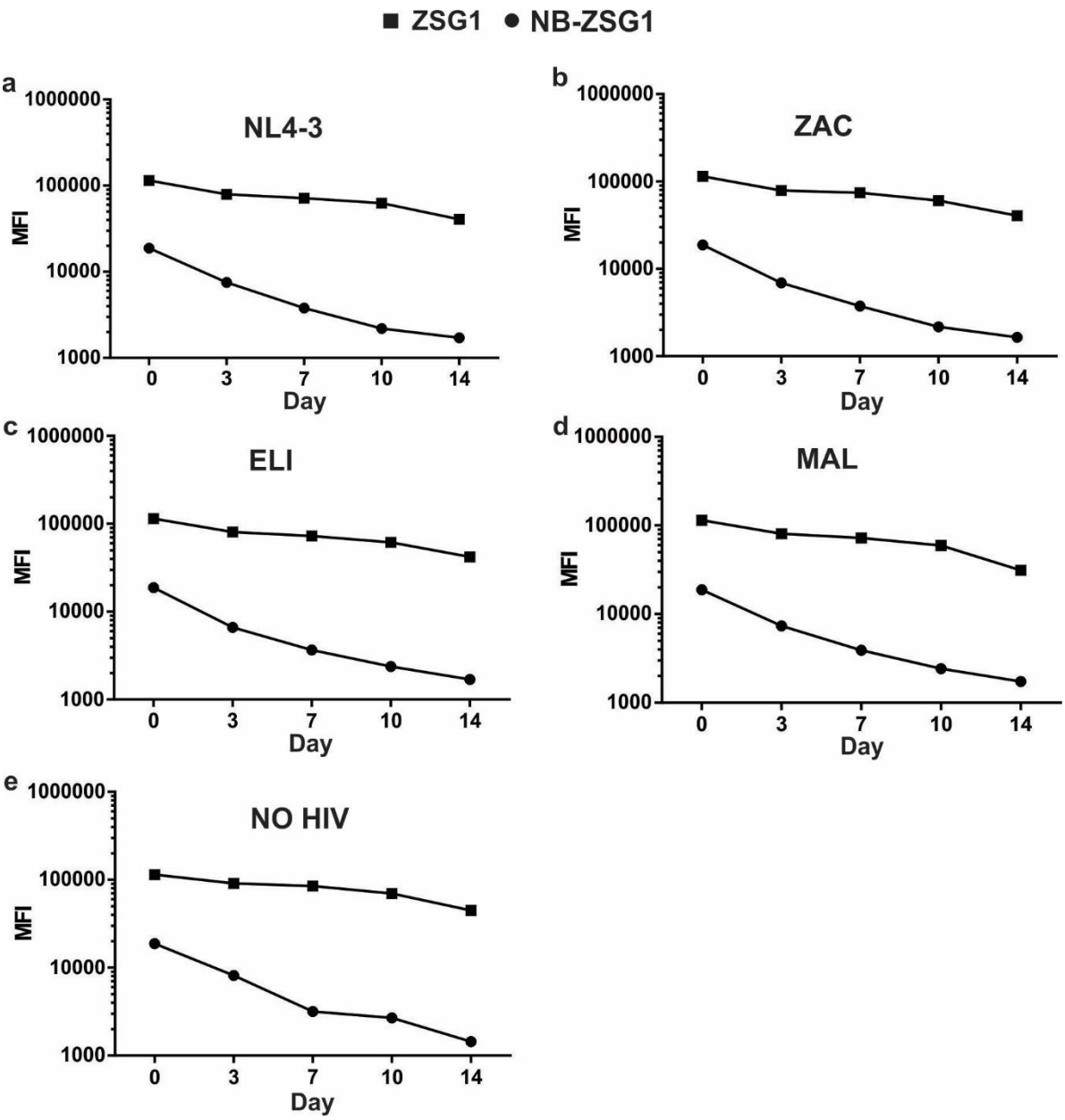

Figure 3-11. Higher rate decrease of MFI of CD4-NB-ZSG1 compared to CD4-ZSG1 MFI
Primary CD4$^+$ T cells expressing NB-ZSG1 or ZSG1 or NT were infected with each HIV-1 strain: (a) HIV-1NL4-3 (subtype B), (b) HIV-1ZAC (subtype C), (c) HIV-1ELI (subtype D), (d) HIV-1MAL (recombinant A/D subtype), and (e) uninfected. MFI was monitored at each time point indicated. The experiment was repeated three times with similar results and a representative experiment is shown.

We investigated if expression of NB-ZSG1 in CD4$^+$ T cells had a measurable detrimental effect that could explain why levels of NB-ZSG1 positive cells declined. First, we evaluated cell viability by MTS colorimetric assay. This assay measures cellular metabolism based on reduction of MTS by NAD(P)H-dependent oxidoreductase enzymes, largely in the cytosolic compartment of dividing cells[152,153]. No significant difference between CD4-NB-ZSG1 and CD4-ZSG1 cells were found, although both NB-ZSG1 and ZSG1 cells had a slightly lower metabolic activity than NT cells, suggesting the transduction, cell purification processes or

ZSG1 were responsible for the decrease level of NB-ZSG1 positive cells (Fig. 3-11a). Second, we measured cell proliferation using a cell permeable and VPD450 dye for monitoring of cell division by flow cytometry. No difference in proliferation was observed between CD4-NB-ZSG1 and CD4-ZSG1 (Fig. 3-11b). Given that HIV-1 wild type Tat is reported to have pro- and anti-apoptotic activities in primary $CD4^+$ T cells[154], an Annexin V assay was used to determine if NB-ZSG1 expression affected cellular apoptosis. All of the cells tested had very little or no apoptosis (Fig. 3-11c). Therefore, we cannot account for a drop in NB-ZSG1 levels in CD4+ T cells due to detrimental effects of NB-ZSG1 on the cellular pathways tested. However, Nullbasic may affect other cellular pathways other than those tested leading to reduced levels of Nullbasic expression in the cells.

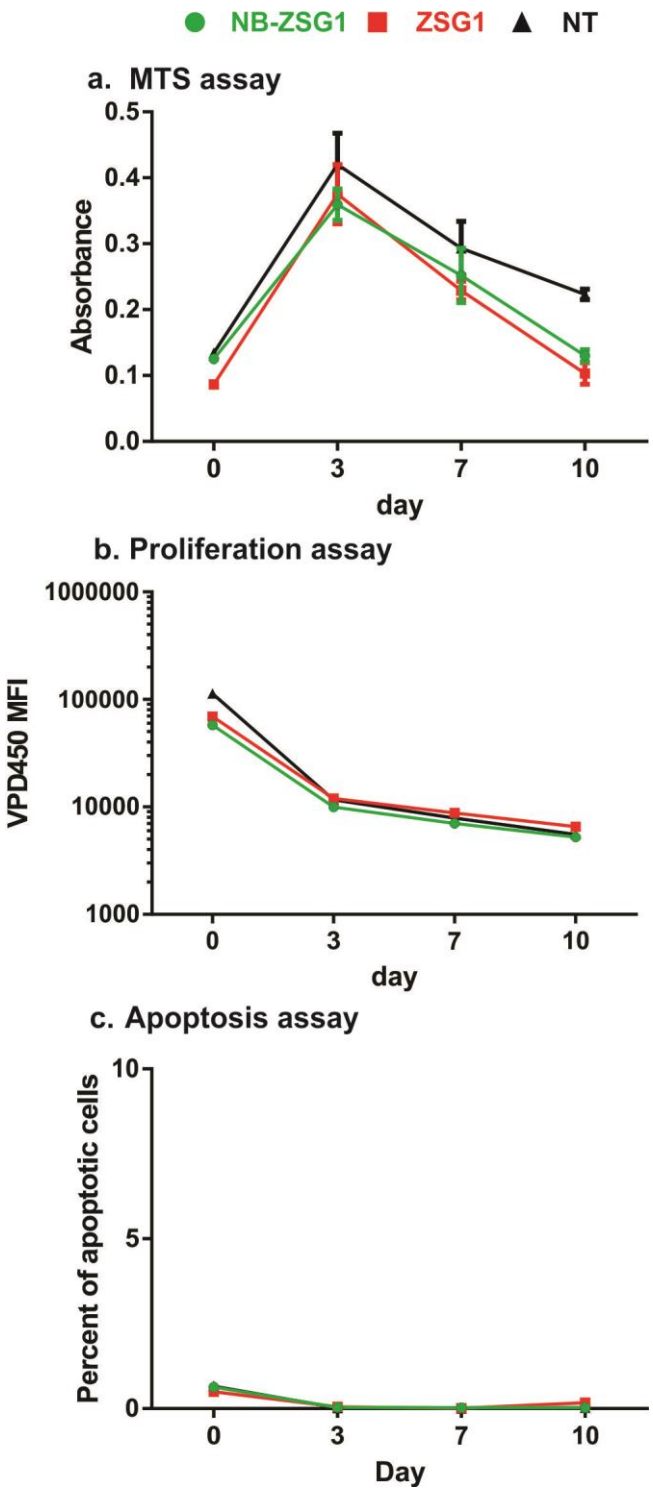

Figure 3-12. NB-ZSG or ZSG expression does not affect CD4+ T cell viability, proliferation or induce apoptosis

(a) An MTS assay was used to measure the viability of CD4+ T cells expressing NB-ZSG1 or ZSG1 or NT. (b) The proliferation of CD4+ T cells expressing NB-ZSG1 or ZSG1 or NT was measured using VPD450 dye assay. (c) An apoptosis assay using PE Annexin V was used to monitor levels of apoptosis in CD4+ T cells expressing NB-ZSG1 or ZSG1 or NT. All assays were performed in triplicate and were repeated three times. Mean values and standard deviations are shown.

In summary, here we show that Nullbasic can inhibit replication of different HIV-1 strains from diverse subtypes. The outcome indicates that the replication pathways affected by Nullbasic are most likely shared by these HIV-1 strains.

3.4 Discussion

In this study, we investigated if Nullbasic could inhibit viral gene expression and virus replication of four different strains representing four HIV-1 subtypes in human cells. We previously showed that Nullbasic has three independent antiviral properties at different stage of HIV-1 replication cycle[15]: 1) inhibition of transactivation of virus gene expression by HIV-1 Tat[2,15,127]; 2) inhibition of HIV-1 Rev activity by sequestration of DDX1[16,18], and; 3) binding to HIV-1 reverse transcriptase in the virion, leading to premature uncoating and defective reverse transcription in newly infected cells[18]. Given that Nullbasic inhibits HIV-1 by binding to both cellular (PTEFb and DDX1) and viral RT targets, we found that NB fusion proteins had antiviral activity against all strains tested, although some differences were observed.

Using TZM-bl cells, the effect of Nullbasic on transactivation and virus replication was examined in three ways. Briefly, wild type Tat mediates HIV-1 transactivation by binding and recruiting the SEC and P-TEFb (composed of Cyclin T1 and CDK9) to nascent viral mRNA, where CDK9 can phosphorylate RNAPII, leading to highly processive RNA transcription[146] [32]. A crystal structure of the Tat-P-TEFb complex showed that Tat tightly binds to P-TEFb because 37% of its folded N-terminal domain (amino acids 1-49) surface is complementary to the kinase[48]. In Nullbasic, amino acids 1-48 are wild type but amino acids 49-57 are mutated. Hence, Nullbasic is able to bind P-TEFb[2,16] but cannot recruit the protein complex to nascent viral mRNA, which requires the RNA binding function of the Tat basic domain (amino acids 49-57)[129].

In TZM-bl transfection experiments where equivalent amounts of each Tat expression plasmid were used, the overall inhibition of transactivation had a similar range (70-90% inhibition) but Tat_{ZAC} was consistently inhibited the least by NB-mCh. Interestingly, a consensus subtype C Tat was reported to have superior transactivation capacity compared to a consensus subtype B Tat[155], whereas Tat_{ZAC} was a poor transcriptional activator here compared to the other Tat proteins tested. This is probably due to a Tat_{ZAC} K41T substitution

that may affect interaction between Tat_{ZAC} and Cyclin T1[155]. TZM-bl cells were infected by each HIV-1 strain and transactivation of the TZM-bl LTR-luciferase reporter was inhibited at similar levels (~90% inhibition of Tat_{ZAC}, Tat_{ELI} and Tat_{MAL}). Furthermore, replication of all four viral strains was strongly inhibited by NB-mCh, which ranged from 97-99%. It is interesting that NB-mCh inhibited virus replication of each strain better than it inhibited trans activation LTR-Luciferase reporter, but the reason for this is yet unclear. This difference inhibition level could be due to multiple round of viral infection and replication would amplify the blockade by Nullbasic because of additional targets during the replication cycle, such as transactivation, RT function and RRE function, while in the transient transfection assay, only the transactivation function of Tat would be affected. Another possibility is differences in the TAR RNAs of the various viral strains. Further work will be required to elucidate the reasons. The data clearly shows that viral transcription and replication mediated by the four different Tat variant proteins, representing different HIV-1 subtypes, was inhibited by NB-mCh under the conditions tested.

A previous study used an MLV-based γ-retroviral vector, pGCsamEN[14], containing NB-ZSG1 or ZSG1 to transduce primary $CD4^+$ T cells. In that study, cells that expressed NB-ZSG1 significantly delayed $HIV\text{-}1_{NL4\text{-}3}$ replication compared to cells expressing ZSG1. However, pGCsamEN is not self-inactivating (SIN) and expression of a transgene is via the MLV-LTR promoter. Therefore, non-SIN γ-retroviral vector can be transcriptionally repressed in cells. This repression can be anticipated in SIN-based γ-retroviral vectors that used strong constitutively expressed internal promoters. Using this vector, a dramatic loss of NB-ZSG1 expression (~50%) was observed in CD4-NB-ZSG1 population by day 7 post infection[156]. Therefore, SIN-based γ-retroviral vectors, which express transgene via SFFV promoter, were used in this study to transduce $CD4^+$ T cells.

It is interesting that in primary CD4+ T cells, NB-ZSG1 had a stronger antiviral activity than NB-mCh. The different levels of the Nullbasic antiviral properties between this two fusion protein forms is possibly due to different oligomerization state of the fluorescent proteins fused to Nullbasic. mCherry forms a dimer while ZS-Green1 forms a tetramer[151], and it is possible these multimeric states may cause different protein conformations that can affect Nullbasic antiviral activity. Perhaps Nullbasic in mCherry fusion protein forms a dimer while in ZS-Green1 fusion protein it forms a tetramer. This result indicates that Nullbasic antiviral effect is more pronounced in the form of a tetramer. A tetramer would bind with higher

avidity to its target than a dimer[151], and thus could induce aggregation of p-TEFb. Further study is required to understand the mechanism behind this. For example, by performing co-immunoprecipitation assay to examine the strength of interaction between NB-mCh or NB-ZSG1 and P-TEFb.

The replication of all HIV-1 strains in stimulated primary $CD4^+$ T cells was also inhibited but differences were noted. The replication capacity of $HIV\text{-}1_{ZAC}$ was below the limit of detection (where an ELISA assay can measure CA as low as ~4 pg/ml), whereas $HIV\text{-}1_{NL43}$ and $HIV\text{-}1_{ELI}$ and $HIV\text{-}1_{MAL}$ were strongly inhibited.

We also noted that although the FACS isolated CD4-NB-ZSG1 and CD4-ZSG1 cells were >97% ZSG1 positive, the percentage of ZSG1 positive cells was maintained by CD4-ZSG1 population over 14 days, whereas the percentage of NB-ZSG1 positive cells in the CD4-NB-ZSG1 population decreased by about 10-20%. We also observed a higher rate of MFI decrease in the CD4-NB-ZSG1 cells compared to that of CD4-ZSG1 cells. However, our data indicates that cell proliferation of CD4-NB-ZSG1 and CD4-ZSG1 are similar, and levels of cytotoxic effects and apoptotic cells were unchanged. We recently reported that NB-ZSG1 strongly suppressed HIV-1 transcription in Jurkat cells[2], so one possible cause of this NB-ZSG1 decreased expression level is that NB-ZSG1 also negatively affects transcription by the constitutively active SFFV promoter. Given that NB-ZSG1 is able to target P-TEFb, it may impede an SEC required for HIV-1 transcription[157], and perhaps SEC complexes that stimulate transcription by the SFFV promoter. Testing these possibilities will require determining if the NB-ZSG disrupts the P-TEFb:SEC complexes, and further understanding of transcriptional activation of the SFFV promoter.

Our data shows HIV-1 replication increased in CD4-NB-ZSG1 cells as the numbers of CD4-NB-ZSG1 cells decreased, as we observed previously[14]. It is possible that alternative promoters used to express Nullbasic in the retroviral vector may provide sustained expression of NB-ZSG1 and lead to a better viral control. In addition, it would be also interesting to introduce a Nullbasic-type mutation into other Tat variants and examine if a strain-specific custom Nullbasic gene is a better inhibitor of specific strains.

Overall, SIN-based gammaretroviral vectors improved expression of Nullbasic. The study shows that Nullbasic can inhibit replication of different HIV-1 strains from four subtypes in

TZM-bl cell line as well as in primary $CD4^+$ T cells. Stable expression of Nulbasic may have utility in a future gene therapy approach applicable to genetically diverse HIV-1 strains.

CHAPTER 4

Establishment of an acute HIV-1 Infected Mouse Model to Examine Nullbasic Antiviral Activity *in vivo*

4.1 Introduction

Nullbasic effects have been extensively studied *in vitro* in human cell lines as well as in primary cells, but the *in vivo* effects have not been studied. *In vitro* studies of Nullbasic provide evidence about various Nullbasic antiviral mechanisms as well as its efficacy in laboratory adapted cell lines and primary $CD4^+$ T cells, but cannot determine if the antiviral activity is similar *in vivo*. As an advance step towards the application of Nullbasic in gene therapy, a preclinical study in an animal model is an important step forward towards an eventual trial in humans.

A humanized mouse model enables studying Nullbasic effects *in vivo* and can also help in understanding the virus reservoir in the body. For the current study, an acute HIV-1 infection model was developed using BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice. BALB/c-Rag2$^{-/-}$γc$^{-/-}$ strain has been used in previous studies to investigate HIV-1 *in vivo*[123,158]. Because this BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mouse strain is immunodeficient, it accepts human cell transplantation. This immunodeficient mouse strain was developed by mutation of recombination-activating gene (*rag*). *Rag* gene is conserved in human and mice[159]. Rag1 and Rag2 proteins play an important role in forming an enzyme complex that joins T and B cell receptor genes. Knock out of *rag* gene results in inability to produce mature T and B cells. The *rag* deficient BALB/c mice were not leaky in T and B cell production and less radiosensitive compared to severe-combined-immunodeficiency (SCID) mice. Therefore, *rag* model is more susceptible to preconditioning by irradiation in order to generate a high rate of engraftment[159]. Engraftment is a result of transplanted cells homing, adapting and proliferating in the recipient body[160]. However, Rag1 and Rag2 deficient mice generate unwanted NK cells, so mutations of common gamma chain receptor (γ_c) to prevent NK cell maturation were required. A gamma chain is an important subunit protein of interleukin receptors, including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Nulling the gamma chain prevents IL-2 and IL-7 signaling, and therefore T

and B cell development as well as NK cell maturation[122]. As a result, BALB/c-Rag1$^{-/-}$γc$^{-/-}$ and BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice are susceptible to human PBL or HSC transplantation and HIV infection. A previous study by Berges and colleagues using this model showed that the engrafted cells were susceptible for HIV-1 infection and the infection led to CD4^{+} T cells depletion[123].

To establish a simple animal model to study Nullbasic activity against acute HIV-1 infection, the current study transplanted mature human CD4^{+} cells into BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice (Fig 4-1). As human CD4^{+} cells are susceptible to HIV-1 infection, our aim in using this model was to examine Nullbasic antiviral activity *in vivo.*

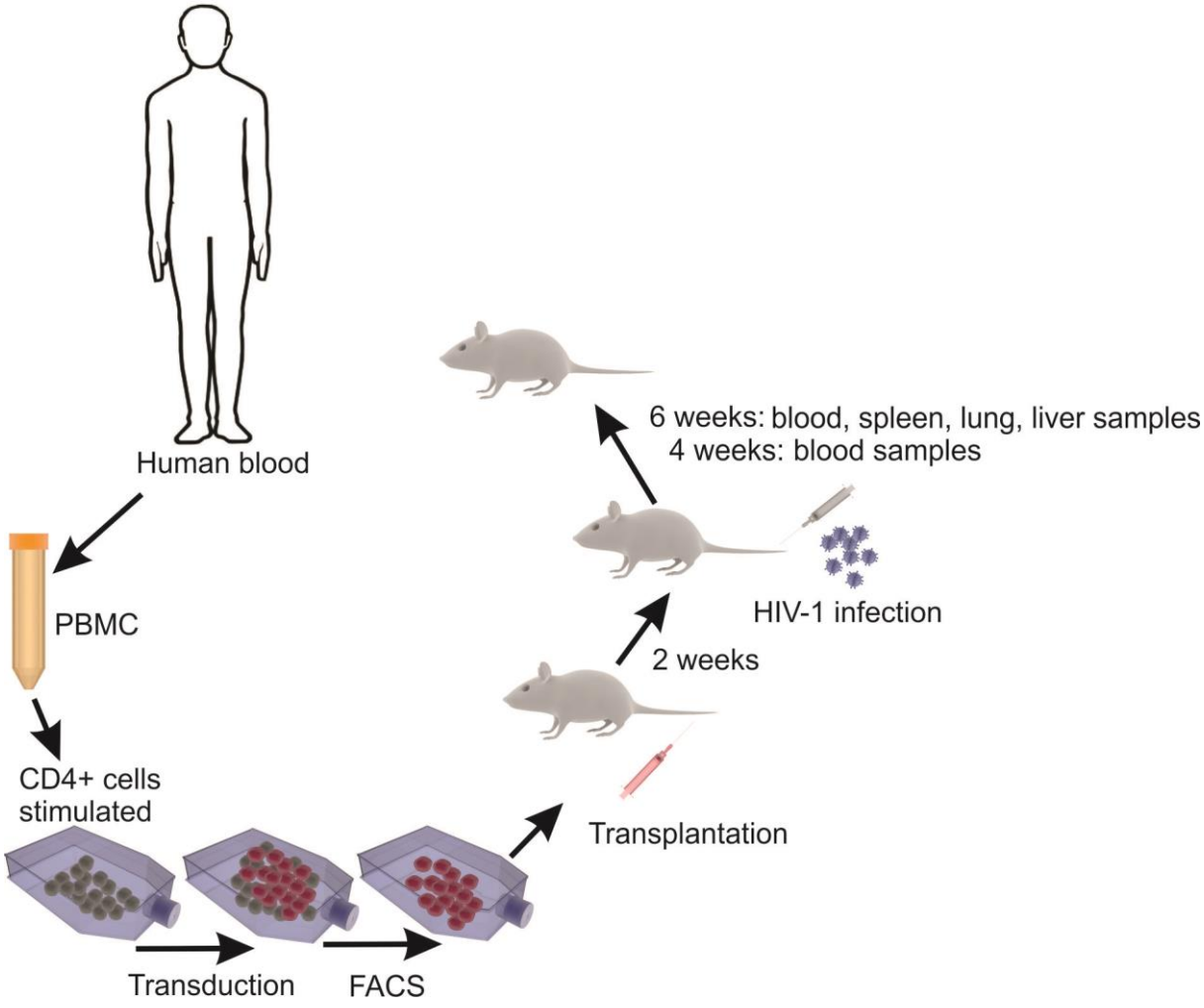

Figure 4-1. Schematic diagram of establishment of an acute HIV-1 infected mouse model
PBMCs were isolated from human buffy coat and selected for CD4^{+} cells. The cells were stimulated using CD3 and CD28 antibodies, transduced by NB-mCh, mCh, NB-ZSG1 or ZSG1 VLPs and sorted by FACS for cells expressing high levels of each fluorescent protein. The cells were then transplanted to previously irradiated mice. Two weeks post transplantation, the mice were infected

with HIV-1_{AD8}. Samples were taken from the blood, spleen, liver and lung then analyzed for human cell engraftment and for viral RNA levels.

This acute model supports investigation of short-term Nullbasic antiviral effects *in vivo* and provides preliminary data for the development of a long-lasting chronic HIV-1 infection model that may be used for testing Nullbasic antiviral effects using human stem cell xenograft.

4.2 Materials Methods

4.2.1 Cell lines and cultures

HEK 293T and Phoenix-Ampho[141] cell lines were grown in DF10 medium composed of Dubelcco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% ($^{v}/_{v}$) FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). PBMCs were isolated from healthy donor's buffy coat supplied by the Australian Red Cross Blood service using Ficoll density gradient centrifugation. $CD4^+$ cells were isolated from the PBMCs by using a MACS human $CD4^+$ cell isolation kit (Miltenyi Biotec) as per the manufacturer's instructions. The selected cells were grown in 6 cm tissue culture dishes and stimulated using plates pre-coated with purified anti-human CD3 (clone HIT3a) and anti-human CD28 (clone CD28.2) antibodies (BioLegend) in RPMI medium supplemented with 20% ($^{v}/_{v}$) FBS and 5 ng/ml IL-2 (hereafter called RF20 IL-2) for 2 days. All cells were grown at 37 °C in humidified incubators with 5% CO_2.

4.2.2 Plasmid constructs

pSRS11-SF-γC-EGFP was a gift from Axel Schambach and Christopher Baum[142]. pSRS11 SF-γC-NB-mCh or pSRS11-SF-γC-mCh construct was made by replacing EGFP in pSRS11-SF-γC-EGFP with NB-mCh or mCh. A proviral plasmid, pAD8, was used to make HIV-1_{AD8}. pAD8-1 was obtained from Allison Greenway (Burnet Institute).

4.2.3 HIV-1 and VLP production

HIV-1 subtype B strain AD-8 was produced from pAD8-1 proviral plasmid. HEK 293T cells were grown on a 10 cm plate at ~80% confluency and transfected with 10 μg of each proviral plasmid, then incubated for 24 hours at 37 °C. On the next day, the transfected cells were gently washed with 1 x PBS and the DF10 media was replaced. The supernatant containing

HIV-1 VLPs was collected 48 and 72 hours post transfection and the amount of CA in each supernatant was measured by ELISA (Zeptometrix) as recommended by the manufacturer.

NB-mCh or mCh or NB-ZSG1 or ZSG1 VLPs were produced in Phoenix-amphotropic retroviral packaging producer cell line by co-transfection of 7.5 μg of SRS11-SF-γC vector expressing NB-mCh or mCh or NB-ZSG1 or ZSG1 and 1.5 μg of Gag-Pol expressing plasmid using X-tremeGENE^{TM} DNA transfection reagent (Roche) in a 10 cm plate. Six hours post transfection, the cells were washed with PBS and the media was replaced. The VLPs were collected 48 and 72 hours post transfection and filtered through a 0.45 µm filter.

4.2.4 Transduction of NB-mCh or mCh or NB-ZSG1 or ZSG1 VLPs in CD4$^+$ T cells

NB-mCh or mCh or NB-ZSG1 or ZSG1 VLPs were concentrated using the precipitation method by adding 20% ($^v/_v$) of 34% polyethylene glycol 8000 (Sigma Aldrich) and 10% ($^v/_v$) of 0.3 M sodium chloride solution. The solution mixture was incubated at 4 °C for 1.5 hours, mixed every 30 minutes and then centrifuged at 1500 × g for 1 hour at 10 °C. The supernatant was discarded and the precipitate was resuspended in 600 μl RF20 IL-2 medium. The concentrated VLPs (150 µl) were added to Retronectin (Takara) coated 24 well plate and incubated at 37 °C for 30 minutes. For each well, 5×10^5 stimulated CD4$^+$ cells were added and incubated for 3 days. The transduced cells were processed by FACS collecting mCh positive cells. The collected cells were grown for a further 3 days. The RF20-IL2 media was replaced every day.

4.2.5 Transplantation of of human CD4$^+$ T cells into BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice

BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice were obtained from Denise Doolan (QIMR Berghofer Medical Research Institute) and bred at QIMR Berghofer animal house. The mice were irradiated with a sublethal dose (350 cgγ) then rested for 6 hours. NB-mCh or mCh or NB-ZSG1 or ZSG1 transduced or non-transduced human CD4$^+$ T cells were injected intraperitoneally or intravenously to the mice. Each mouse required 5 million CD4$^+$ T cells. Every week, the mice were monitored for weight loss and scores were recorded for dull/ruffled coat, hunched posture, reduced intake of food or water and movement.

4.2.6 Infection of of HIV-1 to BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice

Two weeks post transplantation, the BALB/c-Rag2$^{-/-}$γc$^{-/-}$ mice was infected by HIV-1 with a concentration of 50 ng or 100 ng CA p24 via intravenous injection. Blood samples (~100 µl)

were collected every two weeks by tail bleeding in a 1.5 ml tube coated with 10% (v/v) of 0.05 M Na-EDTA. Plasma was separated and frozen at -80 °C. Blood cells were used for flow cytometry. Ten or six weeks post transplantation, the mice were sacrificed. The mice were anaesthetized using isoflurane, then blood was collected via heart puncture (~300-500 µl). Spleen, liver and lung samples were also collected to detect human cell engraftment as well as virus reservoirs. Cells were collected from the spleen and lung tissues for flow cytometry analysis and HIV-1 RNA quantification. For flow cytometry analysis, the cells were stained with CD4-FITC and CD45-APC antibodies. Half number of the cells were suspended in 1 ml Trizol solution containing 5 ng of synthetic Kanamycin RNA and 1 µg of poly-A RNA carrier, and then kept at -80 °C for HIV-1 RNA detection.

4.2.7 Flow cytometry

Single cell suspensions were prepared from whole blood collected in EDTA pre-coated tubes by tail bleeding at weeks 2 and 4, and by heart puncture at week 6. Cells were also collected from spleens, livers and lungs. Red blood cells were lysed twice using BD pharm lyse buffer (BD Bioscience) for 5 minutes each, centrifuged at 500 x g for 5 minutes at RT, washed with 1x PBS, and then stained with anti-human CD4-FITC and anti-human CD45-APC antibodies (Miltenyi biotec) in the dark at RT for 20 minutes. The cells were then fixed with 1% paraformaldehyde and kept in the dark at 4 °C for flow cytometry analysis. At least 10,000 events in the selected population were collected using LSR Fortessa IV (BD Bioscience). The phenotype of engrafted human $CD4^+$ T cell was determined as double positive of CD4 and CD45 expressions. Data analysis was performed using Flow Jo software version 10.

4.2.8 Immunohistochemistry

Spleen and lung samples were collected and fixed in 10% paraformaldehyde. Immunohistochemistry was performed using fluorescent multiplex immunohistochemistry (mIHC) with tyramide signal amplification protocol by Histology Department of QIMR Berghofer Medical Research Institute. The tissues were stained with mouse anti-human CD4-CY3 clone 4B12 (Dako) and counterstained with DAPI.

4.2.9 HIV-1 mRNA isolation and quantification

HIV-1 replication was evaluated at 4 and 6 weeks post transplantation (2 and 4 weeks post infection) by qRT-PCR of the plasma samples. Total RNA was isolated from the plasma

using a high pure RNA isolation kit (Roche) as per the manufacturer's instructions. HIV-1 RNA was also collected from spleen and lung tissues using Trizol. Synthesis of cDNA was performed using Superscript III reverse transcriptase (Thermo Fisher Scientific) as per the recommended instructions. HIV-1 RNA quantification was carried out by real time PCR using iTaq™ universal SYBR green supermix (Bio-rad). A 5'-LTR sequence (GCGACTGGTGAGTACGCCA) was used as a forward primer for HIV-1_{AD8} strain and an LA9 (GACGCTCTCGCACCCATCTCT) was used as reverse primer. PCR cycling condition was set as 95 °C for 5 seconds, 65 °C for 15 seconds and 72 °C for seconds using a real time PCR detection system (Bio-rad). A synthetic kanamycin resistant RNA cassette (Promega) was used as an internal PCR control.

4.2.1 Statistical analysis

Data are presented as means plus and minus standard deviation (SD). Mann Whitney non-parametric analysis was used to compare means between two groups. A confidence interval of 95% was used, therefore p value less than 0.05 is considered to be significant.

4.3 Results

4.3.1 Intravenous administration provided fast and effective human $CD4^+$ T cell engraftment in mice

Human $CD4^+$ T cells isolated from PBMC of healthy buffy coat donors were column purified by positive selection. Flow cytometry analysis shows that 82.5 % of the collected cells are lymphocytes (Fig. 4-2, left panel). Monocytes and granulocytes may also present in smaller amounts as these cells also express CD4 receptor. The purity of isolated $CD4^+$ T cells was 99.4% (Fig. 4-2, right panel).

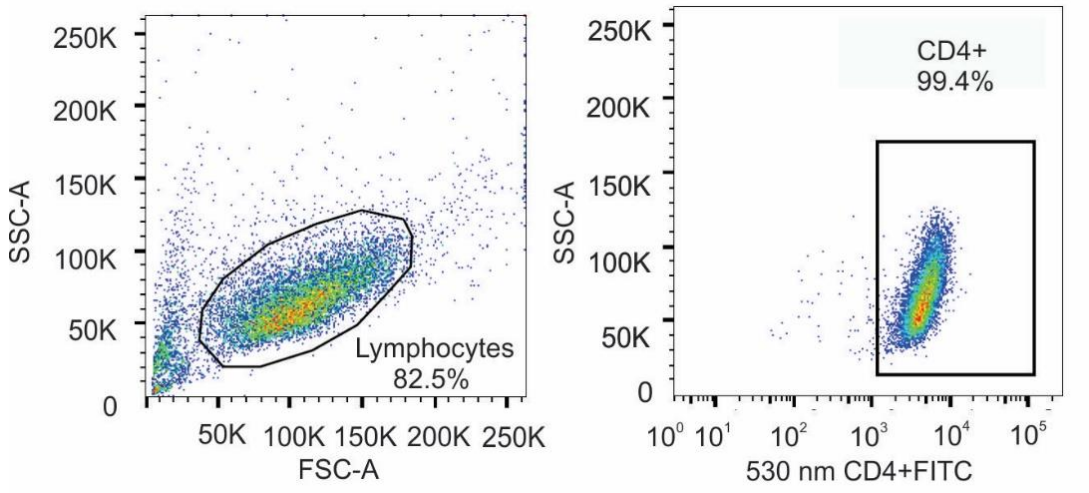

Figure 4-2. CD4⁺ cell purity analyzed by flow cytometry
$CD4^+$ cells were isolated from PBMC and stained with anti-human CD4 FITC antibody then analyzed by flow cytometry. The left panel shows lymphocyte population. $CD4^+$ cells are shown in the rectangular gate (right panel).

We used 8-16 week-old BALB/c-Rag2-/-γ c-/- mice to create an acute HIV-1 infected mouse model. For preconditioning, the mice were irradiated at 350 cGy 6 hours before human $CD4^+$ T cell transplantation. In developing a humanized mouse model using the BALB/c-$Rag2^{-/-}$ $\gamma c^{-/-}$ strain, preconditioning of immunodeficient mice is required to increase the expression of cell factors required for xenograft engraftment and mouse survival[160]. Mouse preconditioning using a sub lethal total body irradiation technique minimized host versus graft disease. It also improved human cell engraftment by inducing proliferative signals of the donor cells post cell homing in the recipient.

For optimization, initially we evaluated the most effective administration route for this model. Firstly, 5 million human $CD4^+$ cells were transplanted by intraperitoneal or intravenous injection to ten mice per group. Blood samples were then taken at week 4 and 6 post transplantation by tail bleeding. The standard method to measure the levels of engraftment of human cells in the recipients was performed by flow cytometry analysis of blood samples to measure the circulating donor cells[160]. In this experiment, we stained the cells with anti-human CD45 and anti-human CD4 antibodies followed by flow cytometry analysis. The engraftment level was measured by calculating the number of cells displaying both human $CD45^+$ and human $CD4^+$ per ml of mouse blood. The result showed that compared to intraperitoneal administration, intravenous administration provided higher engraftment levels in the peripheral blood circulation (Fig. 4-3). The difference was statistically significant at week 4, but not at week 6. This result suggests that intravenous injection resulted in more

reliable human CD4$^+$ T cell engraftment in the mouse. Therefore, we used intravenous administration route for further experiments.

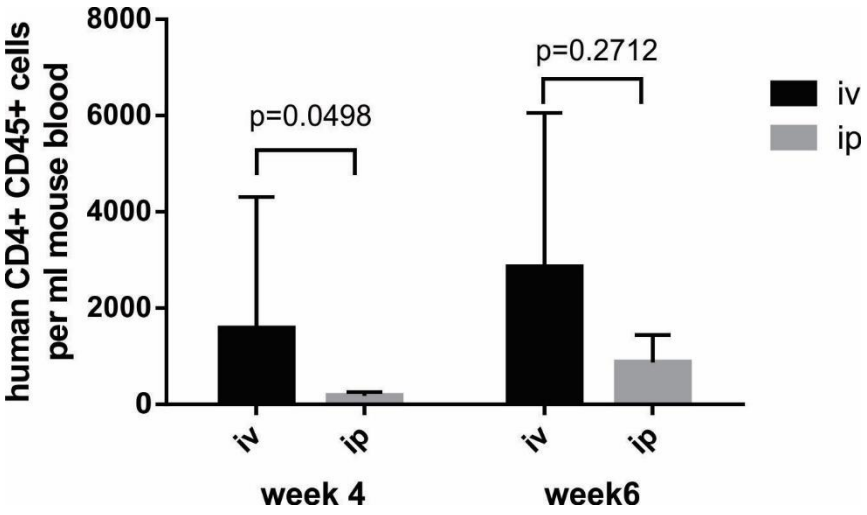

Figure 4-3. Engraftment of stimulated human CD4$^+$ cells in mouse blood after transplantation by intravenous (iv) and intraperitoneal (ip) injections
Blood cells were collected at time point indicated, stained with anti-human CD4 and anti-human CD45 antibodies. The bars indicate mean numbers of human CD4$^+$ and human CD45$^+$ cells per ml of mouse blood (N=10). Errror bars indicate standard deviations.

4.3.2 Engrafted human CD4$^+$ cells can be detected for up to six weeks in mice

To evaluate the duration of human cell survival in the mouse, the mice were observed for ten weeks. Cells recovered from blood samples taken every two weeks, from week 4 to week 10 post transplantation, were monitored by flow cytometry. We observed that levels of human cell engraftment in the blood samples were the highest at week 6 for most of the mice, then the CD4$^+$ cells diminished gradually and were difficult to detect in the blood by week 10 (Fig. 4-4). Only 2 mice maintained a detectable level of the human cells in the blood by week 10. Therefore, we set week 6 as the end-point for the next experiments.

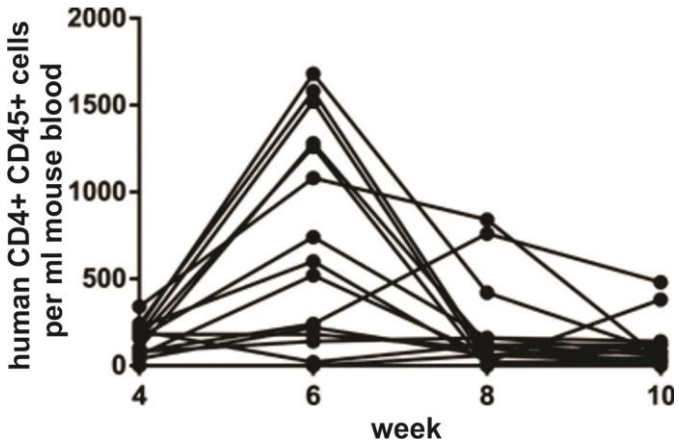

Figure 4-4. Engrafted human CD4$^+$ cells detected in mouse blood
Blood samples were taken at 4 weeks post transplantation, then every 2 weeks up to week 10. The figure shows the number of CD4$^+$ measured by flow cytometry at the time points indicated.

4.3.3 Human CD4$^+$ cells can be detected in the spleen and lung of mice

We evaluated the distribution of the human cells in the mouse organs. To detect where the human cells were located, the mice were sacrificed at week 6 and cells were collected from the mouse organs including spleens, lungs and livers and analysed by flow cytometry. The result shows a very modest level of human cell engraftment in the liver, and high levels of human CD4$^+$ T cell engraftment were detected in the spleen and lungs (Fig. 4-5).

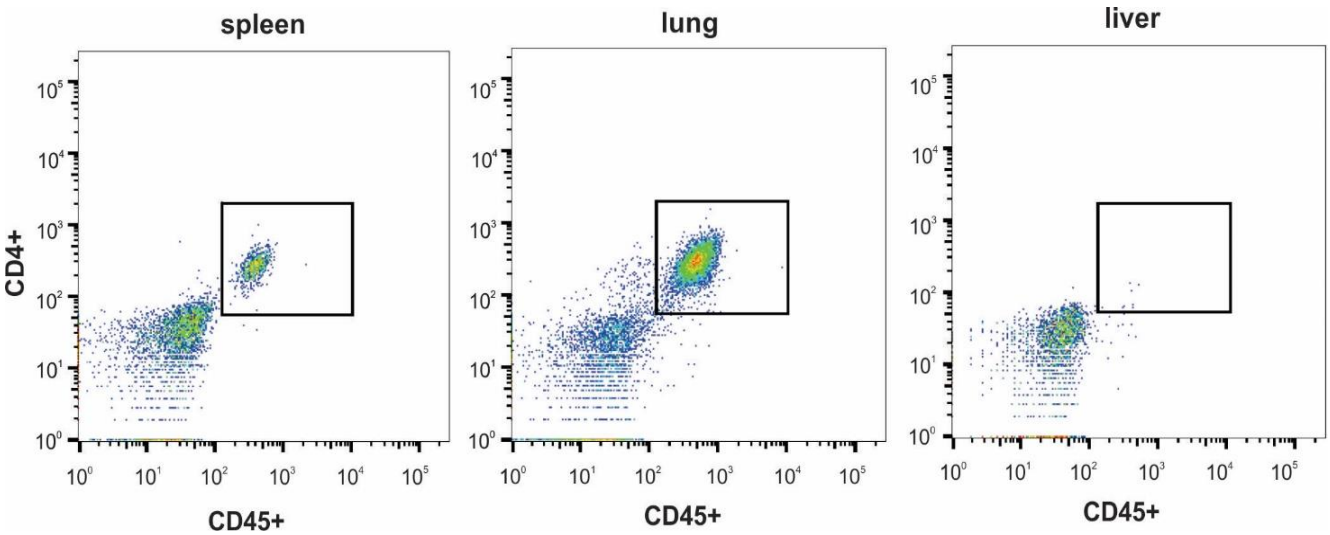

Figure 4-5. Engraftment of human CD4$^+$ T cells in mouse spleen, lung and liver
Cells were collected from mouse spleen, lung and liver at 6 weeks post transplantation. The cells were stained with anti-human CD4 and anti-human CD45 antibodies. Rectangular gates showed cells expressing human CD4 and CD45. The analysis is representative of samples taken from ten mice.

4.3.4 Efficient transduction and expression of NB-ZSG1, NB-mCh, ZSG1 and mCh in CD4$^+$ T cells

After being stimulated with anti-human CD3 and anti-human CD28 antibodies, selected human CD4$^+$ T cells were transduced with SIN gammaretroviral VLP conveying NB-mCh, mC, NB-ZSG1 and ZSG1 (Fig. 4-6).

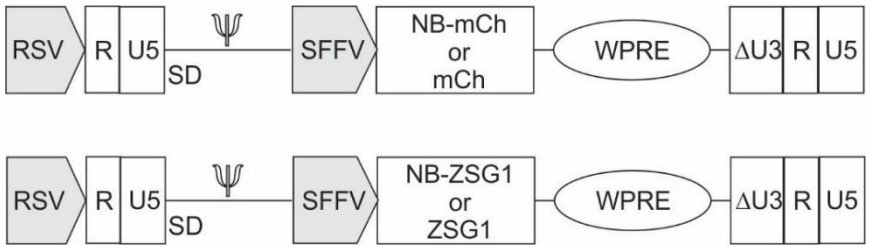

Figure 4-6. A schematic of SIN gammaretroviral SRS11-SF-γC vector containing NB-mCh or mCh or NB-ZSG1 or ZSG1
A spleen forming focus virus (SFFV) promoter is used as an internal promoter. Rous Sarcoma Virus (RSV) promoter is used to transcribe mRNA for packaging in VLPs. WPRE inserted adjacent to NB-mCh or mCh orNB-ZSG1 or ZSG1 enhances gene expression.

The positively transduced cells were selected by FACS. We selected cells that expressed high levels of fluorescent protein (Fig. 4-7), because previous studies indicated that Nullbasic antiviral activity is dose dependent[14,15,18]. Therefore, we selected cells that expressed Nullbasic fusion proteins or the control fusion proteins above a MFI of 10^4. The transduction rate of NB-mCh or NB-ZSG1 ranged between 10 and 40%, while that of mCh or ZSG1 ranged between 20 and 60%.

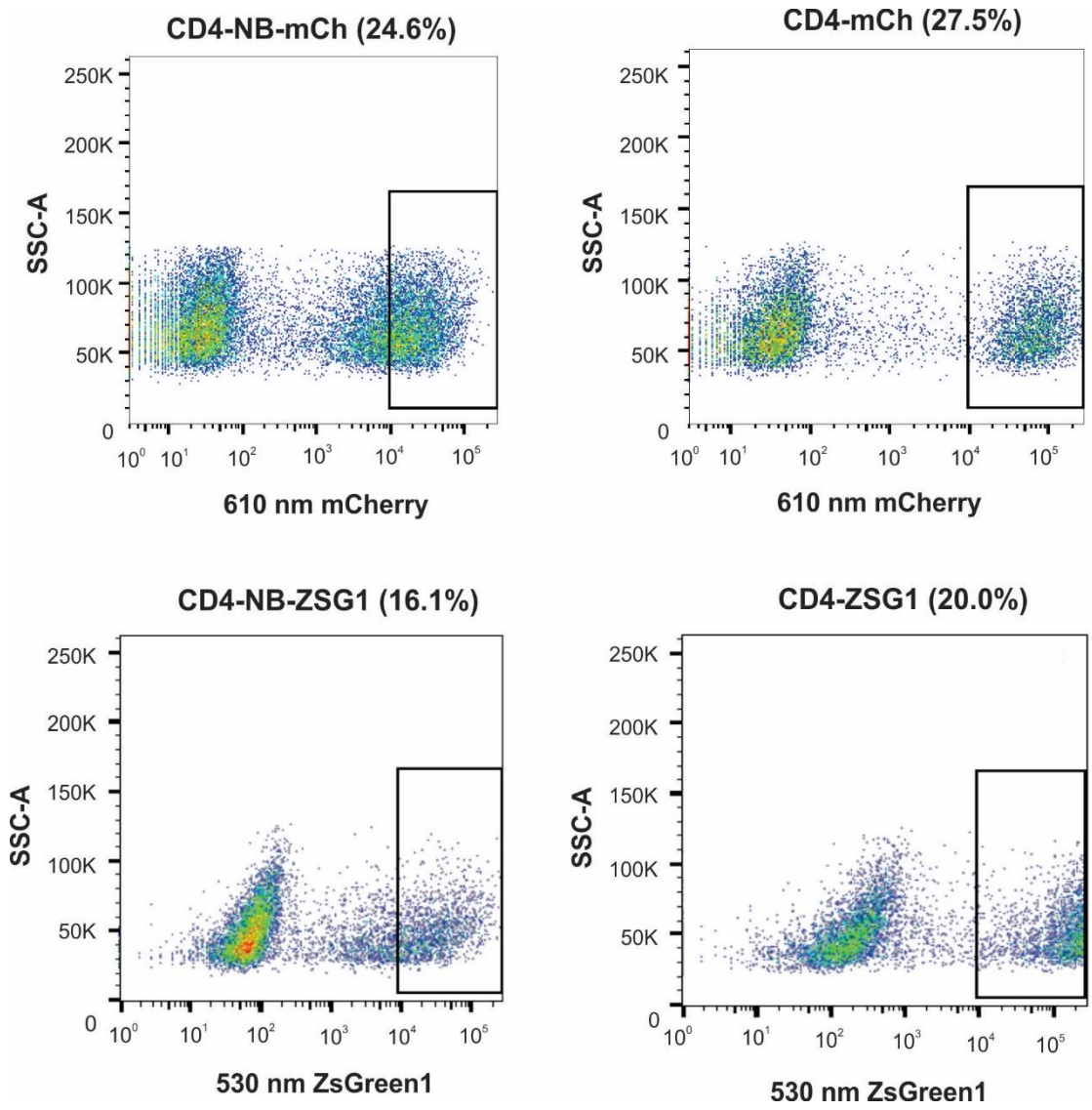

Figure 4-7. FACS of transduced CD4⁺ T cells

The cells were selected based on the fusion protein MFI above 10^4 as shown in the rectangular gates. The result is representative for at least six experiments. The gate was set based on a previous reported study[14].

4.3.5 Human CD4⁺ T cells expressing NB-mCh inhibited HIV-1 replication *in vivo*, partially

In the next experiment, 5 mice were transplanted by intravenous injection with 5 million of either CD4-NB-mCh cells or CD4-mCh cells. Human cell engraftment was monitored after week 2 post transplantation. Both NB-mCh and mCh expressing cells were detectable by flow cytometry in blood samples at week 4 and 6 post transplantation as well as in the spleens and lungs (Fig. 4-8). The data show that there is no significant difference in the

levels of engraftment of mice transplanted with CD4-NB-mCh and mice transplanted with CD4-mCh.

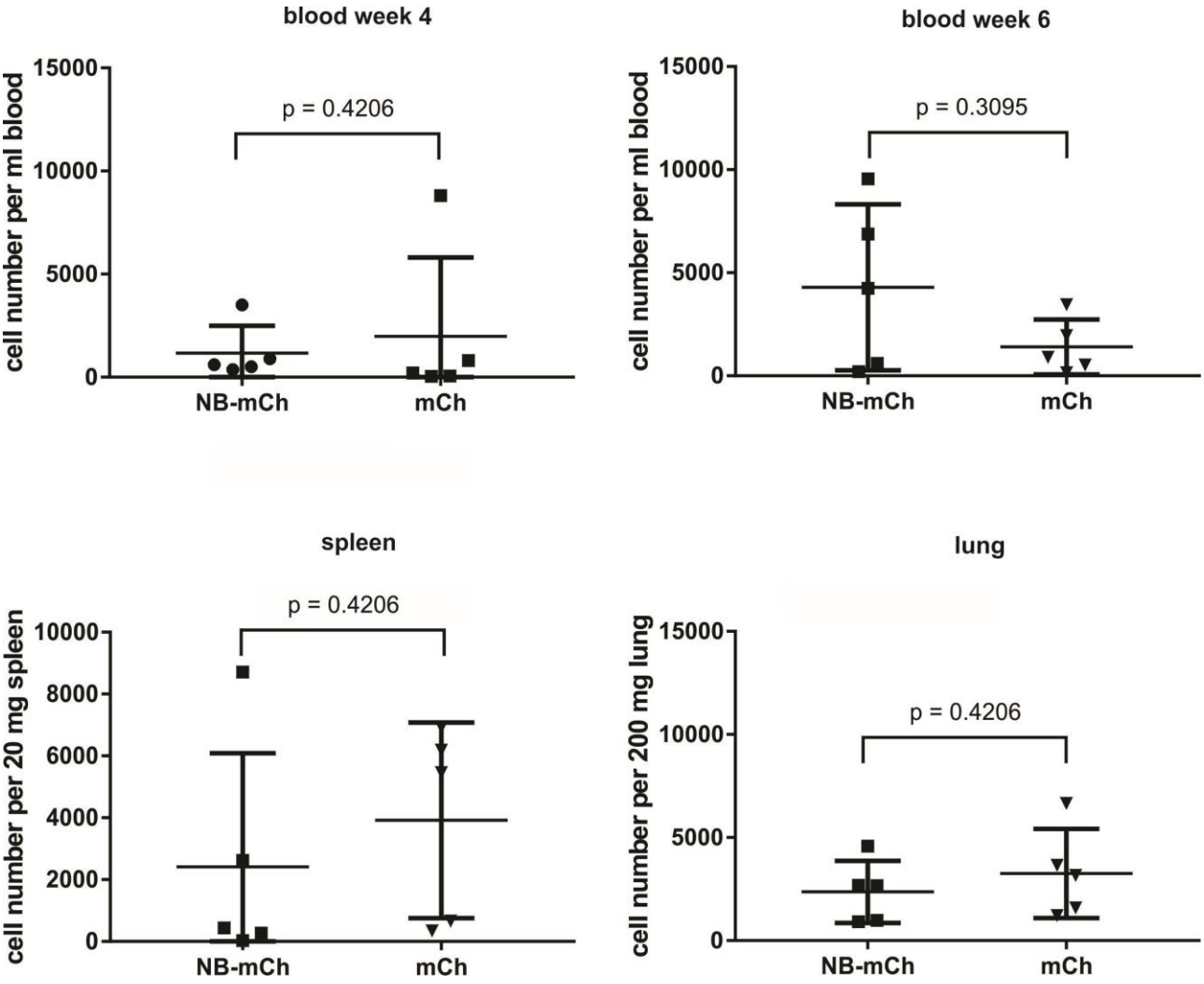

Figure 4-8. Levels of engrafted cells in blood, spleen and lung samples
Top: number of engrafted cells per ml of blood in samples taken at week 4 and week 6 post transplantation. Bottom: number of engrafted cells per 20 mg of spleen or per 200 mg of lung taken at week 6 post transplantation. The mean values and standard deviations from 5 samples are shown.

To evaluate if the human cells engrafted can support HIV-1 infection, HIV-1_{AD8}, a laboratory CCR5 HIV-1 strain of subtype B, was used in this mouse model. HIV-1_{AD8} uses the CCR5 chemokine receptor for viral entry to $CD4^+$ cells.

The mice were injected with a virus stock containing 100 ng of CA (p24) HIV-1_{AD8} by intravenous administration 2 weeks post transplantation. At week 4 post transplantation, blood samples were collected from the mice by tail bleeding. At week 6, the mice were sacrificed and blood samples were taken from the hearts by heart puncture. To detect HIV-

1 reservoirs *in vivo*, we also collected cells from the spleen, lung and liver of mice. Total RNA was isolated from the blood plasma as well as from spleen and lung cells. HIV-1 genomic RNA copy number was measured from the purified RNA samples by qRT-PCR using primers specific for full length HIV-1 RNA.

The level of viral RNA in the infected mice transplanted with CD4-NB-mCh cells was compared to that of CD4-mCh transplanted mice. Figure 4-9 shows the qRT-PCR results from plasma samples collected at week 4. It shows lower viral RNA levels in the CD4-NB-mCh transplanted mice compared to CD4-mCh transplanted mice. The mean value was ~1,800 HIV-1 RNA copies/ml in the former and ~6,200 HIV-1 RNA copies/ml in the later. However, by week 6, one of 5 CD4-NB-mCh mice showed increased HIV-1 RNA copies in the plasma. We also noted that HIV-1 RNA was detected in the spleen and lung tissues at week 6. However, there was no significant difference between HIV-1 RNA in the mice transplanted with CD4-NB-mCh and mice transplanted with CD4-mCh, indicating that NB-mCh antiviral activity in the spleen and lung was not effective at week 6.

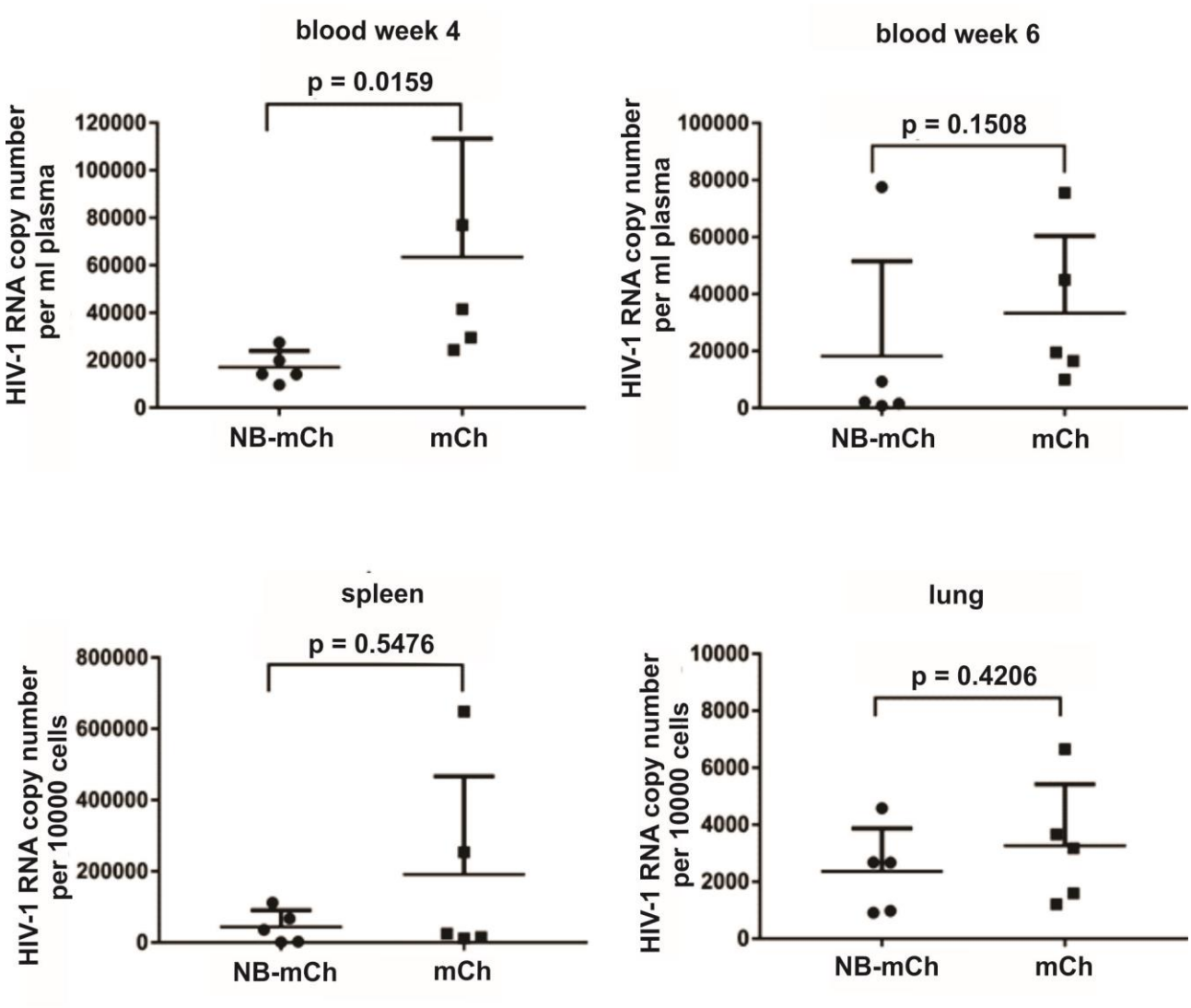

Figure 4-9. Levels of viremia in week 4 and week 6
Top: HIV-1 mRNA copy number per ml plasma at week 4 and week 6. Bottom: HIV-1 mRNA copy number per 10,000 spleen or lung cells on week 6. The mean values and standard deviations from five samples are shown.

We compared week 4 and week 6 blood samples to determine if Nullbasic antiviral activity decreased by time in this *in vivo* experiment. The result indicates that there was no difference of the viral load between week 4 and week 6 blood samples in both NB-mCh and mCh transplanted mice (Fig. 4-9). This means Nullbasic retained its antiviral activity from

week 4 to week 6. When we compared the viremia levels in NB-mCh or mCh transplanted mice at week 4 and week 6, no significant difference was observed.

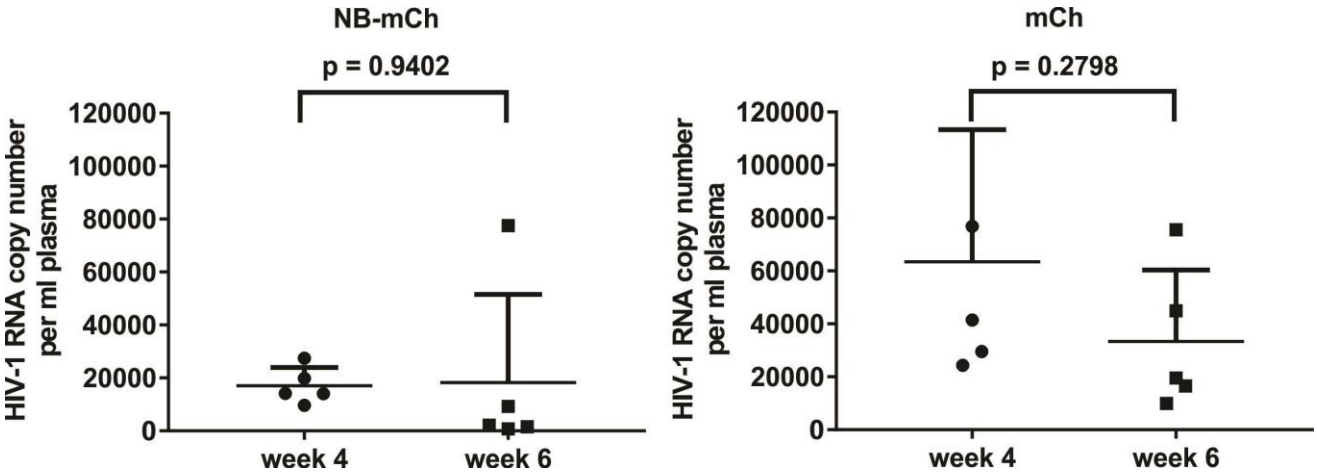

Figure 4-10. Levels of viremia in NB-mCh and mCh

Left panel: HIV-1 mRNA copy number per ml plasma in NB-mCh transplanted mice at week 4 and week 6. Right panel: HIV-1 mRNA copy number per ml plasma in mCh transplanted mice at week 4 and week 6. The mean values and standard deviations from five samples are shown. The mean differences were analyzed by t-test.

Subsequently, in chapter 3, we observed that NB-ZSG1 antiviral activity against HIV-1_{NL4-3} in primary $CD4^+$ T cells *in vitro* was greater than NB-mCh by ~6 fold. This finding drove a change in our mouse model. Based on that result, we performed further experiments by transplanting CD4-NB-ZSG1 cells instead of CD4-NB-mCh in mice.

4.3.6 Human $CD4^+$ T cells expressing NB-ZSG1 inhibited HIV-1 replication *in vivo*, better

To evaluate antiviral activity of NB-ZSG1 *in vivo,* human $CD4^+$ T cells isolated from PBMC were stimulated with anti-human CD3 and anti-human CD28 antibodies and transduced with NB-ZSG1 or ZSG1 VLPs. The transduced cells were selected by FACS for cell expressing high levels of NB-ZSG1 or ZSG1. The cells were grown for three days and then transplanted to irradiated mice intravenously. Four transplanted NB-ZSG1 or ZSG1 mice were infected with HIV-1_{AD8} at week 2 post transplantation. Two transplanted NB-ZSG1 or ZSG1 mice were used as uninfected control. As in previous experiments, we observed human cell engraftment at weeks 2, 4 and 6 by flow cytometry. Figure 4-10 shows human cell engraftment in blood samples collected at week 6 and engrafted cells numbers are

quantified in Figure 4-11. Similarly, human $CD4^+$ T cell engraftment was also detected by flow cytometry in the tissue and spleen tissues (see appendix 1 and appendix 2).

Figure 4-12 shows levels of human cell engraftment in the blood samples at week 4 and 6 as well as in the spleen and lung tissues. The data suggest that in the blood of infected mice, the number of CD4-NB-ZSG1 cells are greater than CD4-ZSG1 cells in both week 4 and 6 samples. At week 6, the two uninfected CD4-ZSG1 mice show higher number of human $CD4^+$ T cells compared to the infected CD4-ZSG1 mice. It is indicative that the number of human cells detected in spleen and lung tissues is higher in CD4-NB-ZSG1 transplanted mice than in CD4-ZSG1 transplanted mice. However, a larger set of mice is required to achieve significant values in order to confirm this result.

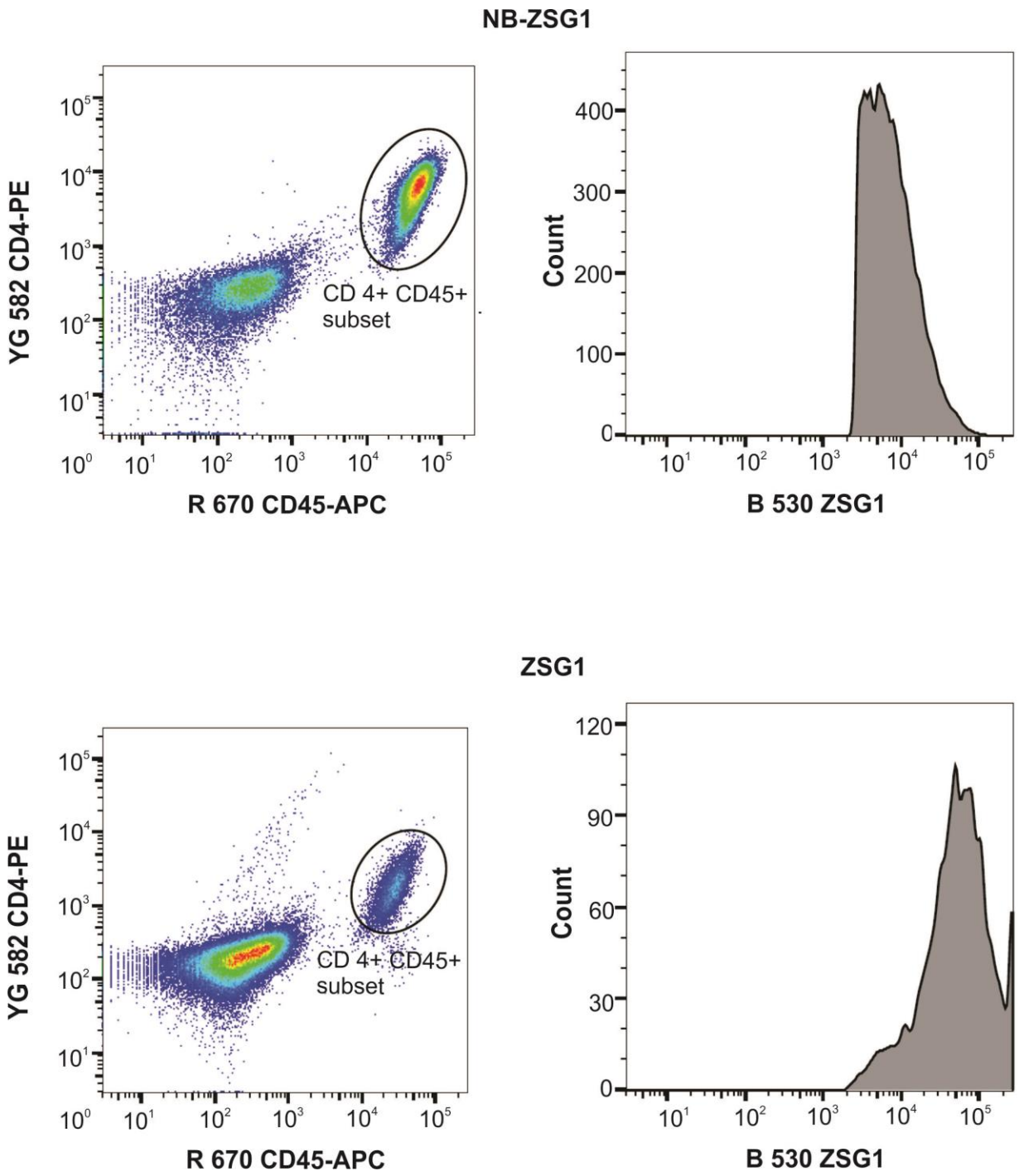

Figure 4-11. Analysis of human CD4-NB-ZSG1 and CD4-ZSG1 cell engraftment in blood sample taken at week 6 post transplantation

Blood cells were stained with anti-human CD4 and anti-human CD45 antibodies and analyzed by flow cytometry. Cells stained by both antibodies, double positives, are shown in the oval gates. The histograms show MFI for the fluorescent protein. The result is representative of four samples in each group.

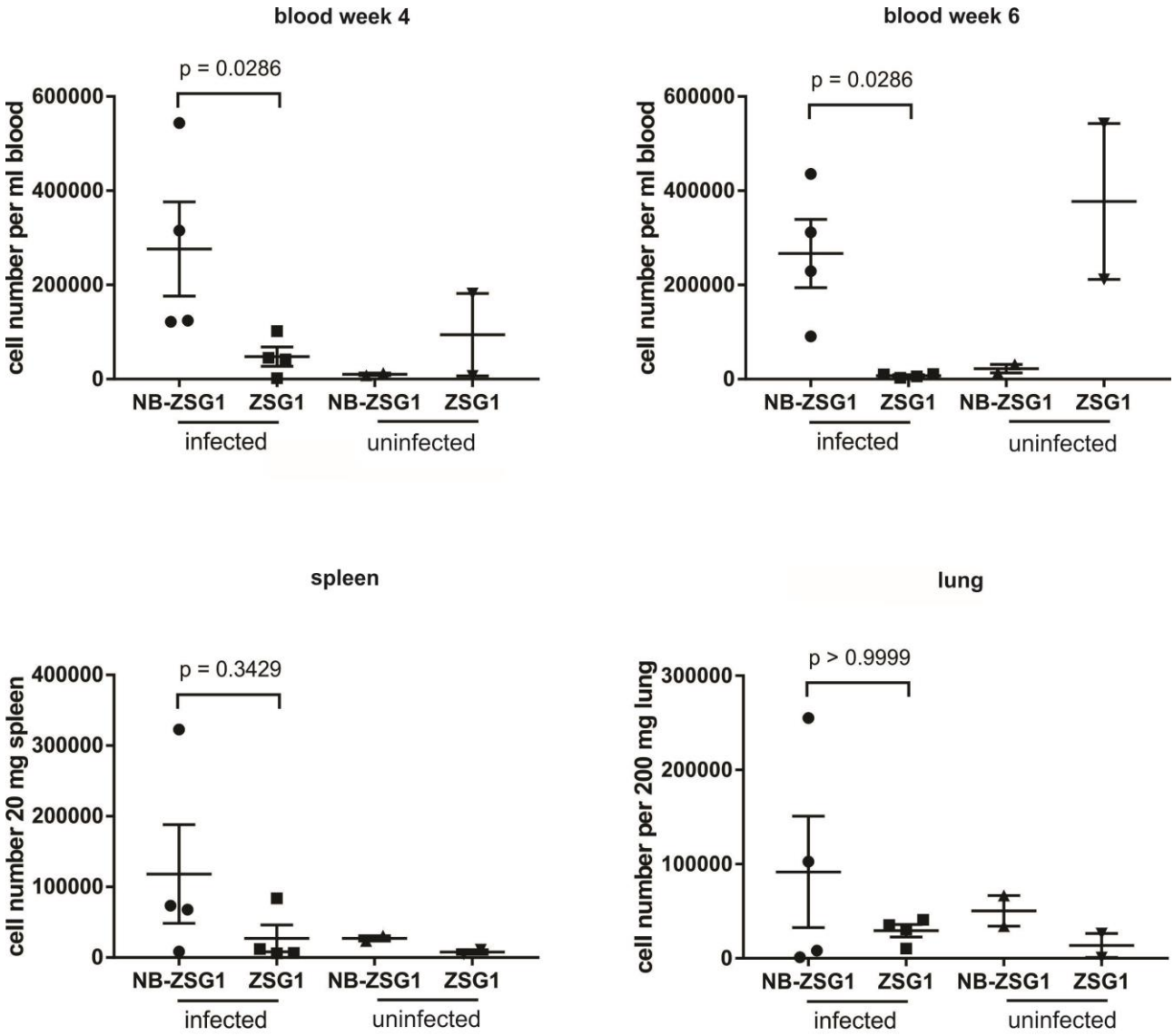

Figure 4-12. Levels of engrafted cells in blood, spleen and lung samples
Top: number of engrafted $CD4^+$ T cells in ml blood samples at week 4 and week 6. Bottom: number of engrafted $CD4^+$ T cells per 20 µg spleen and per 200 µg lung at week 6. Mean values and standard deviations from four samples are shown.

To confirm the human cell engraftment in spleen and lung tissues of the mice, we performed immunohistochemistry on samples taken at week 6 and stained with anti-human CD4-CY3 antibody followed by DAPI as a negative control. The results show that $CD4^+$ cells were present in the spleen and lung tissues in both CD4-NB-ZSG1 or CD4-ZSG1 transplanted mice indicated by green fluorescence. As mock controls, spleen and lung tissues of non-transplanted mice were shown (Fig. 4-12 and 4-13).

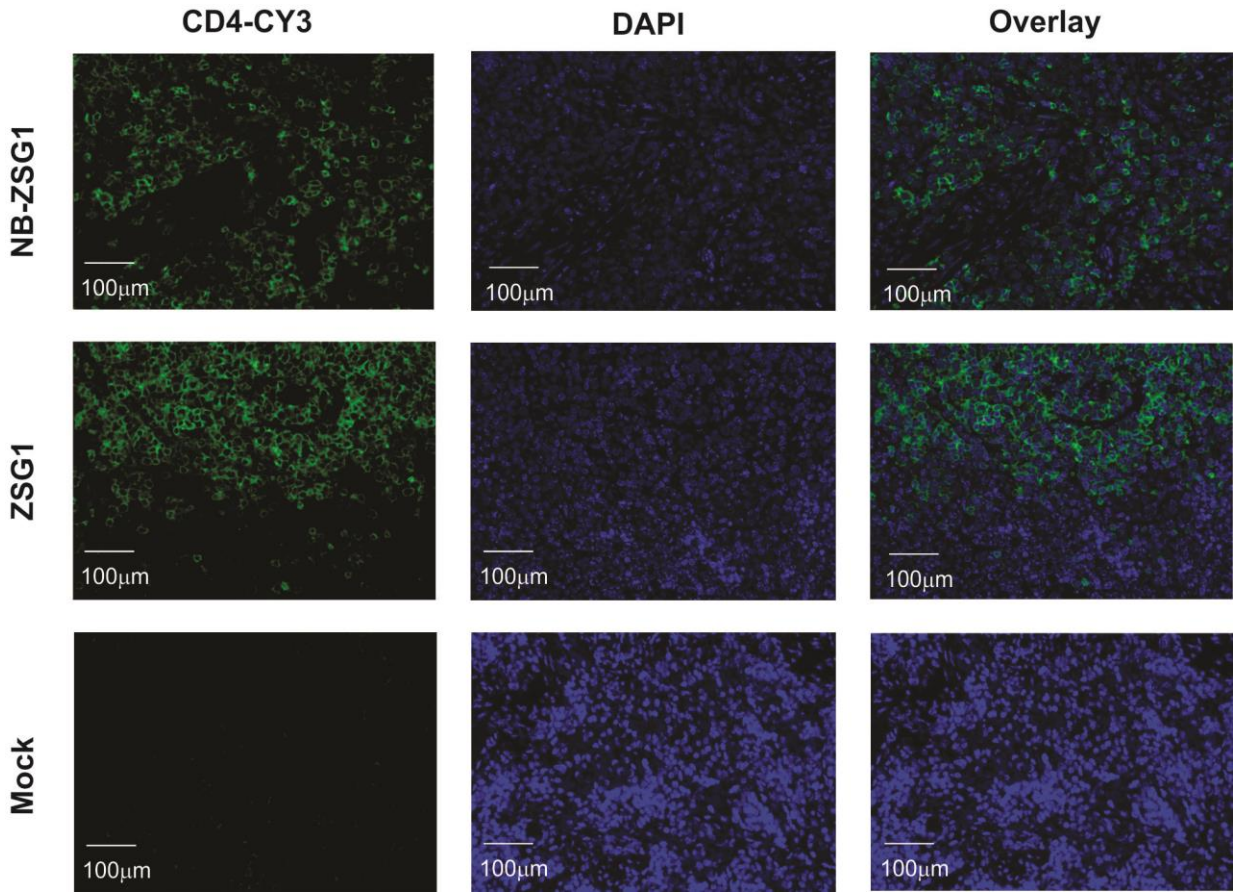

Figure 4-13. Human CD4$^+$ T cell engraftment in spleen tissues
Spleen tissues were collected and subjected to immunostaining with anti-human CD4-CY3 antibody (green). The tissues were also stained with DAPI (blue). Non-transplanted mouse spleen was used as mock control.

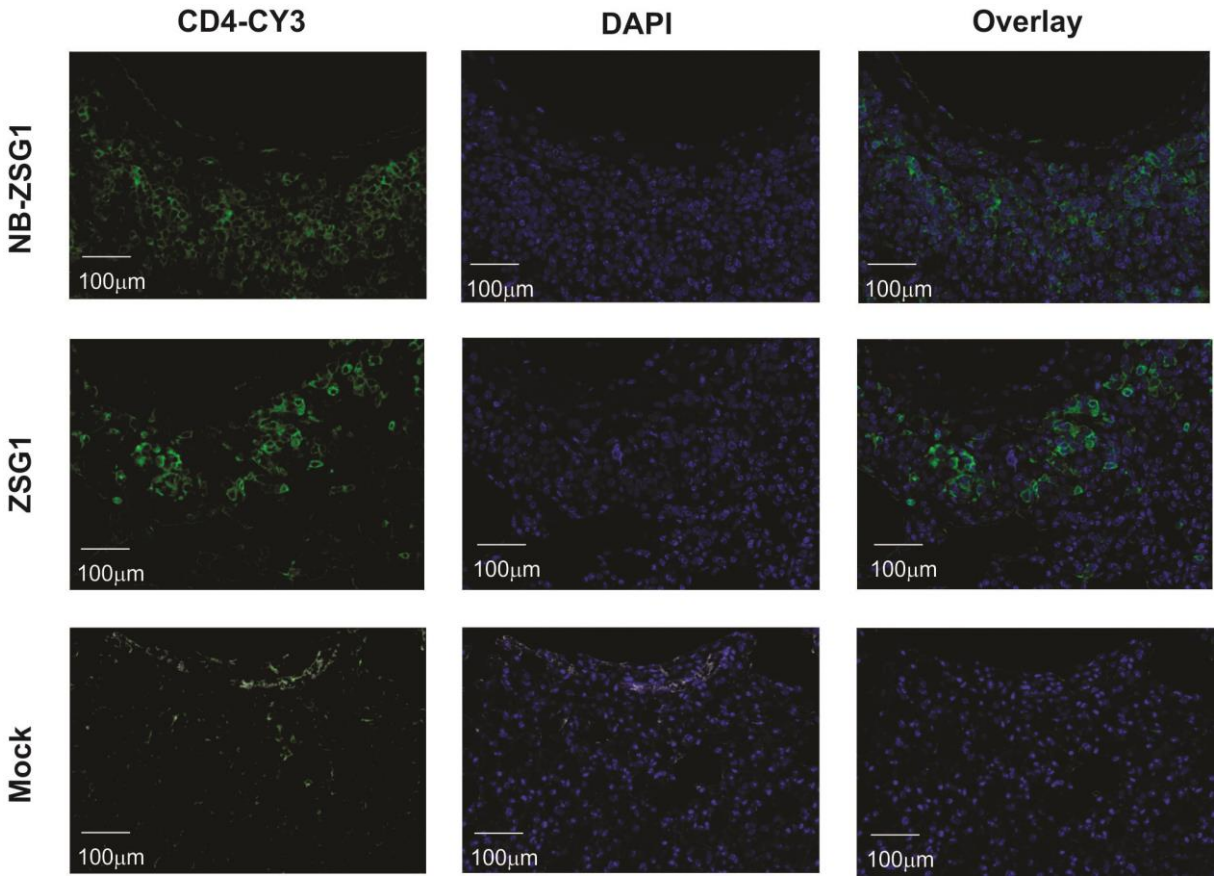

Figure 4-14. Human CD4$^+$ T cell engraftment in lung tissues
Lung tissues were collected and subjected to immunostaining with anti-human CD4-CY3 antibody (green). The tissues were also stained with DAPI (blue). Non-transplanted mouse lung was used as mock control.

HIV-1 RNA levels were measured in the plasma and tissue of all mice at week 4 and 6. We observed that the HIV-1 RNA copies were not detected by qRT-PCR in week 4 plasma samples, but at week 6 high levels of HIV-1 mRNA were detected (Fig. 4-15) in three of four CD4-ZSG1 mice. In three of four CD4-NBZSG1 mice infected with HIV-1_{AD8}, the levels of HIV-1 RNA were undetectable, suggesting that NB-ZSG1 inhibited HIV-1 replication. However, one CD4-NB-ZSG1 transplanted mouse had measurable level of HIV-1 RNA in plasma, spleen and lung samples. Additional sets of HIV-1-infected mice transplanted with CD4-NB-ZSG1 and CD4-ZSG1 cells will be required to determine if the reduced levels of viral RNA measured is significant, but the preliminary results are encouraging. As Nullbasic has not been tested in animal previously, it is not possible to assume the effect size or standard deviation, and therefore the sample size can be calculated based on resource equation method[161].

Using this method, a degree of freedom of analysis of variance (ANOVA) or E value is measured. The E value is calculated as follow, and the value should be in the range of 10 to 20.

$$E = total\ number\ of\ mice - total\ number\ of\ groups$$

To see the difference between Nullbasic and control treatments (2 groups), at least 6 animals per group or 12 animals in total is required to achieve the minimimum E value 10.

$$E = 12 - 2$$

$$E = 10$$

In this study, the sample number was limited to 4 per group because not all the transplanted animals showed detectable human $CD4^+$ T cell engraftment by week 4 post transplantation.

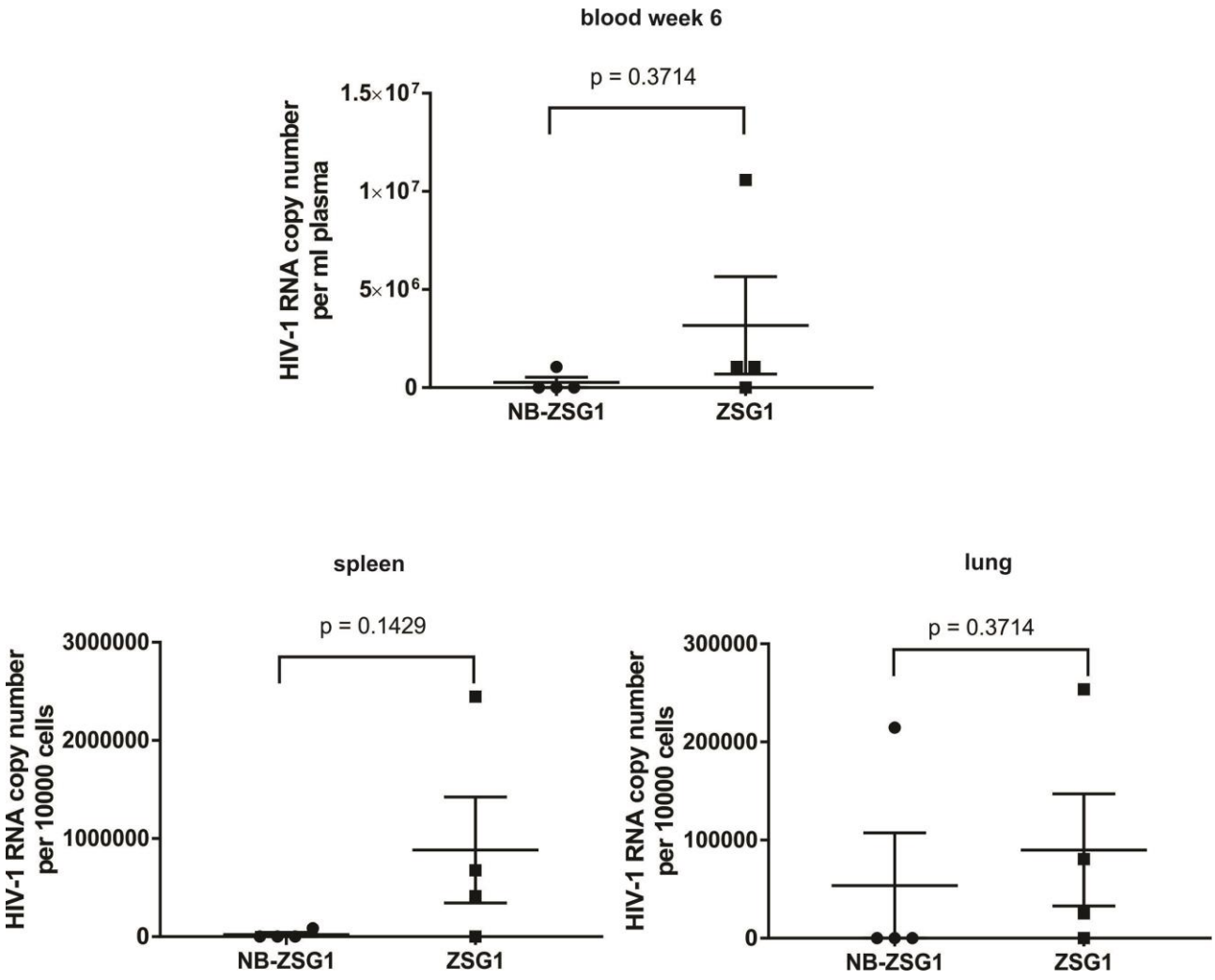

Figure 4-15. Preliminary results showing levels of HIV-1 RNA in week 6 samples post transplantation
Top panel: HIV-1 RNA copy number in plasma samples collected at week 6. Bottom panels: HIV-1 RNA copy number per 10000 cells in spleen and lung tissue taken at week 6. Mean values and standard deviation are shown for four samples.

In summary, transplantation of human $CD4^+$ T cells by intravenous injection in BALB/c-$Rag2^{-/-}\gamma c^{-/-}$ mice resulted in engraftment of the human $CD4^+$ T cells in the mice. Because of fast cell reconstitution in the mice and readily available $CD4^+$ T cells, this humanized model can be used for understanding antiviral activity of Nullbasic in vivo against an acute HIV-1 infection. The preliminary results indicate that NB-ZSG1 should undergo further testing given the encouraging results indicating that HIV-1 replication was inhibited.

4.4 Discussion

In this study, we established a mouse model that can be used to test Nullbasic antiviral activity against an acute HIV-1 infection. An acute HIV-1 infection is defined as the early stage of HIV-1 infection that usually presents between two and twelve weeks after exposure, when the virus is replicating at rapid rate. At this stage, the virus is highly transmissible[162]. Previous studies have reported that early treatment is beneficial to prevent virus replication and spread[163]. Therefore, it is important to treat HIV-1 during the acute stage to prevent virus spread and further disease progression such as chronic infection that may lead to AIDS.

We used isolated $CD4^+$ T cells as donor cells in the BALB/c-$Rag2^{-/-}\gamma c^{-/-}$ mice instead of using whole PBMCs, as in previously reported studies[164,165]. This was done to ensure that the vast majority of $CD4^+$ T cell transplanted were transduced and expressed NB-ZSG1 at a high level. It has been reported that Nullbasic can potently inhibit HIV-1 replication in Jurkat cells[2]. However in primary $CD4^+$ T cells, the virus is able to replicate if Nullbasic expression is too low[14]. This observation is in agreement with the report that Nullbasic inhibits HIV-1 in a dose dependent manner[14,15,18]. So, in this study we used an improved SIN gammaretroviral vector that is able to express genes using the SFFV promoter. The transduced cells were selected by FACS where NB-ZSG1 expression was high. Our preliminary data indicates that these CD4-NB-mCh and CD4-NB-ZSG1 cells can arrest HIV-1 replication in the short term. The preliminary data also indicates that NB-ZSG1 is a better HIV-1 inhibitor compared to NB-mCh. The reason for this is unclear.

Our results indicate the route of administration is also an important factor for efficient delivery of human cells to the mouse. Here we observed that intravenous administration of the transplant cells resulted in improved engraftment in the mouse compared to intraperitoneal administration. Through intravenous injection, the transplant cells are directly available in the blood circulation, while intraperitoneal injection directs the transplant cells to the mouse organs such as the lung and spleen, before they appear in the peripheral blood circulation[166]. We observed human cell engraftment and HIV-1 presence in the lung. This suggests targeting of administrated cells accumulated in the lung, which is a reported site of memory T cells accumulation[167,168].

After infection, we observed that the number of human cells detected in CD4-NB-ZSG1 transplanted mice was higher than in CD4-ZSG1 transplanted mice. This difference was obvious in blood samples taken at week 4 and 6. Considering higher number of human $CD4^+$ T cells in uninfected CD4-ZSG1 compared to infected CD4-ZSG1, particularly at week 6, it is likely that the HIV-1 infection has caused human $CD4^+$ T cell depletion. However, we noticed variations of human cell engraftment levels for each time point in uninfected mice, and thus further investigation using a larger set of mice, at least 6 mice per group, is required to confirm this result.

At 6 weeks post transplantation, high levels of HIV-1 RNA were observed in the lungs when CD4-NB-mCh was used. As we showed that NB-ZSG1 is expressed at higher levels in $CD4^+$ T cells, it is possible that NB-ZSG1 protected cells in the lung better than NB-mCh, but more experiments are required to confirm this. It is interesting to note that studies show that HIV-1 replication and viremia is favoured in lung tissue. For example, a previous study showed HIV-1 viremia in AIDS patients was 7.6-fold higher in the lung compared to the periphery[169]. Another study using bronchoalveolar lavage samples from HIV-1 patients showed residual HIV-1 RNA after six months of cART treatment, while viremia levels were undetectable in their blood. The persistent cellular HIV-1 reservoir in the lung in this study was correlated with a high population of $CD4^+$ lymphocytes in the alveolar compartment. The study also observed that most $CD4^+$ lymphocytes in the lung tissue are long-life memory T cells[170]. In our study, the modest level of antiviral activity by NB-mCh in the lung was likely to be associated with the decreased level of its expression by week 6 post transplantation.

Our *in vitro* experiments discussed in Chapter 3 revealed that NB-ZSG1 provides higher levels antiviral activity than NB-mCh. This finding drove changes in our animal model by transplanting NB-ZSG1 instead of NB-mCh to the mice. The result indicates NB-ZSG1 inhibition for HIV-1 replication in 3 of 4 mice up to week 6. This suggests that NB-ZSG1 is a stronger antiviral than NB-mCh both *in vitro* and *in vivo*. The reason for this is unknown. It might be due differences in protein conformation caused by the fusion partners that affect antiviral functions. In addition, these fluorescent proteins require different multimerization states in order to fluoresce. ZSG1 fluoresces as a tetrameric protein while mCh fluoresces as a monomer[151]; so it is possible that the multimeric state of the fusion protein could somehow influence the antiviral activity of Nullbasic. Other possible reasons include difference in protein half-life or their subcellular localisation. All of these possibilities remain

to be investigated. The observation is important and suggests that an appropriate fusion protein partner will be important to obtain optimal antiviral activity of Nullbasic.

An ideal animal model for HIV-1 gene therapy should: demonstrate a high level of human cell engraftment without triggering host versus graft disease; provide long term and continuous production of human $CD4^+$ cells; allow HIV-1 infection; generate human immune response, and; show $CD4^+$ cell depletion upon infection[171]. The acute model we developed is a reasonable first step but it does not provide long term and continuous production of human $CD4^+$ T cells. However, it did enable reliable human $CD4^+$ T cells availability in the mice and demonstrate a high rate of human $CD4^+$ T cell engraftment. The high degree of variation in results between the animals tested means further testing will be required.

In conclusion, transplantation of human $CD4^+$ T cells in BALB/c-$Rag2^{-/-}\gamma c^{-/-}$ resulted in a short term human cell engraftment that was used to study acute HIV-1 infection. High expression levels of NB-ZSG are required for antiviral potency against HIV-1 infection *in vivo*. Further study will require larger sets of transplanted animals. Although the retroviral vectors used here are very good, a better delivery and improved expression of Nullbasic to target cells is desirable in order to provide long term inhibition of HIV-1 replication. To improve this model, HIV-1 infection could be performed earlier, for example a week post transplantation, because the human $CD4^+$ T cells only survived for 6 weeks in the mice. In addition, a more sensitive PCR method is required to be able to detect viral load during early infection due to a relatively limited number of human $CD4^+$ T cells in the first 2 weeks post transplantation.

CHAPTER 5

Optimization of Nullbasic Lentiviral Virus Like Particle Transduction in T Cells

5.1 Introduction

Gene therapy is a promising option for the treatment of hereditary and acquired diseases. Success in gene therapy requires a safe and effective gene delivery system that can provide and maintain an adequate level of gene expression. Viruses are commonly used as vectors to transfer the desired genes to the targeted host cells. Retrovirus and lentivirus, members of Retroviridae family, have been extensively developed as gene delivery vectors.

Lentiviral vectors have advantages for delivering genes to target cells. Compared to retroviral vectors, which can only transduce dividing cells due to pre-integration complex inability to cross the nuclear membrane, lentiviral vectors can transduce non-dividing cells[172,173]. Lentiviral expression systems provide prolonged transgene expression[174]. Also, lentiviral vectors are considered safer than gammaretroviral vectors in term of theirs reduced genotoxicity risk[175,176]. It is also known that gammaretroviral vectors have risks to the host due to potential oncogenesis caused by retroviral insertional activation of host genes[177]. Phase 1 and 2 clinical trials using a HIV-1 based lentiviral vector to modify CD4+ T cells showed no adverse events following the lymphocyte transplantation. The clinical trials were conducted on 65 HIV-1 patients in total and were followed up to 8 years[178].

In Chapter 3 and 4, a SIN gammaretroviral vector was used to deliver the *nullbasic* gene to target cells. Although this vector can transduce primary $CD4^+$ T cells and the transduction rate was efficient, Nullbasic fusion protein expressions decreased over time. We previously reported that lentiviral vectors expressed Nullbasic in Jurkat cells at stable levels which potently inhibited HIV-1[2]. However, as most lentiviral vectors are based on HIV-1, Nullbasic is also a potent inhibitor of lentiviral vector transduction in all cell types tested, particularly of human primary $CD4^+$ T cells. Therefore, in this chapter we asked if a lentiviral vector could be optimized to deliver *nullbasic* to primary $CD4^+$ T cells.

As stated previously, the vast majority of lentiviral systems used today are derived from HIV-1. An earlier study measured delivery of a Nullbasic-EGFP (NB-EGFP) fusion protein to Jurkat cells by a lentiviral vector called pLOX[14]. pLOX is a second generation HIV-1-based lentiviral vector that can transduced human cells. It is comprised of an Eef1-α promoter and was reported to effectively transduce primary cells including T cell lymphocytes. For biosafety reasons, this vector has a deletion in the 3' LTR to prevent generation of replication-competent viruses as well as interaction between LTR and internal promoter[179,180]. The result showed that delivery of the Nullbasic gene by this HIV-1-based lentiviral vector was inefficient compared to delivery of a EFGP control gene with the same vector (Fig. 5-1)[14].

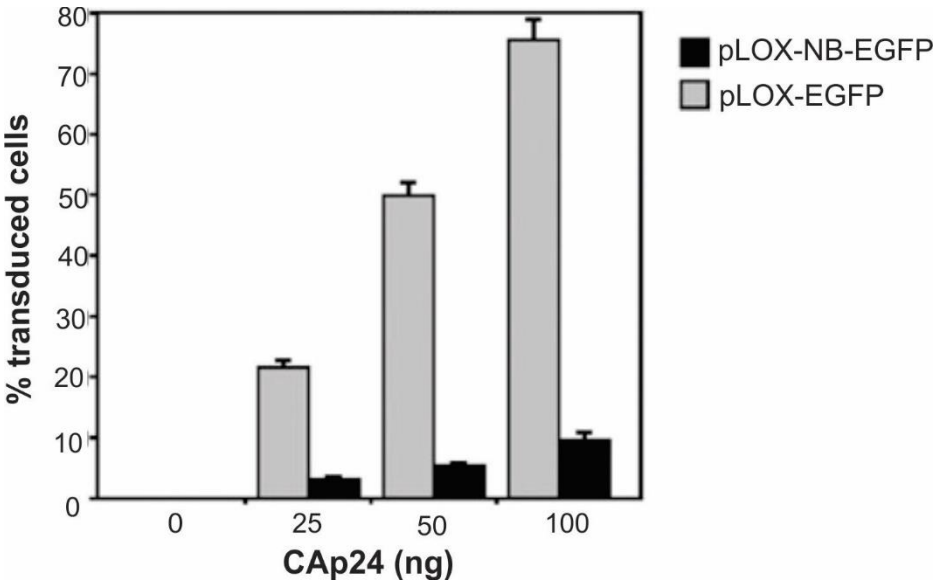

Figure 5-1. Inefficient transduction of Jurkat cells by Nullbasic EGFP (NB-EGFP) VLPs
The NB-EGFP or EGFP was delivered to Jurkat cells using pLOX lentiviral system. The VLPs were transduced in three different CAp24 amounts. Percent of transduced cells was analyzed by flow cytometry by measuring positive EGFP cells[14].

Interestingly in that study, production of NB-EGFP VLPs was not overly effected by expression of NB-EGFP in the packaging cell line but there was a trend towards slightly reduced VLP levels and VLP-associated RNA, which did not attain statistical significant[14]. Nullbasic is packaged in VLP and studies showed that Nullbasic downregulates the VLP infectivity by inhibiting HIV-1 reverse transcription[14,18]. Nullbasic directly binds reverse transcriptase and this leads to instability of the reverse transcription complex and decreased viral DNA synthesis[14,18], and therefore resulted in inefficient transduction of cells by NB-EGFP VLPs.

However, as lentiviral vectors have stable gene expression and the ability to transduce quiescent cells, and are safer than retroviral vectors, it is worth exploring for ways to improve transduction efficiency of T cell lines and primary $CD4^+$ T cells with lentiviral VLPs conveying Nullbasic. To this end, in this study, we investigated if expression of a Tat antagonist or overexpression of viral or cellular proteins targeted by Nullbasic could compensate for poor lentiviral transduction of T cells.

As a Tat antagonist agent, we used a humanized Tat2 intrabody (huTat2) protein. An intrabody is an intracellularly expressed recombinant antibody fragment that can bind a target protein within the cell. This huTat2 protein is highly expressed in eukaryotic cells after transduction. Earlier studies have shown that, when delivered to human immune cells with gamma-retroviral gene therapy vectors, the huTat2 intrabody can protect cells from HIV-1 infection, replication and pathogenesis[23,93,94]. The huTat2 intrabody binds to amino acids +1 to +19 in the N-terminal proline-rich region of Tat[23], which is entirely conserved in the Nullbasic protein. The expressed huTat2 should also bind to Nullbasic at the same amino acid residues. By binding to Nullbasic protein that is expressed in the VLP producer cells but not incorporated in the VLP, huTat2 will prevent Nullbasic inhibitor activity on RT and may block inhibition of lentiviral VLP infectivity.

Acting in a dominant negative manner, Nullbasic inhibits Tat-mediated transactivation by binding to P-TEFb, leading to the inhibition of transcription by RNA polymerase II (RNAPII)[2]. Furthermore, Nullbasic inhibits transport of RRE-containing HIV-1 mRNA through direct interaction with DDX1, a cellular protein that plays an important role in Rev-mediated transport of HIV-1 mRNA from the nucleus to cytoplasm[3]. These actions may also negatively affect transduction efficiencies of HIV-based lentiviral VLPs[14].

Can transduction efficiency of Nullbasic lentiviral VLPs be restored by overexpression of important viral or cellular factors? For example, besides Tat main function as a transcription enhancer, Tat is also important for optimal HIV-1 reverse transcription[128]. A previous study showed that transfection of wild type Tat expression vectors could rescue defects in reverse transcription of HIV-1 with a *tat* gene deletion[128]. Interestingly, a subsequent study showed that overexpression of Tat in cells having Nullbasic could compensate for decreased HIV-1 mRNA levels due to Nullbasic inhibition on Rev activity in nucleocytoplasmic transport of singly spliced and unspliced HIV-1 genomic RNA[3]. Furthermore, overexpression of DDX1 could counteract Nullbasic inhibition on Rev function by rescuing Rev nuclear localization

disrupted by Nullbasic[3]. Here, we hypothesized that overexpression of either wild type HIV-1 Tat, HIV-1 Rev or the RNA helicase DDX1 may improve transduction efficiency of NB-ZSG1 VLPs.

5.2 Materials and Methods

5.2.1 Cell lines and cultures

Human embryonic kidney (HEK) 293T cells (ATCC® CRL-3216) were grown in Dubelcco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% ($^{v}/_{v}$) FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) (referred to as DF10 medium). Jurkat cells were grown in RPMI medium (Life Technologies) supplemented with 10% ($^{v}/_{v}$) FBS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) (referred to as RF10 medium). HEK 293T-mCh-huTat2 and HEK293T-mCh cell lines were established by transduction of pSicoR-Ef1a-mCh-huTat2 and pSicoR-Ef1a-mCh-puro lentiviral systems respectively, and were then selected using FACS for the top 10% of mCherry expressing cells by MFI.

5.2.2 Isolation of of primary $CD4^+$ cells

Human buffy coat was supplied by Australian Red Cross Blood service (negative for HIV-1, hepatitis B and hepatitis C). PBMCs were isolated by Ficoll density gradient centrifugation of the buffy coat. $CD4^+$ cells were isolated from the PBMCs by using a MACS human $CD4^+$ cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. The selected $CD4^+$ cells were grown in 6 cm tissue culture dishes and stimulated using plates pre-coated with purified anti-human CD3 (clone HIT3a) and anti-human CD28 (clone CD28.2) antibodies (BioLegend) in RPMI medium supplemented with 20% ($^{v}/_{v}$) FBS and 5 ng/ml IL-2 (hereafter called RF20 IL-2) for 2 days. All cells were grown at 37 °C in humidified incubators with 5% CO_2.

5.2.3 Generation of of plasmid constructs

A lentiviral transfer plasmid expressing mCherry-T2A-huTat2 (pSicoR-Ef1a-mCh-huTat2) was constructed. Briefly, the gene encoding hu-Tat2 was fused at the C terminal of mCherry-T2A in a pSicoR lentiviral vector using EcoRI and XmaI restriction sites. A huTat2 with C terminal HA tag gene, huTat2-HA, was inserted into a pcDNA3.1+ plasmid (Addgene). Lentiviral plasmids expressing NB-Flag-ZSG1 or ZSG1 were constructed by inserting the

NB-Flag-ZSG1 or ZSG1 genes into a pSicoR vector using NheI and EcoRI restriction sites. pNL4-3.Luc.R$^-$E$^-$ was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH[181,182]. Tat101 Flag, DDX1-HA and Rev expression plasmids were constructed in pcDNA3.1+.

5.2.4 HIV-1 and VLP production

VSV-G-pseudotyped HIV-1 harbouring firefly luciferase reporter gene was produced in HEK 293T cells by co-transfection of pNL4-3.Luc.R$^-$E$^-$ and pCMV-VSV-G plasmids for 48 hours. The supernatant was collected and CAp24 concentration was measured by ELISA (Zeptometrix), as recommended by the manufacturer.

NB-ZSG1 and ZSG1 VLPs were produced in HEK 293T cells expressing mCherry-T2A-sFv-Hutat2 or mCherry alone or parental HEK 293T cells in a 10 cm culture disk by cotransfection of 2 µg of pSicoR-Flag-NB-ZSG1 or pSicoR-ZSG1 plasmids, 2 µg of pCMV-VSV-G expressing plasmid and 6 µg of pCMVΔR8.91 plasmid using 10 µl of X-tremeGENE^{TM} plasmid DNA transfection reagent (Roche) according to the manufacturer's instructions. NB-ZSG1 and ZSG1 VLPs were also produced in HEK 293T cells containing overexpression of Tat, DDX1 and Rev by adding 3 µg of each plasmid in the transfected solution. Six hours post transfection, the cells were washed with PBS and the media was replaced. The VLPs were collected at 48 hours post transfection and filtered through a 0.45 µm filter. CAp24 concentration was measured by ELISA (Zeptometrix), as recommended by the manufacturer.

5.2.5 Western blot analysis

Cell lysates were made from the HEK 293T expressing mCherry-T2A-huTat2 or mCherry or parental HEK 293T cells in cell lysis buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% ($^v/_v$) Triton X-100). The total protein concentration was measured by a Bradford assay using Bio Rad protein assay (Bio Rad) and equivalent amounts of protein were used for analysis. To check the mCherry-T2A-huTat2 expression in the cell lines, the blot was stained with anti α-mCherry rabbit antibody (BioVision) followed by anti-rabbit IgG horseradish peroxidase (HRP)-linked antibody (Cell Signaling Technology) and β-tubulin antibody (Sigma Aldrich) followed by anti-mouse IgG HRP-linked antibody (Cell Signaling Technology). The expression of Nullbasic, Tat, Rev and DDX1 was confirmed by staining

the blot with anti-Tat rabbit (Diatheva), anti-Rev mouse (Santa Cruz Biotechnology) and anti-DDX1 rabbit antibodies (Santa Cruz Biotechnology) respectively.

5.2.6 Transduction of Jurkat cells and primary $CD4^+$ T cells by NB-ZSG1 or ZSG1 VLPs

Transduction was performed in 24 well culture dishes using 4 x 10^5 Jurkat or primary $CD4^+$ T cells in each well. For each well, 50 ng VLPs plus 8 ng/ml polybrene were added to the cells. Spinoculation was performed by centrifugation at 1500 x g for 1 hour at 32 °C. The medium was replaced the next day. Samples were taken at 72 hours post transduction, fixed by 1% paraformaldehyde in PBS, and then analyzed by BD LSR 4 flow cytometer. Data was analyzed by version 9 FlowJo single cell analysis software.

5.2.7 Statistical analysis

Statistical analyses were performed using ANOVA or student t test on the data from at least three independent experiments or measurements where p values are shown. A confidence interval of 95% was used, therefore p value less than 0.05 is considered to be significant.

5.3 Results

5.3.1 An improvement in transduction efficiency with a Nullbasic lentiviral vector using an intracellular antibody (intrabody) to Tat

The gene encoding huTat2 was ligated into the lentiviral vector pSicoR-Ef1a-mCh-puro where huTat2 replaced the *pac* gene encoding puromycin N-acetyl-transferase (PAC) (Fig. 5-2). In this vector, the expression of mCherry acts as a surrogate marker for huTat2 expression, which cannot be detected by Western blot analysis as no anti-huTat2 antibody is available. A stop codon inserted after a ribosomal skipping gene T2A[183] prevented translation of PAC in the control vector. The presence of the T2A element in between mCh and huTat2 genes prevents translation of a peptide bond between the C-terminal glycine of T2A and N-terminal proline of huTat2. Therefore, mCh and huTat2 are expressed as separated proteins.

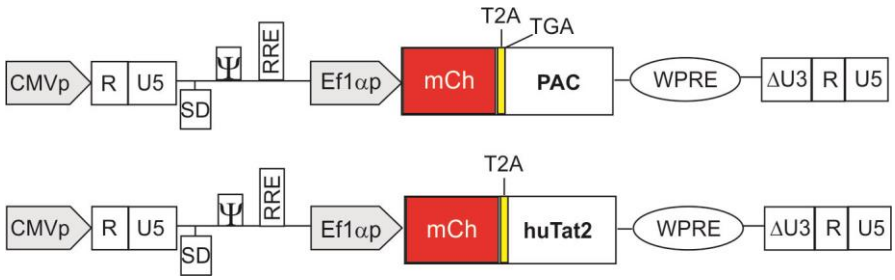

Figure 5-2. Schematic of pSicoR vector harbouring PAC and huTat2 Intrabody
This vector uses cytomegalovirus early immediate promoter (CMVp) hybrid with LTR, cis-acting element psi (ψ), RRE and WPRE. The gene of interest is driven by Ef1α promoter. A ribosomal skipping element T2A was inserted in between mCherry and PAC or huTat2.

VLPs were produced using each vector and used to transduce HEK293T cells. Cells that were mCherry positive (Fig. 5-3a) were collected by FACS and the purity was confirmed by flow cytometry (Fig. 5-3b). Cell lysates prepared from the selected cells were analyzed by Western blot using an anti-mCherry antibody. The mCherry-T2A protein was clearly detectable (Fig. 5-3c, middle panel) and serves as a surrogate indicator of hu-Tat2, which cannot be detected by Western blot analysis. A low amount of the ~65 kDa mCherry-T2A-huTat2 fusion protein was observed following longer exposure of the Western blot (Fig. 5-3c, top panel). A strong mCh band was detected in the mCh-T2A-huTat2 transfected cells as a result of ribosomal skipping gene T2A that stopped translation of a specific peptide that connects T2A and huTat2.

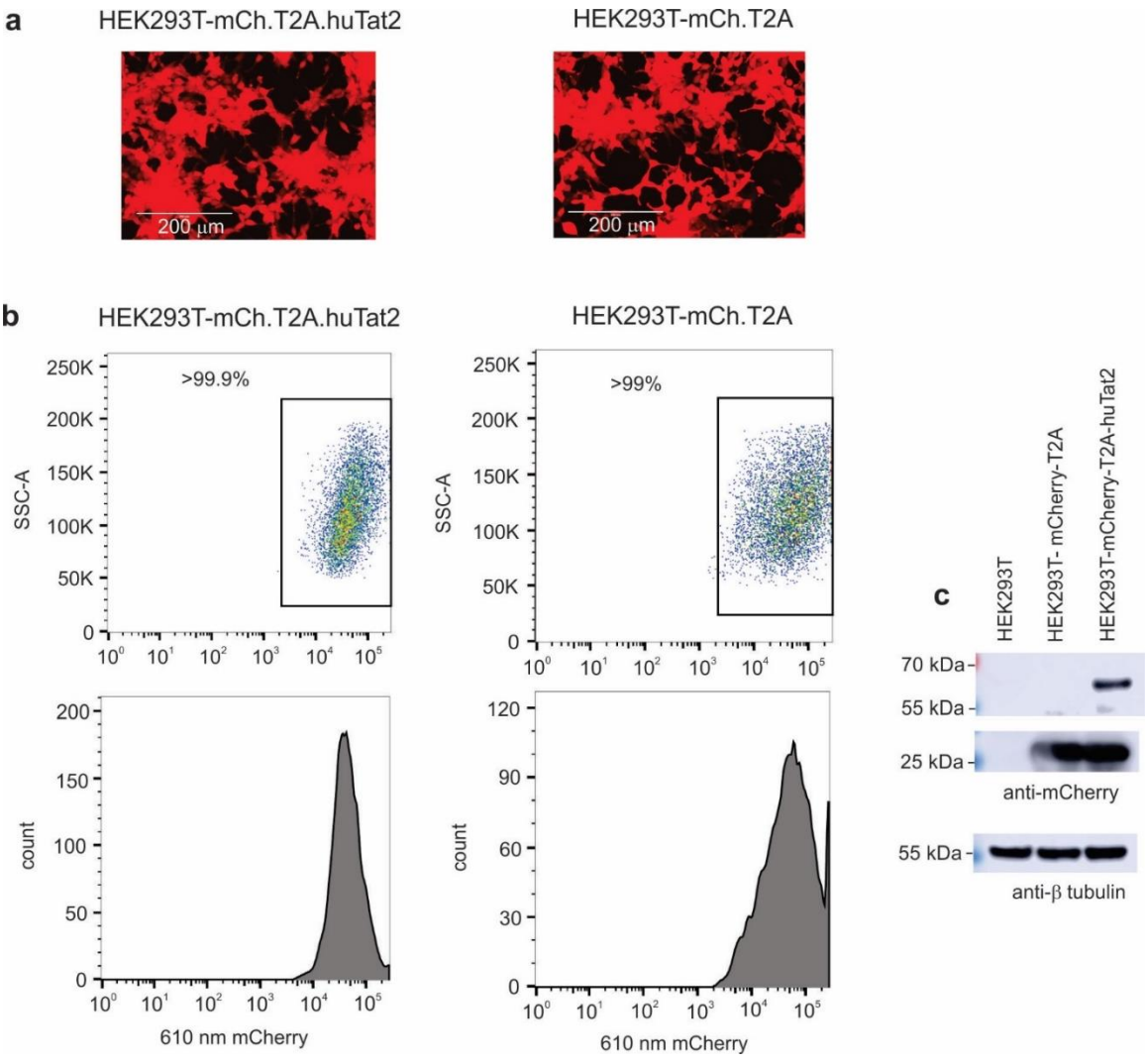

Figure 5-3. MCherry expression in HEK 293T cells.
(a) HEK293T cells transduced with pSicoR-Ef1a-mCh-T2A-hu-Tat2 or pSicoR-Ef1a-mCh-T2A-puro expressed mCherry under EVOS® imaging system. (b). The highly expressed mCherry cells were sorted by FACS and analyzed by flow cytometry. (c). mCherry expression in mCh-T2A-hu-Tat2-Intrabody and mCh-T2A-puro cell lines were detected by Western blot.

The stable cell line HEK293T-mCh-huTat2 and HEK293T-mCh were infected with HIV-1 NL4-3.Luc.R$^-$E$^-$[181,182] pseudotyped with VSV-G envelope. 24 hours after infection, cell lysates were prepared and the level of firefly luciferase was measured. The results showed a statistically significant ~25-30% decrease in firefly luciferase activity in HEK293T-mCh-huTat2 intrabody cells compared to control cells (Fig. 5-4). The data indicate that a Tat-targeting intrabody can inhibit Tat transactivation of the HIV-1 LTR in HEK293T cells, as previously reported in other cell types[91]. PSiCoR vector[184] was chosen as the preffered transduction vector as it uses CMV promoter that can generate strong transgene expression.

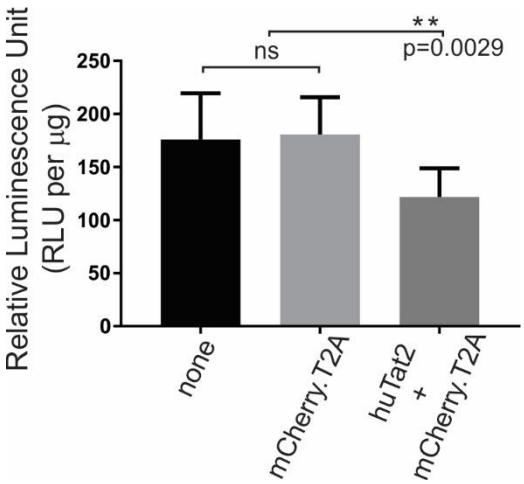

Figure 5-4. HuTat2 intrabody inhibits Tat-mediated transactivation
HEK293T-mCh-huTat2 and HEK293T-mCh or parental HEK 293T cells were infected by VSV-G pseudotype HIV-1 luciferase reporter virus. Bars indicate mean luciferase activity normalized by total protein concentrations and error bars indicate standard deviation. Experiments were performed three times independently in triplicate.

The *nullbasic* gene was fused to ZSGreen1 (NB-ZSG1) and delivered to human T cells using a pSicoR lentiviral system (Fig. 5-5). The pSicoR vector has human cytomegalovirus immediate early enhancer promoter (CMVp), a strong RNA polymerase II promoter, integrated with LTR promoter. NB-ZSG1 or ZSG1 expression is driven by an internal EF1α promoter. This transfer vector has central polypurine tract (cPPT) for assisting reverse transcription and gene translocation into the nucleus of non-dividing cells, RRE for assisting Rev function in transporting mRNA from the nucleus to cytoplasm, and a psi (ψ) packaging signal for viral genome packaging. For safety, this vector has an LTR U3 region deletion, creating a self-inactivating (SIN) virus that prevents transcription of the full length viral genome from LTR post integration. Therefore, this SIN can prevent production of full length viral RNA in target cells. A woodchuck hepatitis virus post-transcriptional modification element (WPRE) in the vector enhances the level of transgene transcripts in the nucleus and cytoplasm of target cells[185,186].

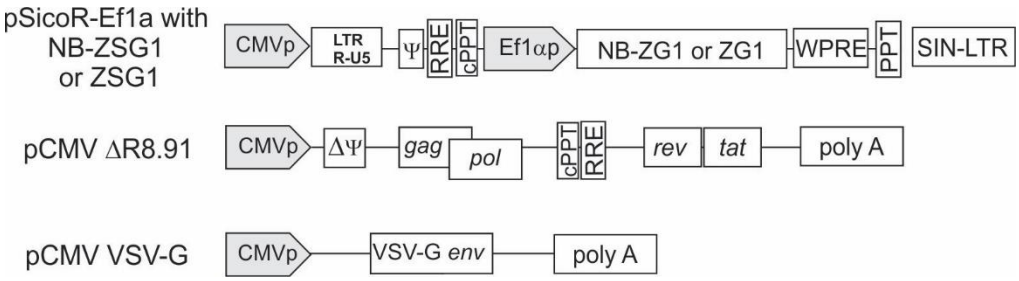

Figure 5-5. Schematic of three expression plasmids used to generate NB-ZSG1 or ZSG1 lentiviral VLPs
The transfer vector, pSicoR, encodes NB-ZSG1 or ZSG1 driven by an Ef1α internal promoter. pCMV ΔR8.91 was used as a packaging vector and pCMV VSV-G was used as an envelope vector.

To produce VLPs, this pSicoR vector was co-transfected with a pCMV ΔR8.91 packaging plasmid and a VSV-G envelope plasmid in HEK 293 T cells. The packaging plasmid construct encodes Gag, Gag-Pol, Tat and Rev to support viral RNA encapsidation, reverse transcription and integration in the target cell chromosome. The VSV-G envelope plasmid encodes VSV-G envelope that facilitates viral attachment and fusion to target cells.

Jurkat cells were transduced with equivalent amounts of each VLP normalized to total HIV capsid (CA), and with or without a spinoculation. Spinoculation can improve rates of infection by retroviruses and transduction efficiency of VLPs[187]. The cells were transduced and then incubated for 72 hours, then the percentage of cells that were positive for ZSG1 or NB-ZSG1 was measured by flow cytometry. The results showed that >99% of Jurkat cells were successfully transduced by ZSG1-VLPs irrespective of spinoculation (Fig. 5-6). This suggests that the concentration of ZSG1-VLPs used was saturating for the number of cells used. However, at the same concentration of CA, only 12% of Jurkat cells were successfully transduced by NB-ZSG1-VLPs without spinoculation (Fig. 5-6a), which increased to 25% with spinoculation (Fig. 5-6b). When huTat2 proteins present, the transduction of Jurkat by NB-ZSG1-VLPs increased by ~2-fold. With spinoculation, transduction of Jurkat cells by NB-ZSG1-VLPs improved up to ~2.5-fold when the VLPs were made in HEK293T-mCh-T2A-huTat2 cells compared to NB-ZSG1-VLPs made in HEK293T-mCh-T2A cells. The transduction rate of Jurkat cells by control ZSG1-VLPs was unchanged irrespective of the cell line used to make the VLPs. Therefore, we conclude that huTat2 intrabody can specifically improve transduction rates of Jurkat cells by NB-ZSG1-VLPs.

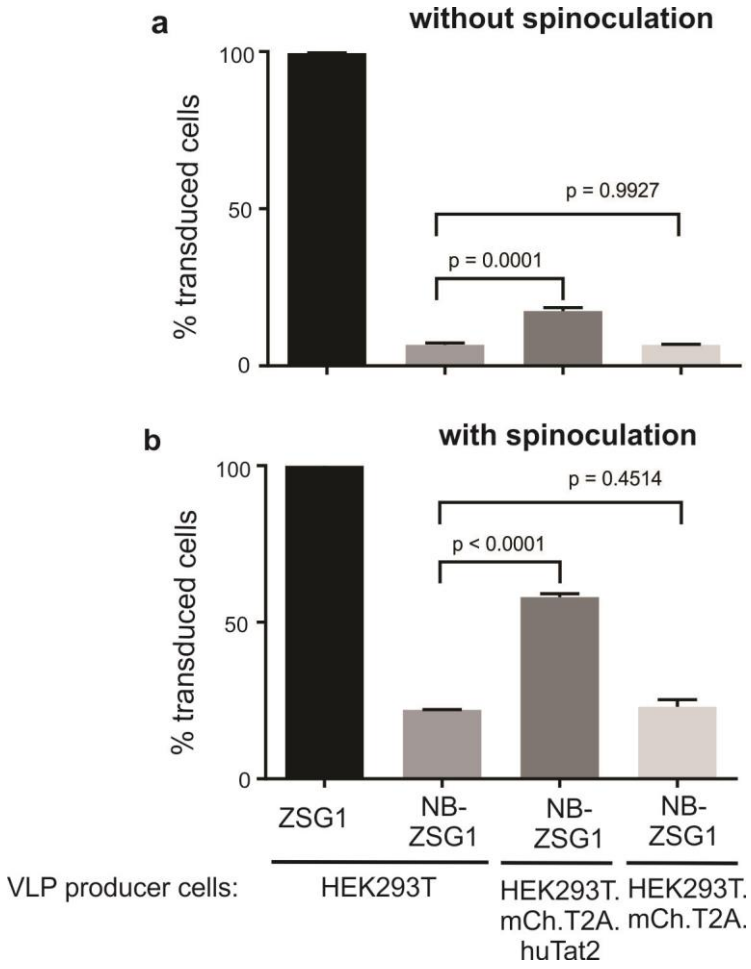

Figure 5-6. HuTat2 intrabody improved transduction efficiency of Jurkat cells by NB-ZSG1 lentiviral VLPs
Jurkat cells were transduced with 50 ng ZSG1 or NB-ZSG1 VLPs produced in VLP producer cell lines, as indicated. Transduction was performed using polybrene without spinoculation (a) and with spinoculation (b). Bars indicate mean percent of transduced cells and error bars indicate standard deviation. Experiments were performed three times independently in triplicate.

5.3.2 Improved transduction of Jurkat cells by NB-ZSG1-VLPs using cellular or viral proteins

NB-ZSG1-VLPs and ZSG1-VLPs were produced in HEK293T cells by co-transfection of pSicoR-Flag-NB-ZSG1 or pSicoR-ZSG1 plasmids, pCMV-VSV-G plasmid and pCMVΔR8.91 plasmid along with an empty pCDNA3.1+ plasmid or with plasmids encoding Tat, Rev or DDX1. Western blots were performed using lysates prepared from the transfected HEK293T cells to confirm expression of the gene products. The expression of NB-ZSG1, Tat, DDX1 and Rev in the VLP producer cells was detected by Western blot (Fig. 5-7a). The VLPs collected were used to transduce Jurkat cells with or without a spinoculation step using an equivalent amount of VLPs normalized to total CA in the supernatant.

Irrespective of spinoculation, ZSG1-VLPs transduced >99.9% of Jurkat cells under the conditions tested (Fig. 5-7b and 5-7c). However, the transduction rate of NB-ZSG1-VLP was reduced to ~25% without spinoculation or ~50% with spinoculation, respectively.

Without spinoculation, the transduction rate was significantly improved when Tat was overexpressed in the VLP producer cells but not when DDX1 or Rev were overexpressed (Fig. 5-7b). With spinoculation, either Tat or DDX1 overexpression improved the transduction efficiency of NB-ZSG1 up to ~70%. Co-expression of Tat and DDX1 (combined) improved transduction; this was most apparent when spinoculation was used, where >95% of Jurkat cells expressed NB-ZSG1. However, co-expression of Tat, Rev or DDX1 had no significant effect on transduction by ZSG1-VLPs (Fig. 5-8). Hence, the results indicate that coexpression of Tat and DDX1 together can improve the transduction ability of NB-ZSG1 VLPs specifically.

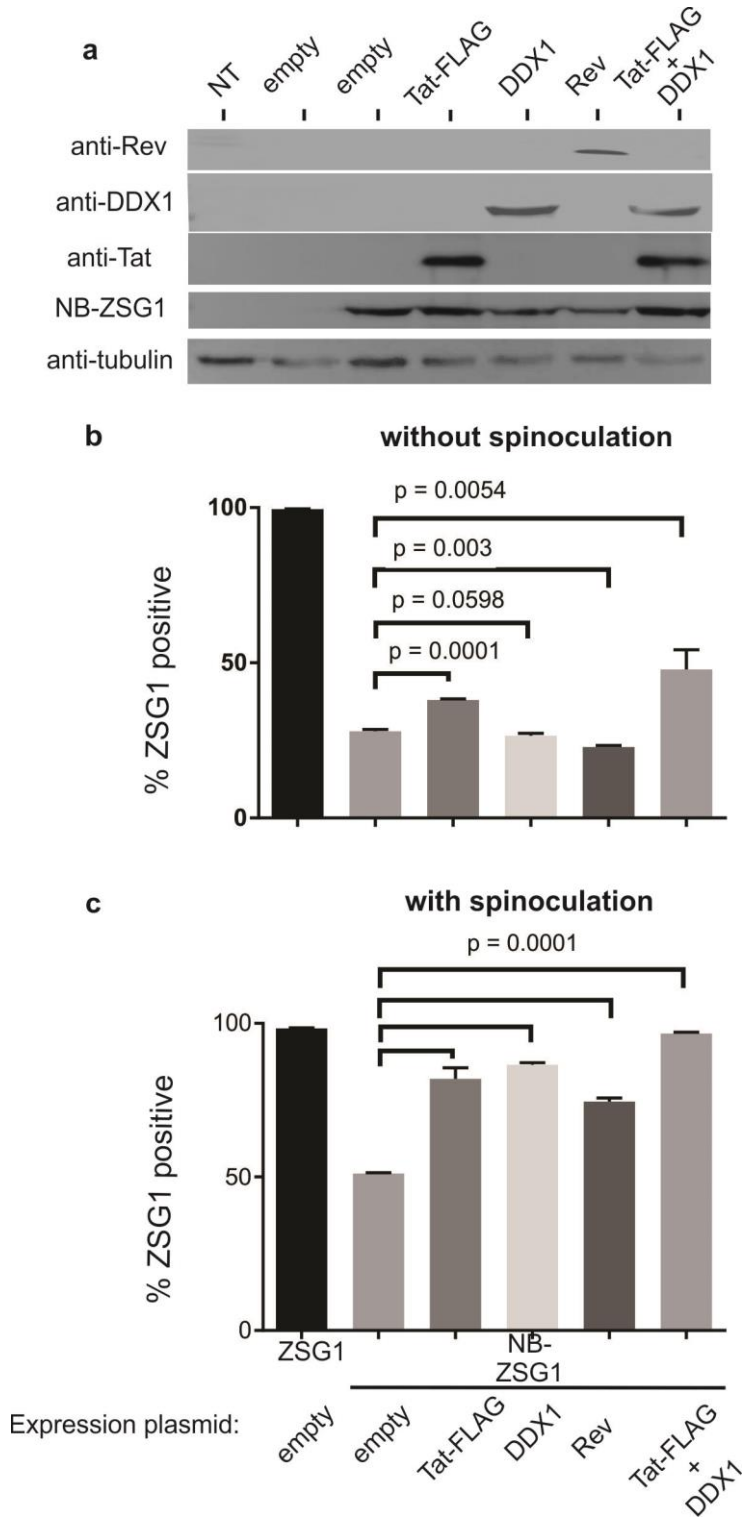

Figure 5-7. Tat, DDX1 and Rev improved transduction efficiency of Jurkat cells by NB-ZSG1 VLPs

(a) Expression of NB-ZSG1, Tat-FLAG, DDX1 and Rev by Western blot. Lysates were collected from the VLP producer cells (HEK 293T cells) and stained with anti-FLAG, anti-Rev, anti-DDX1 and anti β tubulin antibodies. (b) Jurkat cells were transduced by VLPs produced in HEK 293T by cotransfection of VLP producer plasmids and Tat, Rev and/or DDX1. (c) The same VLPs as (a) were used to transduce Jurkat cells by spinoculation. Bars indicate mean percent of transduced cells and error bars indicate standard deviation. Mean of each group was compared to mean of NB-ZSG1 control by t-test. Experiments were performed three times independently in triplicate.

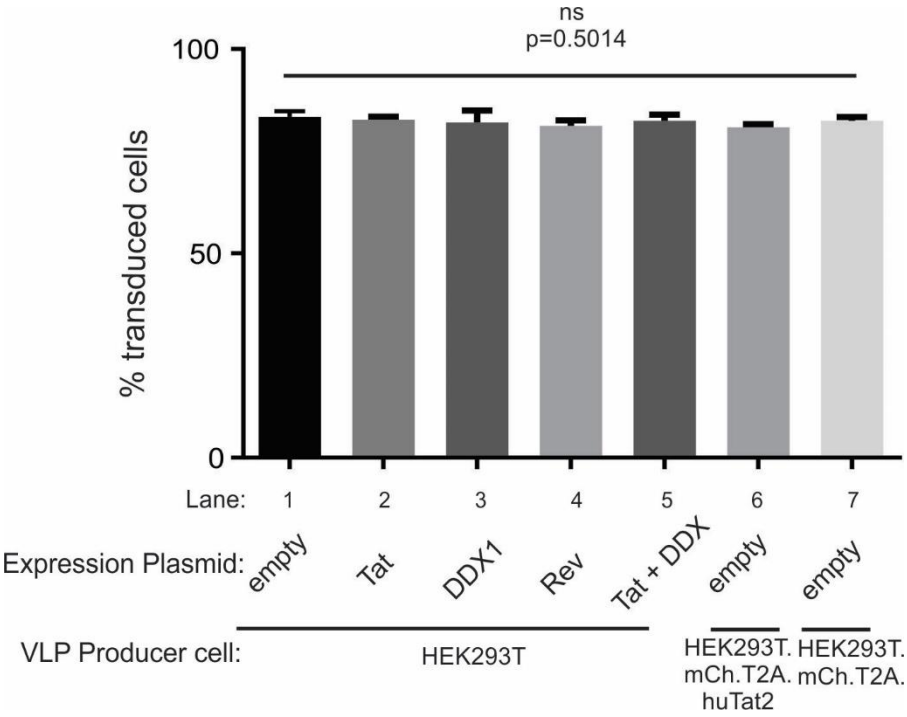

Figure 5-8. No effect of overexpression of Tat, DDX1 and Rev to transduction efficiency of ZSG1 VLPs to Jurkat cells
Jurkat cells were transduced with ZSG1 VLPs produced in HEK 293T cells by cotransfection of ZSG1 VLP producer plasmids and Tat, DDX1 and Rev plasmids. Transduction was performed by spinoculation using polybrene. Bars indicate mean percent of transduced cells and error bars indicate standard deviation. Experiments were performed three times independently in triplicate. Mean comparison between groups was analyzed by ANOVA and p value is indicated.

5.3.3 No improvement in transduction efficiency of NB-ZSG1 VLPs in primary CD4$^+$ T cells

Interestingly, although introduction of Nullbasic antagonist proteins improved transduction efficiency of NB-ZSG1 VLPs to Jurkat cells, this method did not help in transducing NB-ZSG1 to primary CD4$^+$ T cells (Fig.5-9). Hu-Tat2 did not improve transduction of primary CD4$^+$ T cells by NB-ZSG1 lentiviral VLPs, and neither did Tat, Rev and DDX1. However, ZSG1 VLPs transduced ~50-60% primary CD4$^+$ T cells. This transduction rate increased to 70% when polybrene was replaced by recombinant human fibronectin (retronectin).

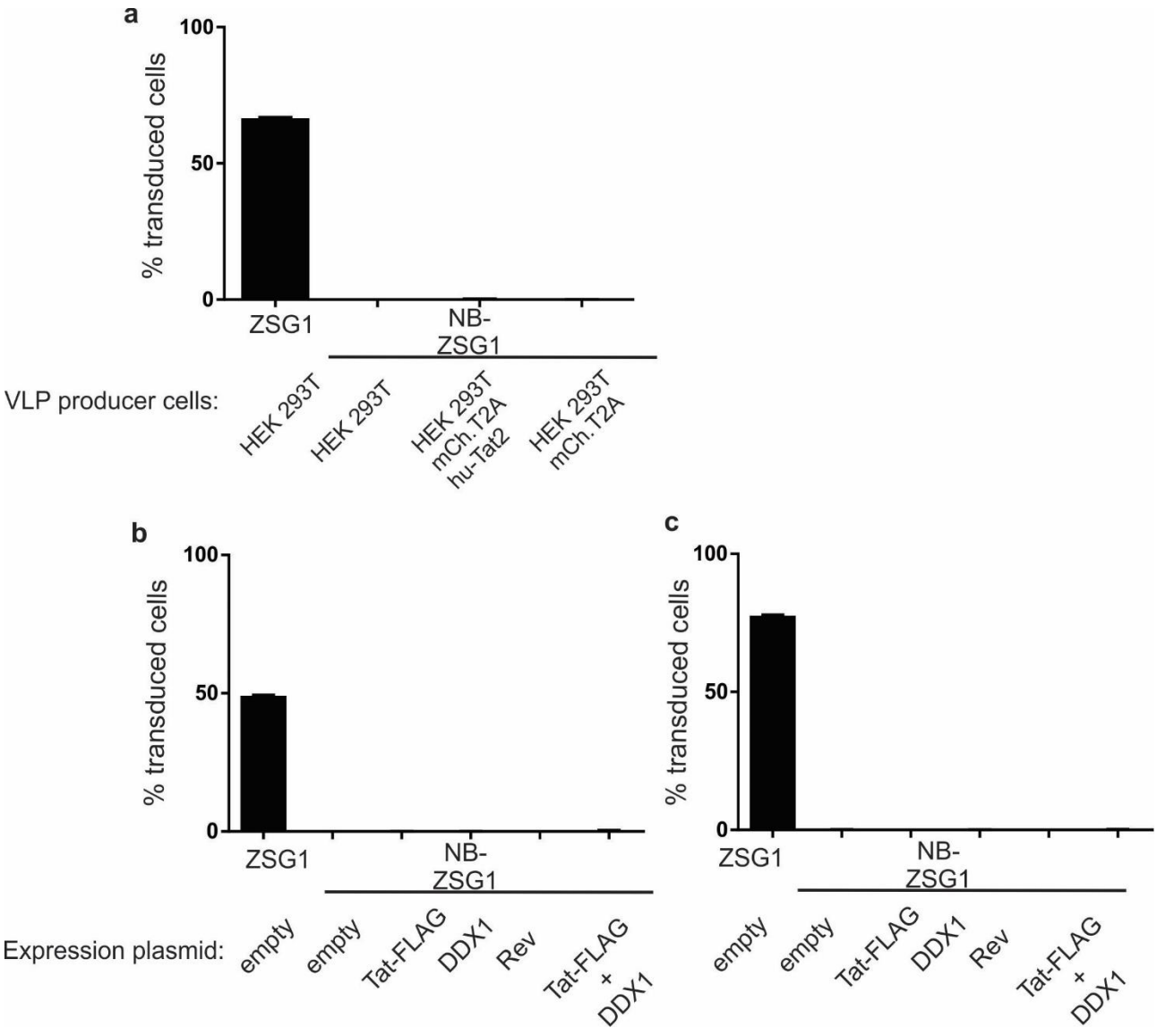

Figure 5-9. No improvement in transduction of primary CD4⁺ T cells by NB-ZSG1 lentiviral VLPs

(a) Primary CD4⁺ T cells were transduced with ZSG1 or NB-ZSG1 VLPs produced in HEK 293T-mCH, HEK 293T-mCh-huTat2 and parental HEK 293T cells. Transduction was performed using polybrene with spinoculation. (b) Primary CD4⁺ T cells were transduced with ZSG1 or NB-ZSG1 VLPs produced in HEK 293T cells by cotransfection of VLP producer plasmids and either Tat, DDX1, Rev or combination of Tat and DDX1. Transduction was performed using polybrene with spinoculation. (c) The same VLPs as (b) were used to transduce Primary CD4⁺ T cells using retronectin with spinoculation.

The fact that NB-ZSG1 transduction efficiency in Jurkat cells but not in primary CD4⁺ T cells was improved by adding Nullbasic antagonist agents suggests that difference in cellular factor in primary cells compared to Jurkat cells may affect lentiviral transduction. Jurkat and primary CD4⁺ T cells may have different levels of important cellular factors that are required during lentivirus transduction.

5.4 Discussion

Lentiviral vectors are useful gene therapy tools for delivering genes of interest into mammalian cells. The ability of lentiviral vectors to transfer genes not only to dividing cells but also to non-dividing cells and to maintain stable gene expression offers advantages over retrovirus vectors. Lentiviral vectors are able to transduce quiescent cells because their pre-integration complex (PIC) can go through the nucleus membrane[173]. Furthermore, lentiviral vectors are considered to be safer than gammaretroviral vectors because of reduced risks of oncogenesis[175].

Nullbasic was delivered to T cells by an HIV-1 derived lentiviral vector called pSicoR. However, Nullbasic delivery by this lentiviral vector was hindered by low transduction efficiency due to the inhibitory effect of Nullbasic on HIV-1. The fact that Nullbasic inhibits reverse transcription causes a shortcoming in the VLP transduction[14].

Nullbasic protein interferes with reverse transcription complex by binding to RT at p51 and p66 subunits, affecting the complex stability and causing premature viral uncoating. A previous reported study suggests that Nullbasic retains these RT binding determinants. [18,188]. To overcome this problem, we used viral and cellular proteins that act as Nullbasic antagonists during VLP production, so that the expressed Nullbasic protein does not bind to RT subunits.

When NB-ZSG1 VLPs were produced in the HEK 293T cell line harboring huTat2 intrabody, transduction efficiency in Jurkat cells was approximately twice as high. Transactivation assay shows that huTat2 intrabody inhibited Tat-mediated transactivation, indicated by decrease of luciferase activity by ~30%. This suggests that hu-Tat2 protein interacts with Nullbasic protein expressed during VLP production and inhibits Nullbasic drawback during transduction, thereby increasing Nullbasic VLP infectivity in Jurkat cells. Nullbasic protein that is not incorporated in the VLP can interact with RT and leads to production of defective VLPs. These VLPs may not contain viral mRNAs and therefore are unable to transduce the target cells. Hu-Tat2 is likely to bind to Nullbasic at amino acids +1 to +19 in the N-terminal proline rich region, as it does to Tat protein. By competitive binding mechanism, hu-Tat2 can inhibit Nullbasic-RT interaction. To determine the exact mechanism behind this protein-protein interaction, further studies need to be done, for example by using co-immunoprecipitation assay.

Overexpression of Tat with or without spinoculation may compensate for Nullbasic inhibition of NB-ZSG1 VLPs transduction of Jurkat cells. Tat has been shown to enhance reverse transcription of HIV-1[127,128]. Both Tat and Nullbasic bind to reverse transcriptase[18,127]. Thus, we hypothesised that overexpression of Tat might compete for interaction with reverse transcriptase and that Tat may partially or completely mitigate Nullbasic negative effect on reverse transcription. Our results suggest this hypothesis is possible.

We hypothesized that transduction efficiency of T cell lines and primary $CD4^+$ T cells may be limited to some extent by the availability of full length lentiviral mRNA in the packaging cell line. This because we have shown that Nullbasic inhibits Rev that is required for RRE-directed export of viral mRNA[3,16]. We showed previously that overexpression of Tat reversed Nullbasic interference of Rev[3]. Likewise, Nullbasic can also interfere with Rev by specifically binding DDX1 thus limiting Rev activity, so that overexpression of DDX1 may also reduce inhibition of Rev. Similarly, DDX1 together with spinoculation also increased the transduction efficiency in Jurkat cells by NB-ZSG1 VLPs. DDX1 is a host cellular protein belonging to the ATP-dependent RNA helicase family. This protein assists the HIV-1-Rev-RRE complex formation by binding to RRE-mRNA, and therefore DDX1 is important in Rev function for nuclear transport of singly spliced and unspliced viral mRNA [3]. Hence, the overexpression of DDX1 compensated for Nullbasic inhibition of the Rev-RRE-NB-ZSG1mRNA transport from the nucleus to cytoplasm. However, Rev overexpression alone was not effective in improving NB-ZSG1 transduction efficiency, suggesting that Nullbasic does not interact directly with Rev. This is consistent with the findings of a previous report showing direct interaction of Nullbasic with DDX1 but not Rev as a mechanism of Nullbasic inhibition of Rev function[3]. The improvement in the transduction rate of Jurkat cells by NB-ZSG1 VLPs was more pronounced after the introduction of combined Tat and DDX1 during VLP production, suggesting that these proteins have additive effects to counteract Nullbasic inhibition of both transcription and viral mRNA nucleocytoplasmic transport. Nevertheless, when the same methods were applied to ZSG1 VLPs, there was no significant difference in the transduction efficiency of Jurkat cells. This suggests that the effect of huTat2 intrabody, Tat and DDX1 on NB-ZSG1 lentiviral VLP transduction efficiency was specific to Nullbasic.

In our study, we noted that some differences in VLP transduction rates were observed using different preparations of VLPs. For example, the transduction rate shown in figure 5-6 was about half that shown in figure 5-7. We believe the is due to difference in VSV-G expression

in the VLP production cell line, as Gag and Gag-Pol expression were consistent in the VLP batches. It is worth noting that irrespective of the VLP batch used, spinoculation increased the transduction rate by ~2-fold.

Spinoculation increased the transduction efficiency of all the tested VLPs in Jurkat cells. Spinoculation has a concentrating effect on VLPs. Also, the centrifugal pressure on the cells stimulates actin and cofilin dynamic activity on non-stimulated Jurkat cells. Actin is a protein located in the cell cytoplasm that forms microfilaments as a primary component in the cytoskeleton, while cofilin is an actin-binding protein responsible for enhancing the treadmilling of actin filaments. Spinoculation modulates actin and cofilin dynamic activity[189]. VSV-G enveloped virus fusion to target cells is increased by actin filament formation. Furthermore, the actin cytoskeleton helps intracellular lentivirus trafficking [190], and thus spinoculation improved lentivirus transduction efficiency.

Although the addition of hu-Tat2 intrabody, Tat, DDX1 and Rev proteins as well as spinoculation improved NB-ZSG1 transduction efficiency of Jurkat cells, these methods did not help transduction of primary $CD4^+$ T cells with NB-ZSG1 lentiviral VLPs. In contrast, up to ~70% transduction efficiency was achieved in primary $CD4^+$ T cells transduced with ZSG1 VLPs, especially when polybrene was replaced by retronectin. This indicates that lentiviral VLPs can transduce primary $CD4^+$ T cells, but not when Nullbasic is present. These results indicate that Nullbasic inhibition of lentivirus was more prominent in primary $CD4^+$ T-cells than in Jurkat cells. There are many possible causes for this result, such as cell factor differences between Jurkat and primary $CD4^+$ T-cells. Those cells may have different levels of important cell factors required during lentiviral vector transduction. One of possible cell factors that is different between primary cells and cell lines is eEF1A. eEF1A plays an important role during reverse transcription. It is possible that primary $CD4^+$ T cells produce less eEF1A proteins compared to Jurkat cells. Lacking eEF1A may cause inefficient reverse transcription complex formation[188]. Further studies are needed to determine different levels of eEF1A proteins in these cells, for example using a gradient concentration Western Blot from the cell lysates. In future, eEF1A gene could be delivered to the primary $CD4^+$ T cells before Nullbasic transduction by using other viral vectors, such as adenovirus based vectors to generate transient eEF1A transgene expression in the cells.

Overall, transduction efficiency of Jurkat cells by Nulbasic lentiviral VLP can be improved by the presence of huTat2, Tat and DDX1 together with spinoculation. Therefore, these

methods can be used to further study Nullbasic activities and interactions using Jurkat cells. However, although lentiviral vectors are preferable delivery system in gene therapy because of their ability to transduce non-dividing cells, provide stable transgene expression and have less oncogenic risks, we cannot use currently available lentiviral vector to deliver Nullbasic to primary $CD4^+$ T cells. The Nullbasic inhibition of lentivirus was so strong in primary $CD4^+$ T cells so that addition of Tat, Rev and DDX1 as well as spinoculation method could not improve Nullbasic lentiviral VLP transduction efficiency. The next chapter will provide conclusion of the thesis, current study limitation and future research direction.

CHAPTER 6

Conclusion

This final chapter summarizes the thesis, highlights its findings and contributions, points out limitations of the current work, discusses general conclusion of the thesis and outlines direction for future research. This chapter is divided into five sections: summary of the thesis, contributions of the thesis, limitation, conclusion and future direction.

6.1 Summary of the thesis

This research focused on investigation of an antiviral agent that may be used in a gene therapy approach. The agent is a mutant of HIV-1 Tat protein, created by substitution of the basic domain in the wild type Tat with glycine and alanine residues. Tat basic domain is composed of arginine and lysine residues, but is referred to as an arginine rich domain (ARD). This ARD is required for Tat to bind TAR RNA during transcriptional activation of the HIV-1 LTR promoter. Under biological condition or neutral pH, arginine is a positively charged amino acid and therefore has hydrophilic characteristic. Alanine and glycine residues have small non-polar aliphatic side chains. Replacing the Tat ARD with alanine and glycine residues created Nullbasic and inactivates its ability to recruit pTEFb to the HIV-1 LTR promoter. Hence, Nullbasic is unable to stimulate HIV-1 transcription. Recent reports indicate that Nullbasic can compete with wild type Tat for binding to P-TEFb and this accounts for one mechanism by which Nullbasic can inhibit HIV-1 replication. In addition, Nullbasic can also inhibit HIV-1 reverse transcription by disrupting reverse transcription complex and this requires Nullbasic packaging in the virion during virus production by infected cells. It also inhibits Rev function in transporting HIV-1 mRNA containing an RRE from nucleus to cytoplasm.

Nullbasic antiviral activity against HIV-1 strains from different subtypes was examined in Chapter 3. Nullbasic is a mutant Tat of HIV-1_{BH10}, a strain of subtype B, and has so far only been tested against other subtype B strains *in vitro*. In chapter 3 we evaluated if Nullbasic

could also inhibit replication of other HIV-1 strains from different subtypes. We tested Nullbasic on HIV-1_{ZAC} that represents subtype C, the most prevalence HIV-1 subtype in world, HIV-1_{ELI} to represents subtype D that is common in Uganda and HIV-1_{MAL} to represent a recombinant form of A/D subtype.

We generated TZM-bl cell lines expressing NB-mCh or mCh by transduction and FACS selection. We observed that Nullbasic strongly inhibits Tat-mediated transactivation of all the HIV-1 strains tested both by transfection and infection assays in those TZM-bl cell lines. Although both assays resulted in similar inhibition of Tat-mediated transactivation by Nullbasic, the assays have small differences. The transfection assay reveals how Nullbasic, a subtype B HIV-1 derived Tat mutant, can inhibit Tat isoforms from different strains that transactivate an LTR-luciferase reporter stably integrated in the TZM-bl cells. The LTR used here is also derived from an HIV-1subtype B strain. The inhibition mechanism will involve competition between the wild type Tat variants and Nullbasic in binding P-TEFb.

In the second transactivation assay, the TZM-bl cell lines were infected with each HIV-1 strain, so the source of Tat is integrated provirus. In this case Nullbasic could inhibit the production of both Tat by the provirus and luciferase production by the LTR-luciferase reporter. To some degree, we had expected Nulbasic to be more effective in the second scenario, which it was not, as we expected inhibition of Tat expression. Unfortunately, Western blots performed to detect Tat post-infection in these experiments were inconclusive, as the number of infected cells was too low.

In a third scenario, the TZM-bl cell lines were infected with the same HIV-1 strains and virus replication was monitored by sampling supernatant and measuring CA levels. This system adds additional complexity. In this experiment, the transactivation involves a Tat - TAR RNA axis of the integrated proviruses and these have subtle difference in the Tat primary sequence and TAR RNA stem loop sequence and structure. This is most evident in subtype A/D that contains mutations in TAR RNA upper stem loop structure. However, the data shows that NB-mCh was an excellent HIV-1 inhibitor under this scenario.

The replication of all the HIV-1 strains tested was inhibited by Nullbasic both in TZM-bl cell lines and primary $CD4^+$ cells. It is interesting to note that even though NB-mCh has a strong antiviral activity in TZM-bl cells against the HIV-1 strains, the antiviral activity was lower in primary $CD4^+$ T cells compared to that in TZM-bl cell lines. It is possible that differences in

cell factors between cell types tested affects Nullbasic antiviral activity. The exact mechanisms underlying these differences requires further investigation. However, our studies show that NB-ZSG1 has a stronger antiviral activity compared to NB-mCh in primary $CD4^+$ T cells, and therefore NB-ZSG1 was used for investigating Nullbasic effects against different HIV-1 strains in primary $CD4^+$ T cells. The results in Chapter 3 demonstrate that Nullbasic can inhibit viral replication of all HIV-1 strains tested in primary $CD4^+$ T cells.

We also observed that NB-ZSG1 showed decreased levels of protein expression over time. Because Nullbasic acts in a dose-dependent manner[14], when the Nullbasic expression levels decrease, the viral load rebounds. We confirmed that expression of Nullbasic in primary $CD4^+$ T cells does not alter cell proliferation, viability or induce apoptosis under the experiment conditions, and therefore we cannot relate the decrease expression levels of Nullbasic to cytotoxic effects. One of possible reasons for this is the gammaretroviral vector we used to deliver Nullbasic to the target cells does not fully support long term and high levels expression of transgenes. It is known that expression of retroviral genes is susceptible to positional effects and transcriptional silencing that can be influenced by the site of integration in the chromosome[191]. Positional silencing effect have been overcome to some degree by chromatin insulators used in retroviral vectors and can block positive and negative positional effects at the site of integration when they flank a transgene[192-194]. New retroviral vectors containing chromatin insulators and NB-ZSG1 are in development.

In Chapter 4, a method to generate an acute HIV-1 infected mouse model was investigated. Both intravenous and intraperitoneal administration route to deliver human $CD4^+$ T cells to the mice resulted in a profound human cell reconstitution in the mouse peripheral blood circulation. However, intravenous delivery through the mouse tails lead to better and more reliable human $CD4^+$ T cells engraftment levels detected in peripheral blood. Therefore, the intravenous delivery was then selected and implemented to our next experiments.

$HIV\text{-}1_{AD8}$, a subtype B molecular clone derived from HIV-1AD-87[195] and HIV-1SG3.1[196], was chosen to challenge the humanized mice because this strain utilizes CCR5 chemokine receptor to infect human cells. CCR5 receptor is commonly used by HIV-1 to enter human cells in early infection.

We examined if Nullbasic can inhibit HIV-1 replication *in vivo.* We initiated mouse experiments using CD4-NB-mCh cells prior to discovering that NB-ZSG1 had superior

antiviral activity compared to NB-mCh. This is because preliminary studies using several different cell lines suggested that NB-mCh was an effective HIV-1 inhibitors[3,16]. Secondly, NB-mCh was selected because some reports suggest that multimeric fluorescent proteins had increased cellular toxicity compared to monomeric fluorescent protein[197]. However, our studies showed no detectable toxicity by NB-ZSG1 in human $CD4^+$ T cells and better antiviral activity than NB-mCh. Hence, we chose to discontinue CD-NB-mCh transplantation experiments in mice. The preliminary results presented in Chapter 4 suggest that CD4-NB-ZSG1 transplantation model in mice is worth further examination.

NB-ZSG1 both *in vitro* and *in vivo* demonstrated strong antiviral activity in a concentration dependent manner[14]. The level of NB-ZSG1 expressed in transduced cells was selected by FACS for cells with a high relative MFI. When the MFI level of CD4-NB-ZSG1 cells decreased, the level of viral RNA in samples tended to increase, a threshold level of Nullbasic protein is required to inhibit HIV-1 replication, which roughly correlates to a relative MFI of $> 3.2 \times 10^3$ under the condition used. Our preliminary results suggest this is achievable in this mouse model for at least 6 weeks.

In chapter 3 and 4 we used SIN gammaretroviral vector VLPs to efficiently deliver Nullbasic to human $CD4^+$ T cells. However, retroviral based vectors are associated with oncogenic risks, although this risk has been greatly reduced in modern SIN-based gammaretroviral vector designs[142]. Also, we observed that using this gammaretroviral vector, Nullbasic expression levels decreased over time. Interestingly, studies of Nullbasic in Jurkat cells showed long term stable expression using third generation lentiviral systems, a pSicoR vector[2]. However, nearly all lentiviral vectors have been derived from HIV-1, which are potently inhibited by Nullbasic. Nullbasic inhibits VLP reverse transcription thus restricting transduction. Therefore, in Chapter 5 we tested if the transduction efficiency of NB-ZSG1 lentiviral VLP could be improved. Using a combination of methods including spinoculation and addition of Nullbasic antagonist cellular and viral proteins, we significantly improved VLP transduction of Jurkat cells by Nullbasic lentiviral VLPs. To transduce Jurkat cells, overexpression of Tat and DDX1 combined with spinoculation method provided the most efficient transduction by NB-ZSG1 VLPs. Nevertheless, transducing primary $CD4^+$ T cells by Nullbasic lentiviral VLPs remained an intractable problem even by implementing the same methods. This could mean that Nullbasic antiviral activity has a stronger effect in primary $CD4^+$ T cells compared to Jurkat cells. It is possible that differences in primary $CD4^+$

T cell factors compared to Jurkat cells may be responsible for this outcome. The exact cell factors associated with difference in Nullbasic lentiviral VLP transduction efficiency are unknown. One of possible cell factors involved in reverse transcription is eEF1A. However, further studies are required to determine if Jurkat cells and primary $CD4^+$ T cells produce different levels of eEF1A protein.

6.2 Contributions of the thesis

This thesis reveals that it is possible to use a mutant of HIV-1 subtype B Tat as an antiviral against other HIV-1 subtypes. In this thesis, Nullbasic derived from HIV-1 subtype B Tat can act as an antiviral against HIV-1 strains from subtype C, D and recombinant A/D. The reason behind this is likely because Nullbasic and Tat sequences from different HIV-1 strains tested are conserved. This means that Nullbasic may have a broad antiviral property applicable for those HIV-1 subtypes and possibly to other subtypes as well. Therefore, Nullbasic can be a potential antiviral agent for future gene therapy for different HIV-1 strains. Nullbasic can inhibit Tat variants from different HIV-1 strains because their basic domains are conserved. In this study, we observed that Nullbasic inhibited transactivation of four different HIV-1 strains. However, the antiviral potency of Nullbasic to other HIV-1 strains should be evaluated further.

This thesis also established a humanized mouse model able to evaluate antiviral potency of a mutant Tat protein *in vivo* against an acute HIV-1 infection. Using human $CD4^+$ T cells isolated from healthy donors and transplanted into the mice by intravenous injection, fast reconstitution of the human $CD4^+$ T cells in the mouse peripheral blood circulation was achieved. Although the human $CD4^+$ T cells were only detected for six weeks in the mouse, this mouse model allowed for quick examination of an antiviral agent against an acute HIV-1 infection. Moreover, this acute mouse model can be used as a basis to further develop a humanised mouse model reconstituted with pluripotent $CD34^+$ human cells to examine longer term expression and antiviral activity of NB-ZSG1 *in vivo.*

Interestingly, in Chapter 3 and 4 we found that different fusion proteins can result in different antiviral potency of Nullbasic. When fused to ZSGreen1 fluorescent protein, Nullbasic provided a stronger antiviral activity compared to when Nullbasic was fused to mCherry. One major difference noted is that ZSGreen1 must form a tetramer to fluoresce, while

mCherry protein will fluoresce as a monomer[151]. It is possible that these difference multimerization state of the fusion protein affects Nullbasic antiviral properties. Differences in multimerization status can affect protein conformation and activity, or possibly the protein half-life. It is possible that Nullbasic in the form of tetramer is more stable and has a stronger antiviral activity than in the form of monomer. However, further research to test these possibilities is required. For example, it would be worth testing if fusion proteins that form in different multimeric states also affect Nullbasic antiviral activity. One possibility is using the yeast GCN4 leucine zipper domain, which can form dimers, trimers and tetramers, which are well characterised[198].

In this thesis, we also developed an effective method to transduce T cells with lentiviral based VLP carrying *nullbasic* gene. A combination of spinoculation method and addition of Nullbasic antagonist proteins worked best in transducing Jurkat cells. This is because Nullbasic can inhibit HIV-1 reverse transcription and this inhibition effect is similar to HIV-1 based lentiviral vectors. Unfortunately, those methods did not improve transduction of human primary cells as expected. This could mean that Nullbasic inhibition on lentiviral VLP reverse transcription is stronger in primary $CD4^+$ T cells compared to T cell lines. To overcome this problem, different cell factors involved during VLP transduction of primary $CD4^+$ T cells and Jurkat cell lines need to be identified. Genome-wide siRNA screening can be performed to identify possible cell factors that inhibit Nullbasic VLP transduction of primary $CD4^+$ T cells[199].

We have not seen resistance issues to Nullbasic in this study. However, resistance to Nullbasic may be an issue in future, especially when Nullbasic is tested againsts different HIV-1 strains with non-conserved Tat sequences. To overcome this, Nullbasic can be modified accordingly to fit the Tat sequences from different HIV-1 strains.

6.3 Limitations of the current study

In Chapter 3, we tested Nullbasic antiviral activity against four strains from four different HIV-1 subtypes, which was a reasonable first step but much more could be tested in the future. Therefore, we cannot conclude that Nullbasic can inhibit replication of all HIV-1 subtypes. It is also possible that some strains of the subtypes could be more resistant to Nullbasic than the HIV-1 strains tested.

Our results suggest that in primary $CD4^+$ T cells, NB-ZSG1 has a stronger antiviral activity than NB-mCh. We compared NB-mCh and NB-ZSG1 against HIV-$1NL_{4-3}$ strain in primary $CD4^+$ T cells. In this thesis, we didn't examine NB-ZSG1 antiviral activity in TZM-bl cell lines. However, the NB-ZSG1 antiviral activity has been tested subsequently in TZM-bl cell lines by other colleague in our laboratory in an unpublished report and the result does not indicate any significant differences compared to that of NB-mCh.

In Chapter 4, a method to develop an acute animal model was investigated. We discovered that expression of NB-mCh provides a short-term antiviral activity against HIV-1_{AD8} strain *in vivo.* We later discovered that NB-ZSG1 provided a stronger and more durable antiviral activity than NB-mCh, and therefore we changed from NB-mCh to NB-ZSG1. However, the small sample number contributed to a high variability in our results in this study; therefore a larger number of animal samples, at least 6 mice per group, is required to confirm the result of this study.

6.4 Conclusion

Overall, Nullbasic either in the form of fusion protein to mCh or ZSG1 fluorescent proteins provided inhibition to HIV-1 replication *in vitro* and *in vivo.* The levels of inhibition were more profound when Nullbasic protein expressions were maintained at high levels. Using primary $CD4^+$ T cells, NB-ZSG1 provided a stronger antiviral activity than NB-mCh against HIV-1_{NL4-3} *in vitro* and HIV-1_{AD8} *in vivo*. The *in vitro* studies of Nullbasic on TZM-bl cell line and primary $CD4^+$ T cells also show replication inhibition of HIV-1_{ZAC} (subtype C), HIV-1_{MAL} (subtype D) and HIV-1_{ELI} (subtype A/D recombinant).

Transplantation of human $CD4^+$ T cells to BALB/c-$Rag2^{-/-}\gamma c^{-/-}$ mice resulted in a short-term human cell reconstitution and HIV-1 replication *in vivo*, allowing a study of Nullbasic antiviral activity against an acute HIV-1 infection for 4 weeks. It was indicative that NB-ZSG1 can provide a stronger and longer antiviral activity *in vivo* than NB-mCh, but a larger number of samples is required to confirm this result. At least 6 animals in each group are required to determine statistical significant of Nullbasic effect.

Although the SIN gammaretroviral vector used in this study was able to deliver Nullbasic gene to the targeted cells, the level of expression decreased over time. Therefore, a better

viral vector, such as lentiviral vectors to deliver Nullbasic is required. We observed that transduction of lentiviral based Nullbasic VLP to Jurkat cells was optimal when Tat and DDX1 proteins were present in the VLP producer cells, and the transduction method was combined with spinoculation. However, the PSicoR lentiviral vector we used was unable to deliver *nullbasic-ZsGreen1* gene to primary $CD4^+$ T cells.

6.5 Future directions

Investigation of a better vector to deliver Nullbasic to target cells is required. An ideal vector for this should provide high levels transgene expression with long term durability. A better means of delivering Nullbasic to target cells may result from changing the internal promoter of the vector. It is important to choose a promoter that will not be downregulated by Nullbasic protein. Towards this goal the lab has identified several constitutively expressed human promoters that are not downregulated in the presence of NB-SG1. Another method to develop a more stable vector to deliver Nullbasic is to introduce an insulator that can prevent silencing of the genes by Nullbasic. An insulator can prevent silencing of viral vectors by transgenes[192,193].

Nullbasic interaction with P-TEFb or DDX1 has been previously reported[2,3]. Nullbasic binds to P-TEFb complex but does not initiate transcription[2]. Nullbasic also binds directly to DDX1, causing inhibition of viral mRNA transport by Rev[3] from the nucleus to cytoplasm. Nullbasic interaction with RT p51 and p66 subunits has been previously revealed by co-immunoprecipitation[18]. New lentiviral transduction vectors for primary T cells should have the ability to overcome Nullbasic inhibition, for example by mutation of the direct targets of Nullbasic such as RT determinant sequences, while retaining the RT functions.

Another approach that may be beneficial for Nullbasic lentiviral VLP transduction is controlling Nullbasic expression in HEK 293 T cells during VLP production by using Tetracycline-controlled transcriptional activation system (Tet-off). Nullbasic expression may be repressed transiently using this Tet-off technology. As Tetracyclin is highly toxic to mammalian cells, its derivatives such as Doxycycline may be used to inhibit Nullbasic expression in HEK 293T cells[200].

NB-ZSG1 showed a stronger antiviral activity compared to NB-mCh *in vitro* and *in vivo.* Investigation of the mechanism underlying this result is outside the scope of this thesis. It could be that by fusion with a tetrameric protein such as ZSG1, Nullbasic forms a tetramer as well. MCherry forms a monomer and therefore NB-mCh is possibly available in the cells in the form of monomer. This difference multimerization states may affect the antiviral activity of Nullbasic as well as its half-life. Further study to discover the mechanism behind the greater and longer antiviral activity of NB-ZSG1 compared to NB-mCh is required. Additionally, it would be useful to investigate other fusion proteins suitable to for Nullbasic detection in future. Ideally, the proteins are human proteins in order to minimize possible off target effect to human cells.

We noticed that Nullbasic antiviral activity against different strains of HIV-1 from different subtypes varied. Since the Nullbasic used in this study was derived from a subtype B HIV-1 Tat, it would be worthwhile to investigate the antiviral activity of Nullbasic variants derived from other subtypes to see if they may provide better antiviral agents specific to each subtype. Further testing of Nullbasic antiviral activity against a number of different strains from each subtype is required to support the use of Nullbasic against all strains in these subtypes. Evaluation of Nullbasic antiviral activity against other HIV-1 subtypes not tested in this research is also required to assess the efficacy of Nullbasic as an HIV-1 therapy. In addition, in will be beneficial to study the mechanisms by which Nullbasic inhibits reverse transcription and Rev function in different HIV-1 strains and subtypes.

In this thesis, we only tested Nullbasic antiviral activity *in vivo* against an acute HIV-1 infection in a very short term. Further study to confirm NB-ZSG1 antiviral activity *in vivo* using a larger number of samples is required. Also, development of a chronic HIV-1 infection model is required to study Nullbasic effect against chronic HIV-1 infection. Using stem cell transplantation technology, a longer survival of human cells that will allow chronic HIV-1 infection study in the mice might be achievable.

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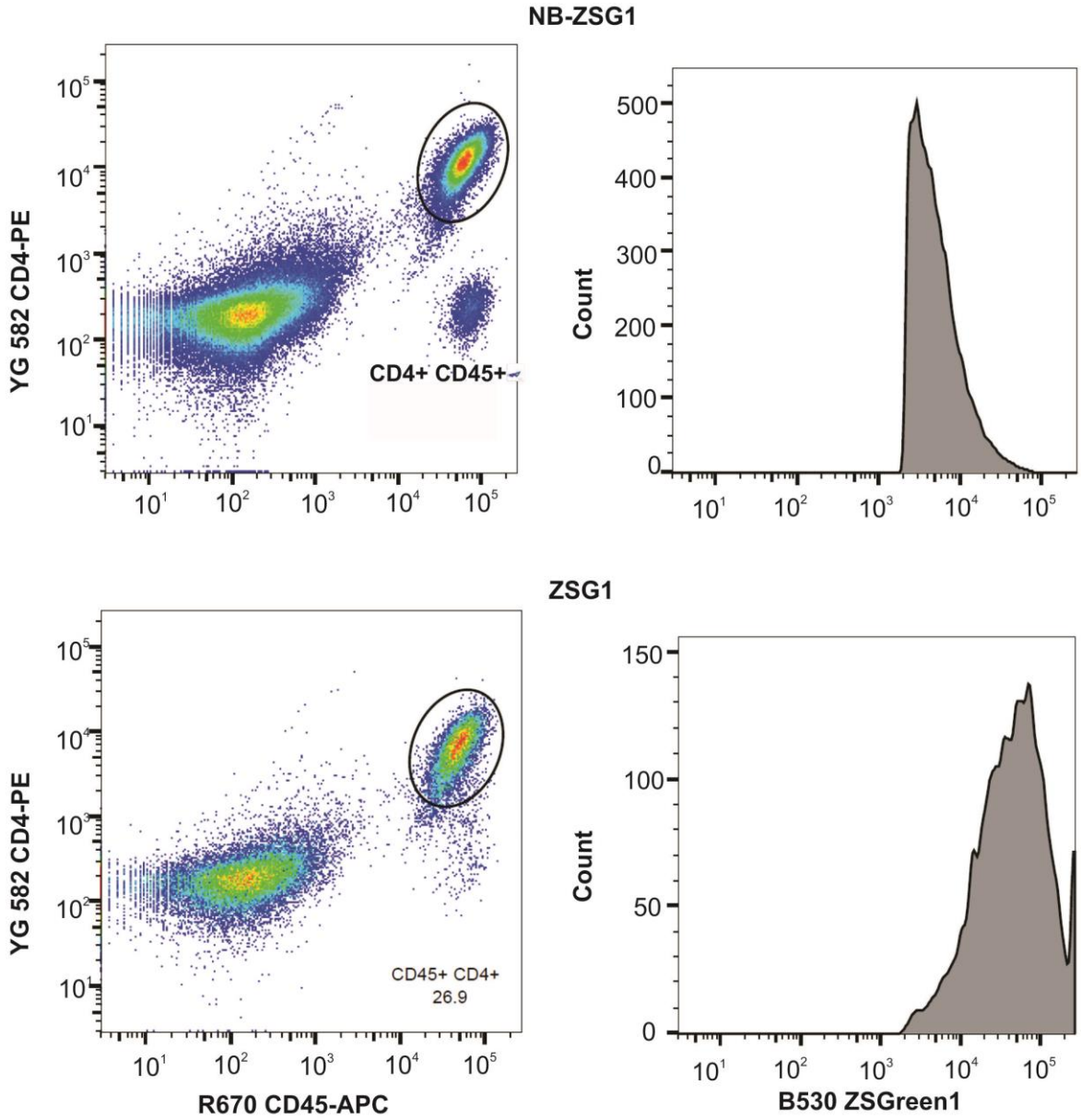
Appendices

Appendix 1. Analysis of human CD4-NB-ZSG1 and CD4-ZSG1 cell engraftment in lung tissue sample taken at week 6 post transplantation

Cells were stained with anti-human CD4 and anti-human CD45 antibodies and analyzed by flow cytometry. Cells stained by both antibodies, double positives, are shown in the oval gates. The histograms show MFI for the fluorescent protein. The result is representative of four samples in each group.



Appendix 2. Analysis of human CD4-NB-ZSG1 and CD4-ZSG1 cell engraftment in spleen tissue sample taken at week 6 post transplantation

Cells were stained with anti-human CD4 and anti-human CD45 antibodies and analyzed by flow cytometry. Cells stained by both antibodies, double positives, are shown in the oval gates. The histograms show MFI for the fluorescent protein. The result is representative of four samples in each group.