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Development of a CD4-independent HIV-1 virus for infection of animals

Dissertation

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Zusammenfassung

Seit der Entdeckung des erworbenen Immunschwächesyndroms (AIDS) im letzten Jahrhundert sind Millionen von Menschen an der Krankheit und ihren Komplikationen gestorben. Diese Krankheit wird durch eine Infektion mit dem **Humanen Immundefizienz-Virus (**HIV) verursacht. HIV und AIDS werden schon seit vielen Jahren intensiv erforscht. Beispiele für Tiere, an denen Virusinfektionen getestet werden, sind nicht-menschliche Primaten, humanisierte Mäuse, das Feline Immunodefizienz Virus (FIV) bei Katzen und das Simiane Immunodefizienz Virus bei Makaken. Aufgrund der Speziesspezifität von HIV-1 können wir jedoch kein geeignetes Tiermodell etablieren; alle bestehenden Tiermodelle für HIV haben mehr oder weniger große Nachteile.

Ein weit verbreitetes Tiermodell ist die Hauskatze und FIV-Infektionen. Aufgrund einschränkender Faktoren kann HIV jedoch Hauskatzen nicht infizieren oder sich in ihnen vermehren. Wenn wir Hauskatzen für HIV-Infektionen nutzen wollen, müssen wir diese einschränkenden Faktoren ausschalten. Es gibt zwei wesentliche Probleme für HIV-1 in feline Zellen: keine funktionellen Rezeptoren und antivirals APOBEC3. Das erste Mittel, das für eine Lösung in Frage kommt, ist ein so genanntes CD4-unabhängiges HIV. Einige Forscher haben einige HIV-1-Hüllen entdeckt, die für die Infektion keine menschlichen CD4-Rezeptoren (Cluster of Differentiation 4) benötigen, sondern nur die Co-Rezeptoren CXCR4 (C-X-C-Chemokinrezeptor Typ 4) oder CCR5 (C-C-Chemokinrezeptor Typ 5), um in Zellen einzudringen. Auf dieser Grundlage konstruierten wir ein HIV-1, das Katzenzellen infizieren kann, um die HIV-Replikation in Tierzellen zu untersuchen. Wir testeten dieses neue Virus an Zellen, die nur den CXCR4-Co-Rezeptor exprimieren. Das Virus konnte solche Zellen infizieren, und diese Infektion konnte durch einen CXCR4-Inhibitor blockiert werden, nicht aber durch einen CD4-Inhibitor. Um die restriktive Aktivität des antiviralen Restriktionsfaktor felines APOBEC3 gegen HIV-1 zu umgehen, konstruierten wir ein chimäres CD4-unabhängiges HIV-1.$_{VifFIV}$, das das *vif* Gen von FIV exprimierte. Wir fanden heraus, dass dieses chimäre HIV-1.$_{VifFIV}$ im Gegensatz zu Wildtyp-HIV-1 feline APOBEC3s

abbauen kann. Unsere Ergebnisse sind viel versprechend für die Etablierung eines HIV-Katzenmodells.

Summary

Since the discovery of the Acquired Immunodeficiency Syndrome (AIDS) in the last century, millions of people have died from the disease and its complications. This disease is caused by infection with the HIV virus. Humans have been studying the disease and the virus for a long time. One research tool is the use of animal models. Examples for animals where virus infections are tested include non-human primates, humanized mice, Feline Immunodeficiency Virus (FIV) in cats, and Simian Immunodeficiency Virus in macaques. However, because of the species specificity of HIV-1, we cannot establish a suitable animal model; all existing animal models of HIV have some disadvantages to a greater or lesser extent.

A widely used animal model is the domestic cat and FIV infections. But because of limiting factors, HIV cannot infect domestic cats or replicate in them. If we want to use domestic cats for HIV infections, we need to to bypass these limiting factors. The first tool that can be used is a so-called CD4-independent HIV. Some researchers have discovered some HIV-1 envelopes that do not require human CD4 (cluster of differentiation 4) receptors for infection but only need co-receptors CXCR4 (C-X-C chemokine receptor type 4) or CCR5 (C-C chemokine receptor type 5) to enter cells. Based on this, we constructed an HIV-1 hat can infect feline cells as a tool to study HIV replication in animal cells. We tested this new virus on cells that only express the CXCR4 co-receptor. This virus could infect such cells, and this infection could be blocked by a CXCR4 inhibitor but not by a CD4 inhibitor. Furthermore, to bypass the restrictive activity of feline apolipoprotein B mRNA editing enzyme catalytic peptide-like 3s (APOBEC3s) against HIV-1, we constructed a chimeric CD4-independent HIV-1.Vif_{FIV}. We found that this chimeric HIV-1.Vif_{FIV}, in contrast to wild-type HIV-1, can degrade feline APOBEC3s. Our findings show promise towards establishing an HIV feline model.

List of abbreviations

AA	amino acid
AIDS	Acquired Immunodeficiency Syndrome
APOBEC	apolipoprotein B mRNA editing enzyme catalytic peptide
APS	ammonium peroxidisulphate
bp	base pair
CCR5	C-C chemokine receptor type 5
CD4	cluster of differentiation 4
CMV	cytomegalic virus
CrFK	Crandell-Rees Feline Kidney
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
dNTP	deoxyribonucleic triphosphate
EDTA	ethylenediaminetetraacetic acid
env	envelope
f	feline
FBS	foetal bovine serum
FIV	Feline Immunodeficiency Virus
Gag/gag	group-specific antigen
gp120	glycoprotein 120
H	hour
h	human
HA	human influenza haemagglutinin
HIV	Human Immunodeficiency Virus
HOS	human osteosarcoma cells
LB	Luria-Bertani
M	molar
mg	milligram
min	minute
ml	millilitre
MLV	Murine Leukaemia Virus
MX2	myxovirus protein
Nef/nef	negative regulatory factor
ng	nanogram

nm	nanometre
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rev/rev	regulator of expression of virion proteins
RIPA	radioimmunoprecipitation assay
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute
s	second
SDS	sodium dodecyl-sulphate
SHIV	Simian/Human Immunodeficiency Virus
SIV	Simian Immunodeficiency Virus
TAE	Tris-acetate
T-cells	thymus lymphocytes
TBST	Tris-buffered saline and Tween-20
TEMED	N, N, N′, N′-tetramethylethylenediamine
TRIM5α	tripartite motif-containing protein 5α
Vif/*vif*	viral infectivity factor
vpr	viral protein R
VSV-G	vesicular stomatitis virus glycoprotein
µg	microgram
µg/ml	microgram per millilitre
µl	microliter
µM	micromolar

Table of Contents

1. Introduction

Viruses can be roughly divided into DNA and RNA viruses. The Human Immunodeficiency Viruses (HIVs) of interest in this thesis belong to the RNA virus family, Lentiviridae, and are further divided into HIV-1 and HIV-2. While HIV-1 is prevalent worldwide, HIV-2 is common only in some parts of Africa [1]. Therefore, we focused our research on the HIV-1 virus.

1.1 Lentiviruses

Lentiviruses are a genus of retroviruses that can cause chronic infections in their host. The name ‘lentivirus’ comes from the Latin word ‘lente’, which means ‘slow’, because lentiviruses are known for their slow replication cycle and long latency period [2].

Lentiviruses are unique among retroviruses in that they can infect nondividing cells [3] such as neurones and macrophages [4], which do not actively replicate. This is because lentiviruses have the ability to cross the nuclear membrane and integrate their genetic material into the host cell’s genome [5]. Once integrated, the viral genome can persist in the host cell and be passed on to daughter cells during cell division.

1.1.1 HIV

HIV is a viral infection that attacks the immune system, specifically targeting CD4 (cluster of differentiation 4) T cells and macrophages, white blood cells crucial for maintaining immune function. If left untreated, HIV can lead to a condition called Acquired Immunodeficiency Syndrome (AIDS), which is caused by a progressive CD4 T cell loss.

Because HIV is a single-stranded RNA virus, it is very susceptible to mutations [6], causing rapid evolution, resulting in different subtypes. The two types of HIV are HIV-

1 and HIV-2. HIV-1, the most common and widespread type of HIV, is responsible for the majority of HIV infections globally. HIV-2, the less common type of HIV, is primarily found in West Africa, although it has been reported in other parts of the world as well. HIV-2 is generally less aggressive and progresses more slowly than HIV-1. Furthermore, the HIV-1 virus can be separated into four different groups: 'M', 'O', 'N', and 'P'. Group 'M' is responsible for the global HIV pandemic and the majority of HIV infections worldwide. It is further divided into subtypes or clades, including subtypes A, B, C, D, F, G, H, J, and K. From the current epidemiological survey, A, B, and C are the subtypes with the largest number of infections and the widest distribution. Moreover, some subtypes are recombinant types, which are also widely distributed [7], [8], [9].

The gene structure of HIV consists of several genes that encode proteins essential for the virus' replication and infection process. HIV is an RNA virus that contains genetic material in the form of single-stranded RNA instead of DNA. The main genes of HIV include: 1. *Gag*: This gene encodes the core structural proteins of the virus, including the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. These proteins are responsible for the formation of the viral core and packaging the viral RNA [10]; 2. *Pol*: The *Pol* gene encodes enzymes necessary for viral replication, including reverse transcriptase (RT), integrase (IN), and protease (PR). Reverse transcriptase converts the viral RNA into DNA, integrase helps integrate the viral DNA into the host cell's DNA, and protease is involved in the maturation of viral proteins [11]; 3. *Env*: This gene encodes the viral envelope glycoproteins, namely gp120 and gp41. These proteins are responsible for viral attachment and entry into host cells. gp120 binds to CD4 and co-receptor on the surface of immune cells, while gp41 assists in the fusion of the viral envelope with the host cell membrane [12]; 4. *Tat*: The *Tat* gene encodes the Tat protein, which plays a crucial role in regulating viral gene expression. Tat enhances viral transcription by interacting with the viral RNA and cellular factors, leading to increased production of viral proteins [13]; 5. *Rev*: The *Rev* gene encodes the Rev protein, which is responsible for the transport of unspliced and partially spliced viral RNAs from the nucleus to the cytoplasm. This facilitates the production of viral proteins

and the packaging of viral RNA into new virus particles [14]; 6. *Nef*: The *Nef* gene encodes the Nef protein, which modulates the host cell immune response and enhances viral replication. Nef helps the virus evade the immune system and promotes the survival of infected cells [15]. In addition, there are *Vpu, Vpr, Vif* genes in the genome of HIV-1, which also have very important functions.

1.1.2 Simian Immunodeficiency Virus (SIV)

SIV is a group of lentiviruses that infect primates, including monkeys and apes. Under natural conditions, SIVs are widely distributed across Africa [16] and have evolved into several different strains. In addition, they have shown the ability to infect monkeys from other continents, with the most notable variant being SIVmac, which was discovered in rhesus macaques that were accidentally infected in captivity with SIV from sooty mangabey monkeys (SIVsmm) [17].

Because of their distance from each other, SIV and HIV viruses are genetically different from each other [18]. However, the two share similarities in some respects [19], [20], [21], [22]. The study of SIV in primates has provided valuable insights into the pathogenesis, transmission, and immune responses to HIV in humans [22], [23], [24]. However, in their natural primate hosts, SIV infections generally do not lead to severe immunodeficiency and AIDS-like symptoms. However, HIV is believed to have originated from the cross-species transmission of SIV to humans [25]. The HIV-1 virus is closely related to SIVcpz (SIV from chimpanzees), and HIV-2 is closely related to SIVsmm (SIV from sooty mangabey monkeys) [26]. For the above reasons, research on SIV can provide us with a lot of knowledge related to HIV. We can gain a better understanding of the mechanisms of HIV transmission, viral replication, and immune responses by studying SIV. SIV infection in non-human primates (NHPs) is widely used as an animal model to study HIV/AIDS [27], [28], [29].

1.1.3 Feline Immunodeficiency Virus (FIV)

The feline lentivirus FIV primarily affects domestic cats, and some wild felids. It is also very susceptible to mutations, resulting in different subtypes [30]. FIV is thought to have originated in Africa but has now spread to all continents of the world, except Antarctica [31], [32]. Unlike SIV, when domestic cats are infected with FIV, their immune system is attacked, leading to a gradual decline in their immune function over time due to T cell depletion, similar to HIV in humans. This also makes FIV-infected cats a suitable animal model to gain some knowledge about HIV in humans. FIV also shares lots of similarities with HIV in terms of gene structure. Therefore, researchers are trying to use the FIV virus as a tool to study HIV infection [33], [34], [35]. In the present study, we aimed to construct a new HIV virus that can infect domestic cats, making these cats a new animal model for HIV/AIDS.

1.1.4 Other lentiviruses

Besides HIV, SIV, and FIV, the lentivirus genus also has other members. EIAV can infect horsess [36], BIV can infect cattles [37], and CAEV and OMVV mainly infect goats/sheep [38]. Interestingly, all of the lentiviruses identified so far have used mammals as hosts. The genomes of these lentiviruses have been studied, and a genealogy of lentivirus evolution has been developed [39].

While lentiviruses may cause diseases in their host species, they can be used in several applied technologies. Firstly, lentiviruses have been engineered to serve as efficient gene delivery vehicles for introducing genetic material into target cells [40]. These lentiviral vectors can deliver genetic cargo into cells, offering potential applications in gene therapy for the treatment of genetic disorders, cancers, and other diseases [41] Secondly, lentiviruses can help in the development of vaccine vectors [42] By removing disease-causing genes and incorporating genes encoding antigens from specific pathogens, lentiviral vectors can stimulate immune responses against

those pathogens. Thirdly, lentiviruses have been used in cell reprogramming and stem cell research [43]. Fourthly, lentiviruses have been employed as tools for introducing or modifying specific genes in cells to study gene function and disease mechanisms. Researchers have now combined lentiviral vectors with CRISPR/Cas 9 as an efficient and accurate method of gene editing [44]. This is also a hot topic of research.

Overall, the emergence of HIV poses a massive challenge to human health. On the other hand, the adaptation of lentiviruses, especially HIV, has improved the efficiency of biological research.

1.2 Dependency and restriction factors in HIV infection

HIV is a species-specific virus that can only infect humans and chimpanzees, and the symptoms of AIDS only appear after infection in humans [45]. The main reason for single-species infections is that several restriction factors are at play in the infection process.

1.2.1 HIV entry receptor

The HIV replication cycle encompasses attachment, entry, reverse transcription, integration, transcription, translation, assembly, and release stages. The initial constraint on the inability of HIV to infect species beyond humans and chimpanzees pertains to cell-surface receptors [12]. As elucidated, the primary step in HIV cell entry involves its binding to the human CD4 receptor. In humans, this receptor is predominantly expressed on the surface of CD4+ T cells [46] with additional expression in monocytes/macrophages, dendritic cells, and a subset of natural killer cells [47]. Upon binding of the gp120 outer glycoprotein to the CD4 receptor, HIV-1 Env glycoproteins undergo structural changes. The distinct layers of the gp120 inner domain play a role in facilitating the shift of Env towards the conformation associated

with CD4 interaction. The interaction between CD4 and gp120 involves the insertion of phenylalanine 43 (Phe43) of CD4 into a cavity known as the ‘Phe43 cavity‘ [48]. This cavity is situated at the junction of the inner and outer domains of gp120. While CD4 is not variable in humans, primates express often different alleles of CD4 [48]. To illustrate this, chimpanzees exhibit a spectrum of nine distinct CD4 receptor variants. A study conducted by Russell et al. [49] identified diverse residues of CD4 across 24 of 29 African primate species. The origin of this CD4 diversity among primates remains unclear, with ongoing speculation that lentiviral interactions with CD4 might contribute to this phenomenon [49].

The interaction between CD4 and HIV is highly selective [50]. However, cells expressing only the human CD4 receptor are still unable to be infected with HIV, and the process of HIV entry into the cell requires the involvement of another surface receptor (called co-receptor). Well-known examples of co-receptors include CXCR4 (C-X-C chemokine receptor type 4) and CCR5 (C-C chemokine receptor type 5) [51]. Following the binding of HIV to CD4, the virus necessitates engagement with a co-receptor for subsequent cellular entry. Various HIV strains exhibit distinct preferences for these co-receptors. Strains that utilize the CXCR4 receptor are designated as X4-tropic strains, whereas those favouring the CCR5 receptor are categorized as R5-tropic strains [52]. Both receptors belong to the G protein-coupled receptor (GPCR) family; however, they possess distinct attributes. While CXCR4 is widely expressed in a multitude of cell types [53], CCR5 predominantly graces the surface of immune cells [54]. CXCR4 receptors are highly conserved across mammalian species, maintaining primary protein structures that are remarkably similar among different species. For instance, the amino acid identity shared between the human and cat CXCR4 proteins is an impressive 94% [55].

In contrast, CCR5 exhibits variations in expression within diverse populations. Notably, Picton et al. noted significant variance in peripheral blood expression between South Africans and Caucasians [56]. The 32-bp deletion in the CCR5 open reading frame,

known as CCR5Δ32 or Δ32, represents a noteworthy variant. This deletion is particularly prevalent in Europe, with an average allele frequency of 10%, while it is absent in Africans. Furthermore, intraspecific diversity within CCR5 is also observed, adding to its complexity [54].

While the interplay of the CD4 receptor and co-receptor typically defines the infectious specificity of HIV viruses in natural settings, laboratory investigations have revealed instances of CD4-independent HIV envelopes, termed CD4-independent HIV viruses. These deviations from the norm have been identified in both HIV-1 and HIV-2 [57]. One such variant, HIV-1 m7NDK, serves as a tool in these experimental contexts. This virus was first reported by Dumonceaux et al. [58]. They used the HIV-1 NDK virus to infect human CEM cells chronically. After long-term culture, the NDK isolate mutant m7NDK (m7 means seven specific variants in the envelope gene) was obtained by limited dilution cloning of the chronically infected CEM cell line. This mutant exhibits the ability to infect cells independently of CD4. They also describe the specific mutation sites (described in the "Results" section). These mutations empower the virus to infiltrate cells, even when only the CXCR4 receptor is present.

Notably, given the striking similarity between the CXCR4 receptor in domestic cats and the human CXCR4 receptor, and the comparatively lower likeness of CCR5, this virus presents itself as a promising research instrument for our experiments.

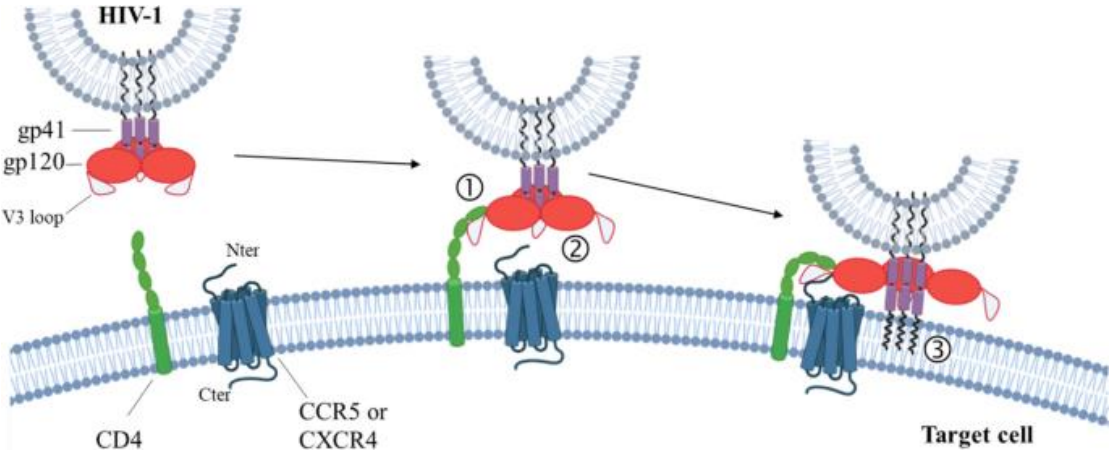

Figure 1. Schematic view of the early steps of HIV infection. The HIV envelope (Env),

composed of gp120 and gp41 subunits, initially binds to the CD4 receptor on the target cell's plasma membrane. This binding triggers a conformational shift in gp120, enabling its V3 loop to interact with a co-receptor, CCR5 or CXCR4, determining the virus's tropism. This subsequent interaction causes another conformational change in gp120, exposing the fusion peptide of gp41. This peptide then inserts its N-terminal domain into the target cell's plasma membrane, initiating the fusion process that ultimately releases the viral content into the cytoplasm of the infected cell [59].

1.2.2 SERINC5 (serine incorporator 5)

SERINC5 is an intrinsic membrane protein with multiple transmembrane domains that acts to reduce the infectivity of budding HIV-1 virions when it becomes part of their envelope during assembly. The precise mechanism by which SERINC5 impedes HIV-1 infection still needs to be explained. Nonetheless, available evidence implies that this inhibition could be linked to SERINC5's ability to induce alterations in the conformation of the HIV-1 envelope protein [60]. Additionally, the HIV-1 Nef protein may counteract SERINC5 [61], conceivably through a mechanism associated with the formation of Nef homodimers during infection [62]. Notably, similar to HIV-1, certain other viruses also exhibit anti-SERINC5 properties [63], [64]. For instance, a research team noted reduced SERINC5 levels in COVID-19 patients with SARS-CoV-2 infection [65].

Nef, a protein of the HIV-1 virus that resists the antiviral capacity of SERINC5, varies in its antiviral capacity among different isoforms. However, the ability of Nef to target SERINC5 is highly conserved among all primate lentivirus-derived alleles [66]. Moreover, the Nef protein of HIV can exert its ability to resist feline SERINC5 [67].

1.2.3 TRIM5α (tripartite motif containing 5 alpha)

TRIM5α is a cellular protein characterised by a tripartite structure consisting of a RING structural domain, a B-box structural domain, and a coiled-coil structural domain [68]. This protein plays a crucial role in preventing the cross-species transmission of HIV among primates. For example, TRIM5α in rhesus monkeys has shown effectiveness in limiting HIV-1 infection [69]. However, human TRIM5α has limited to negligible activity against HIV-1, while HIV-2 is relatively more susceptible to TRIM5α [70].

Although the exact mechanism of human TRIM5α resistance to HIV-1 has not been fully elucidated, it has been determined that the protein binds directly to the HIV-1 capsid [71]. Upon viral entry, rhesus TRIM5α accelerates the fragmentation of the capsid-formed core, leading to disruption of the core, which in turn blocks reverse transcription and nuclear entry [72]. Notably, the antiretroviral effects of TRIM5α do not appear to be dependent on the accelerated vesicle fragmentation process. The SPRY domain at the amino terminus of TRIM5α is essential for its antiviral function, and the RING structural domain of TRIM5α possesses E3 ubiquitin ligase activity [73]. The ubiquitin or proteasome machinery does not appear to be essential for antiviral efficacy. Therefore, it can only be hypothesised that TRIM5α may hinder infection through multiple mechanisms. On the other hand, in order to overcome human TRIM5α restriction, HIV-1 M employs the strategy of binding cyclophilin A (CYPA; also known as peptidylprolyl isomerase A) to the designated CYPA-binding loop on its capsid protein. The identification of huTRIM5α-susceptible residues by Twizerimana et al. [74]. revealed the importance of residue 88 in the CYPA binding loop. HIV-1 M uses alanine at this position, resulting in resistance, while non-M HIV-1 variants use valine or methionine, making them susceptible to huTRIM5α.

Although TRIM5α specialises in blocking the cross-species transmission of HIV-1 in primates, domestic cats do not have the same ability. Studies have shown that the TRIM5α gene in cats is truncated, expressing a protein that has no antiviral activity. Therefore, cats have no TRIM5 restriction of HIV-1 [75].

1.2.4 MX2 (myxovirus resistance protein 2)

MX2 (also called MxB) is a member of the dynamin-like GTPase family. It is an innate antiviral factor that is induced by interferons in the human body [76]. Notably, MX2 inhibits several viruses, including HIV-1, HBV, HSV-1, and HSV-2 [77], [78]. Functionally, MX2 binds to the HIV-1 capsid, preventing viral DNA from entering the host cell nucleus [79].

Integral to its antiviral role is the amino-terminal structural domain, and its effectiveness is influenced by the phosphorylation of serine residues at positions 14, 17, and 18 within this domain. Alterations such as aspartic acid substitutions at Ser306 or Thr334, and alanine substitutions at Thr343, lead to notably reduced antiviral activity. Conversely, the presence of aspartic acid residues at Ser28, Thr151, or Thr343 augments its antiviral activity [80].

Within the GTPase family, MX1 represents another member with antiviral capabilities, although it does not exert influence on HIV-1 [80]. Intriguingly, MX2, a counteracting force against HIV-1 in felines, is conspicuously absent [81].

1.2.5 APOBEC3 (apolipoprotein B mRNA editing enzyme catalytic peptide-like 3)

APOBEC3 belongs to a class of cytosine deaminases that are widespread among mammals and play crucial roles in the innate immune response. In humans, chromosome 22 encodes seven distinct APOBEC3 enzymes (A3A/B/C/D/F/G/H). Among them, A3A, A3C, and A3H are single-domain deaminases, whereas the remaining four (A3B, A3D, A3F, and A3G) are double-domain deaminases [82].

Among these enzymes, at least four (A3D, A3F, A3G, and A3H) exhibit anti-HIV activity and are capable of restraining HIV-1 replication. They achieve this by inducing C>U hypermutations in the viral genome and/or utilising deaminase-independent mechanisms [83]. Notably, A3G has emerged as a particularly potent antiviral factor

that has garnered significant attention as the most extensively studied HIV-1 inhibitor of the APOBEC3 family.

Beyond their role in inhibiting HIV-1, APOBEC3 proteins extend their antiviral influence to various other viruses, including retroviruses, such as SIV, FIV, and EIAV [84], through their capacity to target single-stranded DNA intermediates. Additionally, APOBEC3A and B exhibit associations with tumorigenesis [85], as mutational patterns linked to APOBEC3 activity have been identified in more than half of all known cancers.

Domestic cats also possess APOBEC3 proteins, including four single-domain A3 variants (A3Z2a, A3Z2b, A3Z2c, and A3Z3) and two double-domain A3 variants (A3Z2bZ3 and A3Z2cZ3) [86]. Similar to their human counterparts, feline APOBEC3 proteins (A3) reduce retroviral infectivity, including viruses such as HIV-1 and FIV. Interestingly, it is noteworthy that feline A3Z2 lacks antiviral activity against FIVΔ*Vif*, which warrants further investigation [87].

To counteract the antiviral effects of APOBEC3 proteins, lentiviruses, such as HIV and FIV, have evolved strategies to evade their activity. These viruses contain *Vif* genes that encode proteins capable of degrading host APOBEC3 proteins. Specifically, the Vif protein directs APOBEC3 proteins to an E3 ubiquitin ligase complex, leading to their polyubiquitination and subsequent proteasomal degradation [88].

Similarly, FIV Vif proteins were able to resist domestic cat A3 proteins, and even feline A3Z3 and A3Z2Z3 were sensitive to domestic cat FIV Vif. Surprisingly, HIV-2 Vif was able to resist feline A3Z2Z3 [87].

These findings underline the complex interplay between viruses and host defence mechanisms, shedding light on how viruses have developed tools to neutralise the antiviral effects of APOBEC3 proteins. The dynamics between APOBEC3 proteins and viral countermeasures remain an area of active research, contributing to our understanding of virus-host interactions [89], [90].

1.2.6 BST-2/tetherin

BST-2/tetherin is a type II transmembrane protein with significant antiviral properties. Its primary function is to inhibit the release of viral particles from infected cells, thereby impeding the spread of the virus [91]. However, viruses have developed strategies to counteract the inhibitory effects of BST-2.

Regarding HIV-1, its Vpu protein plays a crucial role in antagonising BST-2. It can do so by enhancing BST-2 degradation [92], inhibiting retrograde transport, clearing lipid rafts, and inhibiting antibody-dependent cytotoxicity. These four aspects influence the activity of BST-2 [93].

Interestingly, the cat tetherin protein also affects the release of HIV-1, albeit with a different outcome. While it hinders the release of viral particles, this inhibition does not significantly impact the replication of the virus. Notably, due to the formation of virological synapses, where infected and uninfected cells establish close contact, blocked virions can still be transmitted directly from one cell to another. This mechanism potentially contributes to the dissemination of the virus, even in the presence of tetherin-mediated inhibition [88].

1.3 Animal models of HIV

Animal models play a vital role in facilitating our understanding of disease development, progression, and potential treatments. However, in the case of HIV/AIDS, a number of constraints, including ethical issues and economic limitations, have made it challenging to develop an entirely appropriate animal model, due to the various limiting factors mentioned above. Despite these challenges, researchers have managed to develop a variety of animal models that provide valuable information for understanding specific aspects or stages of HIV/AIDS.

These animal models provide an opportunity to study certain aspects of HIV/AIDS, although they may not fully replicate the complexity of the disease in humans [94]. By using these models, researchers can explore a variety of factors, such as viral replication, immune responses, and potential therapeutic interventions. While these models may not fully mimic the human condition, they provide important information that can guide research efforts and inform clinical approaches to HIV treatment.

1.3.1 Humanised mice

Due to the inherent resistance of normal mice (e.g., receptor limitations and restriction factors between species) (see the section above [Section 1.2] for details) to HIV-1 infection and their possession of various HIV-specific restriction factors, directly infecting them to create HIV/AIDS animal models is not feasible. To address this issue, researchers have developed humanised mice [95]. In these mice, human cells and tissues are transplanted, allowing them to become susceptible to HIV-1 infection. However, the robust immune system of mice can lead to the destruction of directly transplanted human cells and tissues. To circumvent this problem, researchers employ immunodeficient mouse strains for transplantation. Examples include nude mice, mice with severe combined immunodeficiency (SCID), and immune-related gene knockout mice. The humanization of these mice is broadly classified into four basic types: the hu-PBL model, in which human peripheral blood leukocytes (hu-PBLs) are injected into mice (SCID is achieved by implanting immature cells or human hematopoietic stem cells [HSCs] into immunodeficient mice that are differentiated into mature cells); the human thymus/liver (SCID-hu-Thy/Liv) model; the human HSC (hu-HSC) model; and the bone marrow/liver/thymus (BLT) model [96]. These humanised mouse models have been pivotal in expanding our understanding of various aspects of HIV infection, including gene function, replication, pathogenesis, and potential therapeutic strategies. Depending on the research goals, different methods are used to construct these models, and updates continue to refine their accuracy and utility [97], [98]. These

models provide a unique platform to study HIV/AIDS in a controlled environment, contributing significantly to advancements in HIV research.

The benefits of using human-derived mouse models are apparent. They contain all major HIV host cell types, they can be stunned for genetic manipulation when transplanting stem cells, and they can test human-specific gene or cytokine therapies, clinically relevant drugs, neutralising antibodies, etc. However, the drawbacks are just as numerous [99]. First, there are specific requirements for the breeding environment of immunocompromised mice; second, the transplantation of human cells and tissues requires surgery; third, the source of particular tissues may also involve ethical requirements; fourth, the constructed immune environment is quite different from that of the human body; and, fifth, the metabolism of mice is very different from that of humans. In conclusion, mouse models have unique advantages over other animal models of HIV infection; however, their limitations cannot be ignored.

1.3.2 Non-human primate (NHP) models

NHPs share numerous physiological similarities with humans, making them valuable models for scientific research. Given that HIV is believed to have originated from the cross-species transmission of SIV from infected monkeys [100], NHPs are particularly pertinent for studying HIV/AIDS. There are two scenarios in which the natural hosts of SIV, African monkeys, do not develop AIDS after being infected with species-specific SIV. This is not because the SIV virus is less virulent but rather because, over the course of a long evolutionary period, these natural hosts have evolved excellent defence mechanisms [101]. Asian macaques, on the other hand, as non-natural hosts of SIV, can develop simian AIDS after infection with SIV under experimental conditions. The most commonly used experimental NHP models are rhesus monkeys, pigtailed macaques, and crab-eating monkeys [102].

While studies of SIV-infected macaques have yielded valuable insights into the

pathogenesis of lentiviruses, applying these findings directly to HIV presents challenges due to the genetic distinctions between SIV and HIV. The genetic differences between these two viruses complicate the direct translation of SIV research to HIV. To bridge this gap and create models that more closely resemble HIV, researchers have developed chimeric viruses known as SHIVs (Simian-HIVs) [103],[104]. SHIVs are essentially SIVs that incorporate segments of the HIV genome. By introducing HIV-specific genetic elements into SIV, these chimeric viruses offer a means to better mimic HIV infection. These new viruses are better able to help us test drugs and vaccines against HIV [105].

In addition, chimpanzees can also be infected with HIV, but fail to develop AIDS-like clinical signs. There are also ethical considerations and the disadvantage of high maintenance costs, making this an unsuitable animal model for HIV [106].

1.4 Feasibility of an animal model of HIV-1 infection in cats

The quest for an ideal animal model for HIV research remains a challenge due to the limitations of existing models, such as humanised mice and NHPs. In this context, the domestic cat is a promising candidate for supplementing HIV research efforts. Extensive studies have led researchers to consider domestic cats as a potential model due to several compelling factors [107].

Based on previous studies, we believe that the domestic cat is appropriate. Domestic cats have been used as a model to study HIV [108]. Domestic cats can be infected with FIV, which is very similar to HIV in terms of its genetic structure. Moreover, cats can also develop AIDS after being infected with FIV. By studying the process of cats being infected with FIV and developing AIDS, we can analyse the cycle of HIV infection in humans. At the same time, FIV can utilise the feline CXCR4 receptor [108], which is 94.9% similar to the human CXCR4 receptor. Moreover, feline CXCR4 is as efficient as human CXCR4 in supporting cell fusion between CD4-expressing murine fibroblast

cells and HIV-1 or HIV-2 Env-expressing human cells [55]. However, the feline CD4 receptor is too different from the human CD4 receptor to be utilised by HIV. Other HIV-1 limiting factors, such as SERINC5 and TRIM5α, do not work against HIV-1 in cats. MX2 is not expressed in cats, and tetherin does not entirely prevent cell-to-cell transmission of HIV-1 by cell contact. This means that most of the inhibitors can be bypassed. The biggest problem remaining is the APOBEC3 protein [109]. This is the crucial factor in stopping HIV-1 replication in cat cells. Therefore, if the problem of HIV-1 entry into cat cells and the killing of HIV-1 by the cat APOBEC3 protein can be solved, then it will be possible to infect domestic cats with HIV-1.

Domestic cats also possess practical advantages. As small animals, they are cost-effective to manage and study, facilitating research logistics. However, it is important to acknowledge that, while domestic cats offer compelling parallels to HIV infection, there are inherent differences between the two viruses and species. Despite these differences, domestic cat models have the potential to enhance our understanding of HIV pathogenesis, transmission, and potential interventions.

1.5 Aim of our thesis

Using the domestic cat as an animal model of HIV-1 infection can help us obtain a small, economical model for testing drugs against HIV-1 and developing vaccines, among other things. However, at least two problems need to be solved to reach this goal.

On the one hand, HIV-1 cannot utilise feline CD4 as a receptor, and feline CD134, operated by FIV, cannot be used by HIV-1 [110]. Therefore, we need to find a way to get around this limitation. We plan to use an HIV-1 virus that is not CD4-dependent, but can use CXCR4 as a receptor, to address this problem.

On the other hand, one of the fundamental reasons why HIV-1 cannot replicate in cat

cells *in vivo* is the killing of the virus by the feline APOBEC3 protein. Therefore, we must find a way to nullify this killing function [111]. Then, as the key to counteracting the effects of feline A3, the FIV *Vif* gene, if integrated into the HIV genome, may be able to accomplish our goal.

2. Materials

2.1 Chemicals

All listed chemicals were stored and used according to the manufacturer's instructions.

Table 1. Chemicals

Chemical	*Company*
Agar	Applichem GmbH (Darmstadt)
Agarose	Bio&Sell eK (Feucht near Nürnberg)
Ammonium peroxidisulphate (APS)	Applichem GmbH (Darmstadt)
Ampicillin	Sigma-Aldrich (Carlsbad, USA)
Casein peptone	Sigma-Aldrich (Carlsbad, USA)
D-glucose	Applichem GmbH (Darmstadt)
Dulbecco's Modified Eagle's Medium (DMEM)	PAN-Biotech GmbH (Aidenbach)
DPBS	PAN-Biotech GmbH (Aidenbach)
Dimethyl sulphoxide (DMSO)	Applichem GmbH (Darmstadt)
Ethanol	Carl Roth (Karlsruhe)
Foetal bovine serum (FBS)	PAN-Biotech GmbH (Aidenbach)
Isopropanol	Carl Roth GmbH (Karlsruhe)
L-glutamine	PAN-Biotech GmbH (Aidenbach)
Methanol	Carl Roth (Karlsruhe)
Penicillin-streptomycin	PAN-Biotech GmbH (Aidenbach)
PolyJet™	Sigma-Aldrich (Carlsbad, USA)
TEMED	VWR, Radnor (PA/USA)
Puromycin	Sigma-Aldrich (Carlsbad, USA)
Roswell Park Memorial Institute (RPMI)-1640	Thermo Fisher Scientific, (Waltham, USA)
SDS	Applichem GmbH (Darmstadt)
Trypsin-ethylenediaminetetraacetic acid (EDTA)	PAN-Biotech GmbH (Aidenbach)

2.2 Enzymes

All listed enzymes (Table 2) were stored at -20°C and used according to the

manufacturer's instructions.

Table 2. Enzymes

Enzyme	*Company*
Restriction endonucleases (10 U/µl)	Thermo Fisher Scientific, (Waltham, USA)
Polymerase chain reaction (PCR) enzyme	
Q5® high-fidelity DNA polymerase	New England Biolabs (Ipswich, USA)
T4 DNA ligase	New England Biolabs (Ipswich, USA)

2.3 Reaction systems

The listed reaction systems (Table 3) were used according to the manufacturer's instructions.

Table 3. Reaction systems

Reaction system	*Company*
DNeasy Blood & Tissue Kit	QIAGEN (Venlo, Netherlands)
ECL Western Blotting Detection System	Amersham Bioscience Europe GmbH (Freiburg)
Pure Yield™ Plasmid Maxiprep System	Promega (Madison, USA)
Steady-Glo® Luciferase Assay System	Promega (Madison, USA)
ZR Plasmid Miniprep™ Classic	Zymo Research (Irvine, USA)

2.4 Plasmids

All plasmids were divided into storage plasmids and stored at -20°C. Working plasmids were present at 4°C.

Table 4. Plasmids

Plasmid	*Resistance gene*	*Reference/Origin*
Chimeric HIV-1 m7NDK+FIV,vif*	Ampicillin	This work
Feline APOBEC3z3-HA	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany

FIV pC-34TF10*Vif*	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
FIV pCT-C36*Vif*	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
huAPOBEC3C-HA	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
huAPOBEC3G-HA	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
HIV-1 m7NDK full length	Ampicillin	This work
HIV-1 NDK full length	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pBABE-fusin (hCXCR4)	puromycin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pcDNA3.1(+)	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pHit.60	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pL102 (BH10 env)	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pMDLg/pRRE	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pMSCV-neo-fCD4	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pMSCV-neo-hCD4	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pMSV-puro-fCXCR4	Puromycin/ Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pNL4-3*Env*	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pNL4-3 full length	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pNL4-3*Vif*	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pRSV*Rev*	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany

pSIN PPT Luc	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pMD.G(VSV-G*Env*)	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
pJD34(m7NDK*Env*)	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany
Puma APOBEC3z3-HA	Ampicillin	Carsten Münk, Heinrich-Heine-Universität Düsseldorf, Germany

*HIV-1 chimeric plasmids will be explained in the 'Results' chapter.

2.5 Oligonucleotides

Single-stranded oligonucleotides were initially designed using *in silico* methods (Vector NTI® Advanced 10; Invitrogen). Following the design phase, the oligonucleotides were then ordered from Eurofins MWG Operon (Ebersberg, Germany).

Subsequently, the obtained oligonucleotides were dissolved and further diluted to achieve a final concentration of 100 pmol/µl using distilled water (dH2O).

2.5.1 Primer sequences used for m7NDK and m7NL4-3 establishment

We utilised overlapping PCR to introduce the target variants.

Table 5. PCR primers used for side-directed mutagenesis from NDK to m7NDK

Name	*Nucleotide sequence 5´→3´*	*Function*
Forward primer 1'	TTTAACATGTGGAAAAATAACATG	Imported into the 5' terminal AflIII sticky end
Reverse primer 1'	CTGTGTAATGGCTGAGGTGTTACA ATTTAT	

Forward primer 2'	ATAAATTGTAACACCTCAGCCATT ACACAG	Resulting in NDK envelope: AA192 D to N, AA195 T to A.
Reverse primer 2'	TGATTGCCCTAGTCCTATCGATGTC CTTTGCCTTATATTGTTGTAGGG	
Forward primer 3'	CCCTACAACAATATAAGGCAAAGGA CATCGATAGGACTAGGGCAATCA	Resulting in NDK envelope: AA296 K to N, AA297 Y to N, AA298 T to I.
Reverse primer 3'	CCATTCTACTCTGCTAATTTTACA	
Forward primer 4'	TGTAAAATTAGCAGAGTAGAATGG	Resulting in NDK envelope: AA333 A to V.
Reverse primer 4'	GCTGGTGCACTATACCAGACATTAA	Imported into the 3' terminal ApaLI sticky end

Based on the seven amino acid variants of m7NDK, an attempt was made to introduce these variants into the envelope of NL4-3 at their corresponding positions.

Table 6. Primers used for side-directed mutagenesis from NL4-3 to m7NL4-3

Name	*Nucleotide sequence 5´→3´*	*Function*
Forward primer 1´	TAAGAATTCTGCAACAACTGCTG	Imported into the 5' terminal EcoRI sticky end
Reverse primer 1´	GGCCTGTGTAATGGCTGAGGTG TTACAAC	
Forward primer 2´	GTTGTAACACCTCAGCCATTACA CAGGCC	Resulting in NL4-3 envelope: AA197 V to A.
Reverse primer 2´	CTCTTCCTGGTCCCCTCTGGATAC GGATACTTTTTCTTATATTG	
Forward primer 3´	CCCTACAACAATATAAGGCAAAGG ACATCGATAGGACTAGGGCAATCA	Resulting in NDK envelope: AA301 T to I.
Reverse		

primer 3´	CCATTCTACTCTGCTAATTTTACA	
Forward primer 4´ Reverse primer 4´	TGTAAAATTAGCAGAGTAGAATGG GCTGGTGCACTATACCAGACATTAA	Resulting in NDK envelope: AA334 A to V. Imported into the 3' terminal BamHI sticky end

2.5.2 Primer sequences used for the creation of the HIV chimera.

Overlapping PCR was also utilised to establish our HIV chimera of interest. The specific viral structure will be described in the 'Results' chapter.

Table 7. Primer sequences for HIV-1 chimera creation

Name	*Nucleotide sequence 5´→ 3´*	*Function*
Forward primer 1´ Reverse primer 1´	TAGTAGCTAGCTGTGATAAATGTCA GCTAA TTACCTGCCAATCTTCTTCACTCAT GGCTCATTCTCCTGTATGCAGACCCC	Imported into the 5' terminal NheI sticky end. Fragment I, for chimera A.
Forward primer 2´ Reverse primer 2´	GGGGTCTGCATACAGGAGAATGAGCC ATGAGTGAAGAAGATTGGCAGGTAA TGATGTAGTGGAACCTGCCAC	First part of FIV34TF10*Vif* Fragment II
Forward primer 3´ Reverse primer 3´	GTGGCAGGTTCCACTACATCA GGTTAGGGATAGGCTTACCTAA TTCTCCCGACCATAACAG	Second part of FIV34TF10*Vif*. Fragment III. Fragments II and III together form FIV34TF10*Vif*, while changing one nucleic acid at the intermediate expression enzyme

		cleavage site BamHI, not the translated amino acid.
Forward primer 4´ Reverse primer 4´	GGTAAGCCTATCCCTAACCCTCTCCTC GGTCTCGATTCTACGTGATCTCCATAG AATGGAGGAAAAGGAGATA ACTGGATCCATTTCTTGCTCTTCTCTGT CG	Imported into the 3' terminal BamHI sticky end. Includes V5 tags. Fragment IV.
Forward primer 2.1´	GGGGTCTGCATACAGGAGAAGCCA TGAGTGAAGAAGATTGGCAGGTAA	Create fragment II with reverse primer 2´ for chimera A1.
Reverse primer 1.1´	TTACCTGCCAATCTTCTTCACTCAT GGCTTCTCCTGTATGCAGACCCC	Create fragment I with forward primer 1´ for chimera A1.
Forward primer 2.2´	AGTAGACAGGATGAGGATTAAGCC ATGAGTGAAGAAGATTGGCAGG	Create fragment II with reverse primer 2´ for chimera A2.
Reverse primer 1.2´	TTACCTGCCAATCTTCTTCACTCA TGGCTTAATCCTCATCCTGTCTACT	Create fragment I with forward primer 1´ for chimera A2.
Forward primer 4.3´	GTTATGGTCGGGAGAATTATGATCT CCATAGAATGGAGGAA	Create fragment IV with reverse primer 4´ for chimera A3.
Reverse primer 3.3´	TTCCTCCATTCTATGGAGATCATAATT CTCCCGACCATAAC	Create fragment IV with forward primer 3´ for chimera A3.
Reverse primer B1	CCTGCCAATCTTCTTCACTCATTTAA TCCTCATCCTGTCTACT	Create fragment I with forward primer 1´ for chimera B.

Forward primer B2´ Reverse primer B2´	AGTAGACAGGATGAGGATTAAATGA GTGAAGAAGATTGGCAGG TCCTTTTCCTCCATTCTATGGTCATAGC TCTCCTAACCATA	Create fragment II for chimera B.
Forward primer B3´ Reverse primer B3´	TATGGTTAGGAGAGCTATGACCATAGA ATGGAGGAAAAGGA CCAAATACTGTAAGCACCTTACCTTGTT AT	Create fragment III for chimera B. Fragment II + III = FivC36*Vif*. Replacement of the first endonuclease BamHI in the rest part of HIV *Vif*.
Forward primer B4´	ATAACAAGGTAAGGTGCTTACAGTATTT GG	Create fragment IV for chimera B with reverse primer 4´.
Forward primer C1´ Reverse primer C1´	ACATCGATAGGACTAGGACAATCACTC TAT TACCTGCCAATCTTCTTCACTTTTTGA CCATTTGCCACCCAT	Imported into the 5' terminal ClaI sticky end. Fragment I, for chimera C.
Forward primer C2´ Reverse primer C2´	ATGGGTGGCAAATGGTCAAAAAGT GAAGAAGATTGGCAGGTA GGTCTCGAGTCACTTGTCATCGTC GTCCTTGTAATCTAATTCTCCCGACCA TAACAGGTT C	Create fragment II for chimera C, contains FIV *Vif,* flag-tag, XhoI in the 3´ terminal.

2.5.3 Primers for CD4 knockout by CRISPR/Cas 9

In addition, we also designed four primers according to the requirements to knock down the cell surface CD4 receptor using the CRISPR/Cas 9 method.

Table 8. Primers for CD4 knockout

Name	*Nucleotide sequence 5´→3´*
SgRNA1	GGCAAGGCCACAATGAACCG

SgRNA2	AGTGCAATGTAGGAGTCCAA
SgRNA3	GGGGTAAAAACATACAGGGG
SgRNA4	GGGCAAAAAAGGGGATACAG

2.6 Sequencing primers

Table 9. Primers for sequencing

Name	*Nucleotide sequence 5´→3´*
m7NDK forward primer 1	CAATAGTTGTGTGGACCATA
m7NDK forward primer 2	GAAGAAGAGATCATAATTAG
m7NDK reverse primer 1	GGGCATTAAACAGCTCCAGG
m7NL4-3 forward primer 1	TAAGAATTCTGCAACAACTGCTG
m7NL4-3 forward primer 2	GTTGTAACACCTCAGCCATTACACAGGCC
Chimera A and B forward primer	TAGTAGCTAGCTGTGATAAATGTCAGCTAA
Chimera A and B reverse primer	ACTGGATCCATTTCTTGCTCTTCTCTGTCG
Chimera C forward primer	TATTGGAGTCAGGAACTGAG
Chimera C reverse primer	GGGGACTGGAAGGGCTAATTTGG

2.7 Bacterial strain

The ligated DNA constructs were transformed into Top10 and Stbl2 (Thermo Fisher Scientific, Waltham, USA) or XL2-blue (Agilent Technologies, CA, USA) chemically competent cells, according to the manufacturer's instructions.

2.8 Antibiotics

Antibiotics were stored and diluted according to the manufacturer's recommendations.

The antibiotics used to select bacteria are described in Table 10.

Table 10. Antibiotics used in bacterial culture media

Antibiotic	*Working Concentration*
Ampicillin	50 µg/ml
Kanamycin	50 µg/ml

Antibiotics were also used for cell screening, when establishing permanent cell lines.

Table 11. Antibiotics used in cell culture media

Cell line	Antibiotic	Concentration
CrFK.hCXCR4	Puromycin	1 µg/ml
HOS.hCXCR4	Puromycin	0.5 µg/ml
HOS.hCD4.hCXCR4	Puromycin	0.5 µg/ml
Thp-1.CD4.KO	Puromycin	2.0 µg/ml

2.9 Cell lines

Both human and feline adherent cell lines were cultured in DMEM. Suspension cells were cultured in RPMI-1640 medium.

DMEM and RPMI-1640 are standardised media for cell culture and contained the following components:

L-glutamine 2 mM

Penicillin 100 U/ml

Streptomycin 50 mg/ml

Foetal calf serum 10%

The foetal calf serum was previously heat inactivated at 56°C for 30 min.

The following cell lines were used during the experimental work of this thesis. The origin and characteristics are described in Table 12.

Table 12. Characteristics of the cell lines

Cell line	*Organism*	*Tissue*	*Characteristics*
CrFK	*Felis catus*	Kidney, cortex	Adherent
HEK-293T	*Homo sapiens*	Kidney	Adherent
HOS	*Homo sapiens*	Bone	Adherent
Thp-1	*Homo sapiens*	Blood	Suspension

2.10 Preparation of gels for electrophoresis

Table 13. Preparation of 1% agarose gel

Name	*Amount*
Agarose	X g
1× TAE buffer	X ml

Table 14. Preparation of SDS gel

Name	*Running gel (10%)*	*Stacking gel*
Rotiphorese® Gel 30 (37.5:1) (Carl Roth, Karlsruhe, Germany)	5 ml	840 µl
1.5 M Tris-HCl pH 8.6 (PanReac AppliChem, Chicago IL, USA)	3.75 ml	/
1 M Tris-HCl pH 6.8 (PanReac AppliChem, Chicago, IL, USA)	/	630 µl
10% SDS (PanReac AppliChem, Chicago, IL, USA)	150 µl	50 µl
20% APS (Sigma-Aldrich, Carlsbad, USA)	75 µl	25 µl
TEMED (VWR, Radnor, PA/USA)	15 µl	5 µl
dH_2O	6.1 ml	3.5 ml

2.11 Buffers and solutions

2.11.1 Buffers for gel electrophoresis

20× TAE buffer

Tris (hydroxymethyl)-aminomethane 9.68 g

Ice acetic	2.28 ml
EDTA 0,5 M	4 ml
dH_2O	ad 1,000 ml

6× DNA loading dye

6× sample buffer	100 µl
Glycerine	150 µl
dH_2O	1,250 µl

2.11.2 Buffer for flow cytometry

FACs buffer

PBS	500 ml
FBS (1%)	5 ml
EDTA (500 mM)	5 ml

2.11.3 Buffer for immunoblotting

RIPA buffer

Tris pH 8.0	25 mM
NaCl	137 mM
SDS	0.1%
Glycerol	0.1%
Na deoxycholate	0.5%
Nonidet P-40	1%
EDTA	2 mM
dH_2O	ad 100 ml

Aliquots of 10 ml were prepared and stored at 4°C. After thawing of the RIPA-buffer, a

protease inhibitor (Roche, Mannheim, Germany) was added.

10× electrophoresis buffer (pH 8.8)

Tris	25 mM
Glycine	192 mM
SDS	0.1% (w/v)
dH_2O	ad 1,000 ml

1× stacking gel buffer (pH 6.8)

Tris	0.5 M
dH_2O	ad 200 ml

1× running gel buffer (pH 8.8)

Tris	1.5 M
dH_2O	ad 200 ml

20× TBS buffer (pH 8.0)

Tris	25 mM
NaCl	150 mM
KCl	3 mM

TBST buffer

Tween-20 diluted in 1× TBS buffer 0.2% (v/v)

5% blocking solution

5 g milk powder dissolved in 100 ml TBST buffer

2.12 Protein ladder

To identify the molecular weight of proteins after gel electrophoresis, Fermentas PageRuler™ prestained protein ladder (11–170 kDa) was used.

2.13 DNA ladder

To identify the length of DNA fragments after gel electrophoresis, Fermentas 1 kb GeneRuler^{TM} was used.

2.14 Culture media for *Escherichia coli*

Before use, all prepared media were autoclaved at 200 kPa and 121°C for 30 min. Heat-unstable components were added after autoclaving.

Luria-Bertani (LB) media, liquid

Casein peptone	10 g
Yeast extract	5 g
NaCl	10 g

made up with 1 L dH_20, autoclaved.

LB media plates, solid

Casein peptone	10 g
Yeast extract	5 g
NaCl	10 g
Agar	15 g/l

made up with 1 L dH_20, autoclaved, cooled down to 60°C, if required, addition of antibiotics, allocated to Petri dishes, and allowed to dry.

2.15 Culture media for cryo-conservation

Media for cryo-conservation

FBS	90%

DMSO 10%

2.16 Antibodies

Proteins were used for immunoblotting or flow cytometry analysis depending on requirements.

Table 15. Antibodies for western blotting

Antibody	*Company*	*Dilution*
Anti-tubulin	T6074 (Sigma-Aldrich, Carlsbad, USA)	1:10,000
Anti-V5	V8137 (Sigma-Aldrich, Carlsbad, USA)	1:5,000
Anti-HA	51064-2-AP (Proteintech, Rosemont, USA)	1:5,000
Anti-flag	F1804 (Sigma-Aldrich, Carlsbad, USA)	1:7,000
Anti-HIV-1-P24	30168-1-AP (Proteintech, Rosemont, USA)	1:5,000
HRP-conjugated sheep anti-mouse IgG	NA931V (GE Healthcare, Chicago, USA)	1:10,000
HRP-conjugated donkey anti-rabbit IgG	NA9340V (GE Healthcare, Chicago, USA)	1:10,000
HRP-conjugated mouse anti-goat IgG	Sc-2354 (Santa Cruz Biotechnology, Dallas, USA)	1:10,000

In general, the reaction conditions for the primary antibody were incubation at 4°C with overnight shaking. Secondary antibody conditions were incubation for 2 h with shaking at room temperature. All antibodies were dissolved in 5% non-fat milk.

Table 16. Antibodies for flow cytometry and incubation conditions

Antibody	*Amount*	*Incubation conditions*	*Company*
α-hCD4 PE mouse	10 µl	30 min at 4°C	Dako (Lot.: 20069495)
α–hCXCR4 PE	10 µl	15 min at 37°C	BD Pharmingen (Cat.: 555974)

2.17 Inhibitors

We treated the cells with the corresponding inhibitors to silence the function of cell surface receptors (human CD4 or CXCR4).

Table 17. Inhibitors of cell surface receptors

Name	*Company*
BMS-663068 (fostemsavir, human CD4 inhibitor)	Adooq Bioscience (Irvine, USA), Cat No.: A15025
AMD3100 (CXCR4 inhibitor)	Sigma-Aldrich, (Carlsbad, USA), Cat No.: 239820

2.18 Equipment

The devices used are listed in the table below, and were used according to the manufacturer's instructions.

Table 18. Equipment

Device	*Company*
Table centrifuge	Fisher Scientific
Heating block	Bioblock Scientific
CO2 incubator BBD 6220	Heraeus, Hanau
Vortexer, Top Mix FB 15024	Fisher Scientific
PCR thermocycler, 96 Universal Gradient peQSTAR	PeQLab
NanoDrop ND-1000	Peqlab, Erlangen
Ultra centrifuge	Beckmann Coulter
Thermocycler	Eppendorf
BD FACSCanto	BD Bioscience
SDS gel running and semidry western blot apparatus	BioRad

2.19 Software

We analysed the data obtained from the experiments using the following software.

Table 19. Software

Software	Company
GraphPad Prism 8.0	GraphPad Software
Vector NTI® Advance 10	Invitrogen
FlowJo 10.4.0	Tree Star, Inc.

3. Methods

3.1 Microbiological methods

3.1.1 Transformation of bacteria

Chemical transformation is a method of transferring plasmid DNA of interest to active bacteria for expanded culture. We used three transformation methods to get the plasmid we were interested in: ‘Top10’, ‘STBL 2’, and ‘XL2-blue’. ‘Top10’ is suitable for the amplification of plasmids created based on the pcDNA3.1 vector; ‘STBL 2’ is ideal for the amplification of lentiviral plasmids; and ‘XL2-blue’ is ideal for lentiviral plasmids that are difficult to amplify using ‘STBL2’.

Top10: First, we thawed aliquots of cells stored at -80°C on ice and then added 100–150 ng of the target plasmid or ligation product to the competent cell tubes. The mixture was incubated on ice for 10 min. Then, the bacteria were heat-shocked on a heat block at 42°C for 45 s and immediately incubated on ice for another 10 min. Next, 200 µl of antibiotic-free LB medium was added to the mixture and shaken at 550 rpm at 37℃ for 45–60 min. Finally, 20–200 µl of the mixture was spread onto LB culture plates, containing the appropriate antibiotics, and incubated at 37℃ overnight.

STBL 2: Aliquots of cells were thawed on ice and 100–300 ng of target plasmid was added to the competent cell tubes. The mixture was incubated on ice for 30 min, heat shocked at 42℃ for 40 s, and incubated on ice for another 10 min, before adding 500 µl of antibiotic-free LB medium and incubating at 30℃ for 90 min at 225 rpm. Finally, 200 µl of the mixture was spread onto LB plates and incubated at 30℃ overnight or room temperature for 24 h.

XL2-blue: First, we thawed the aliquots of XL2-blue competent cells on ice while preparing β-mercaptoethanol (β-ME) at a concentration of 1.22 M. We added 2 µl of β-ME to the aliquots of cells and incubated them on ice for 10 min, swirling the tubes every 2 min. Next, 1–50 ng of plasmid DNA was added to one aliquot of cells, and the

tube was incubated on ice for 30 min. We heat-shocked the tube at 42℃ for 30 s and incubated it on ice for 2 min. Then, we added 900 ml of preheated (30℃) LB medium and incubated it at 37°C for 1 h with shaking at 225–250 rpm. After that, we plated 200 µl of mixture on an LB agar plate and incubated it at 30℃ overnight.

In order to isolate the plasmid DNA, grown bacterial colonies were picked and used to inoculate a bacterial culture for mini or maxi DNA preparation. Cultures were incubated overnight at the appropriate temperature (30 or 37°C). Next day cultures were used for the following DNA preparation.

3.2 DNA techniques

3.2.1 Plasmid DNA preparation

We used the Miniprep programme to isolate plasmid DNA from <5 ml of overnight grown bacterial cultures. For the mini preparation, we followed the protocol of the ZR Plasmid Miniprep Classic kit from Zymo Research.

We used the Maxiprep programme to isolate plasmid DNA from 200–250 ml of overnight grown bacterial cultures. For the maxi preparation, we follow the protocol of the Pure Yield™ Plasmid Maxiprep System kit from Promega.

3.2.2 Estimation of DNA

Nucleic acid concentration was assessed by spectrophotometry using the NanoDrop ND-1000 device from PeQLab. A 1-µl aliquot of the sample was applied to the device, and the absorbance at 260 nm was determined. Additionally, the device computed the 260/280 nm absorbance ratio to evaluate DNA purity. A ratio of approximately 1.8 indicates the presence of pure DNA.

3.2.3 DNA digestion

For the analysis and evaluation of targeted DNA sequences, as well as for the generation of DNA fragments intended for subsequent procedures, the nucleic acids of interest were hydrolysed using type II restriction endonucleases. These endonucleases selectively identify particular sequences within the DNA and catalyse their hydrolysis.

The composition of the restriction endonuclease mixture is given in the following table.

Table 20. DNA restriction mixture

Agent	*Amount*
DNA	Variable µl (equivalent amount of DNA is 400–800 ng)
Restriction endonuclease	5–10 U
10× buffer	2 µl
dH_20	Made up to a total volume of 20 µl

The buffer was selected according to the web tool provided by Thermo Fisher Scientific, same as the reaction conditions. The hydrolysed DNA was used for further work or analysed on a 1% agarose gel.

3.2.4 Gel electrophoresis

Gel electrophoresis is an analytical technique that is employed to separate macromolecules, such as DNA and proteins, based on their inherent charge and molecular dimensions.

Regarding DNA molecules, gel electrophoresis was utilised to ascertain the lengths of the DNA fragments. This determination relies on the fact that negatively charged DNA molecules migrate towards the anode when subjected to an electric field. For agarose gel electrophoresis, a 1% agarose gel was prepared in TAE buffer. The gel mixture

was heated until boiling and subsequently cooled to approximately 60 °C. To the cooled agarose solution, 0.1% (v/v) ethidium bromide was added, and the resulting liquid agarose was then cast into a plastic chamber for subsequent use.

Ethidium bromide inserts itself between the major and minor grooves of DNA and emits light in the visible spectrum (590 nm) when exposed to UV light.

DNA samples were mixed with 6× loading dye, applied to the gel, and subjected to electrophoresis at 130 V in an electric field. The gel was placed in a running chamber filled with 1x TAE buffer. Following separation, the DNA fragments were visualised under UV light and photographed for analysis and record keeping. A separate tracking marker was employed to determine the lengths of the DNA fragments, with smaller molecules migrating more quickly towards the anode compared to larger fragments.

For protein analysis, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted to determine protein molecular weights. In contrast to agarose gel electrophoresis, SDS-PAGE involves both a separating and a stacking gel, each serving a distinct purpose. The stacking gel gathers all proteins from the sample, at the start of the run, before allowing them to enter the separating gel, ensuring proper separation. The gel was formed by combining components, as detailed in Section 2.11. Initially, a running gel was created. Once its lower part was polymerised, the upper stacking gel was added after blending the components specified in Section 2.11. These gels were constructed using the horizontal SDS gel system from Bio-Rad.

Before loading onto the gel, protein samples were heated with 5× Roti-Load containing 2-ME at 100°C for 5 min. This step is essential for imparting a negative charge to the proteins and denaturing them, thereby enabling their migration towards the anode under the influence of an electric field. The proteins were then separated on SDS gels within an electrophoresis chamber filled with SDS running buffer at 130 V until they reached the lower end of the gel. Following SDS-PAGE, the SDS gels were retained for further experimentation.

3.2.5 Side-directed mutagenesis

Side-directed mutagenesis is a method to introduce point mutations into a nucleotide sequence of interest. For our experiments, we used overlapping PCR to introduce mutations into the plasmids.

Overlapping PCR is a specialised technique used in molecular biology to create DNA sequences with specific modifications or combinations of DNA fragments. This method is beneficial for constructing DNA molecules with desired features, such as introducing mutations, adding tags, or combining different DNA fragments.

The process involves designing two separate DNA fragments with overlapping regions at their ends. These overlapping regions contain complementary sequences that allow the fragments to anneal or bind together during PCR. The primer pairs amplifying these fragments have extensions corresponding to the overlapping areas.
The experimental mixture for PCR was composed according to the following table.

Table 21. Composition of the PCR mixture

Agent	*Amount*
Q5 high-fidelity DNA polymerase	0.5 µl
5× Q5 reaction buffer	10 µl
10 mM dNTPs	1 µl
Template DNA	5 µl (DNA concentration 1 ng/µl) or fragment I (2.5 µl) + fragment II (2.5 µl)
10 µM forward primer	2.5 µl
10 µM reverse primer	2.5 µl
Nuclease-free water	Made up to 50 µl

The components were mixed thoroughly, and the samples were placed in the PCR instrument to start the program. The general program is shown in the table below.

Table 22. PCR programme

Process	*Duration*	*Temperature*
Lid heating	X s	100°C
Denaturation	5 min	95°C
25× cycle		
Denaturation	45 s	95°C
Annealing	30 s	Based on the Tm values of the two primers
Elongation	Based on the target DNA length	72°C
Final elongation	5 min	72°C
Storage	∞	4°C

When we amplify a DNA product using PCR, we use gel electrophoresis to detect the size of the product. The gel containing the correct size band was cut, and DNA fragments were collected using DNA extraction. For the extract method, we followed the protocol of the QIAquick Gel Extraction Kit from Qiagen.

After collecting all of the bands, we combined them using the overlapping PCR approach. Next, both the prepared vector and the PCR product were exposed to bilateral sticky ends by digestion. Again, we get one vector and one insert through gel electrophoresis and DNA extraction. Finally, they are connected using the method of DNA ligation, as shown in the following table.

Table 23. DNA ligation programme

Component	*20-µl reaction*
T4 DNA ligase buffer (10×)	2 µl
Vector DNA	50 ng
Insert DNA	37.5 ng
Nuclease-free water	Made up to 20 µl
T4 DNA Ligase	1 µl
	Room temperature for ≥2 h

Next, we amplified the DNA ligase product by transformation and miniprep to obtain a newly amplified plasmid. The new plasmid was then miniprepped. The plasmid was digested again with the same restriction endonuclease as before, and gel electrophoresis was carried out to check whether the size of the cut bands was as expected. If so, the plasmid was sent for sequencing.

In addition, we encountered difficulties in the PRC procedure when the Tm values of the forward and reverse primers differed too much, affecting the success of the PCR. The solution to this problem is the touchdown PCR method, which is described in Table 24.

Table 24. Touchdown PCR programme

Step	*Phase*	*Temperature (°C)*	*Time*
1	Initial denaturation	95	3 min
2	Denaturation	95	30 s
3	Annealing*	70–60 or 60–50	45 s
4	Extension	72	Based on length
	Repeat steps 2–4(8 cycles)		
5	Final extension	72	10 min
6	Final hold	4	∞

*T_a is reduced by 1°C per cycle.

3.2.6 DN sequencing

The DNA samples were sequenced by Eurofins to determine the DNA sequence of interest. The samples were prepared as described in Table 25. The received nucleotide sequence was analysed using Vector NTI Advanced 10.

Table 25. DNA sequencing approach

Agent	*Amount*
400–700 ng DNA	X µl
Primer (10×)	2 µl
dH_2O	Made up to 17 µl

3.2.7 CRISPR/Cas 9 knockout

The CRISPR/Cas 9 system is an immune system for prokaryotes, which has been utilised to develop a set of gene-editing tools. It has the advantages of high efficiency, relatively low operating difficulty, and low cost. In this experiment, this system was utilised to knock out the human CD4 receptor expressed on the surface of human Thp-1 cells, thus creating a human cell line that does not express hCD4. The knockout was performed using a genome-scale CRISPR knockout (GeCKO) library. Successfully knocked out cells were screened under antibiotic stress or subjected to flow cytometry. In our experiments, we used this system to knock down the CD4 receptor on the THP-1 cell membrane. The steps are as follows:

sgRNA design

The sgRNAs used for CD4 (NCBI gene ID: 960) knockdown were designed using CHOCHOP, and the following were picked for the experiments:

sgRNA	**Sequence**
CD4.sgRNA1-Forward	**CACCG**GGCAAGGCCACAATGAACCG
CD4.sgRNA1-Reverse	**AAAC**CGGTTCATTGTGGCCTTGCC**C**
CD4.sgRNA2-Forward	**CACCG**AGTGCAATGTAGGAGTCCAA
CD4.sgRNA2-Reverse	**AAAC**TTGGACTCCTACATTGCACT**C**
CD4.sgRNA3-Forward	**CACCG**GGGGTAAAAACATACAGGGG
CD4.sgRNA3-Reverse	**AAAC**CCCCTGTATGTTTTTACCCC**C**
CD4.sgRNA4-Forward	**CACCG**GGGCAAAAAAGGGGATACAG
CD4.sgRNA4-Reverse	**AAAC**CTGTATCCCCTTTTTTGCCC**C**

a. **Lentiviral vector digestion**

Five micrograms of lentiCRISPRv2 plasmid were digested using BsmBI restriction enzyme (Thermo Fisher Scientific, Germany), for 30 min at 37°C, and then phosphorylated using FastAP enzyme (Fermentas, Germany), as follows:

Item	Amount (μl)
plentiCRISPRv2 (5 μg)	7
FastDigest BsmBI	3
FastAP	3
10× FastDigest buffer	6
100 mM DTT	0.6
ddH_2O	31.4
Total	50

Following digestion, the digested plasmid was subjected to agarose gel electrophoresis for separation of the vector (~12.5 kb) from the non-vector fragment (~2 kb filler piece), followed by vector purification using a QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany).

b. **Oligo pair phosphorylation and annealing**

Item	Amount (μl)
Oligo 1 (100 μM)	1
Oligo 2 (100 μM)	1
10× T4 ligation buffer	1
ddH_2O	6.5
T4 PNK (NEB M0201S)	0.5
Total	10

The reaction tubes were put in a thermocycler using the following parameters: 37℃: 30 min; 95°C: 5 min, with a ramp down to 25℃ at 5℃/min. This was followed by

annealing of the oligos at a 1:200 dilution, using nuclease-free water.

c. **Ligation of sgRNA oligos into BsmBI digested plentiCRISPRv2**

The complementary oligonucleotide sgRNAs were ligated into BsmBI digested plentiCRISPRv2, as follows:

Item	*Amount (μl)*
BsmBI digested plentiCRISPRv2 (50 ng)	1.5
Diluted sgRNA oligo duplex	1
10× T4 DNA ligase buffer	2
Nuclease-free water	14.5
DNA ligase	1
Total	20

The reaction was incubated at room temperature for 2 h, followed by transformation in Stbl2 *Escherichia coli*, on LB media agar plates containing ampicillin, because the vector has an ampicillin resistance gene. LB plates were incubated at room temperature for 2 days and individual bacterial colonies were picked and allowed to grow in 4 ml of LB liquid medium for 2 days at room temperature for small-scale plasmid propagation. The plentiCRISPRv2 plasmid, with ligated sgRNA, was then extracted from bacteria using a Zymo Research kit (Zymo Research, Freiburg, Germany).

To confirm successful cloning, the extracted plasmids were sent for sequencing, and the results confirmed successful ligation of sgRNA into plentiCRISPRv2. For large-scale plasmid propagation, 0.5 ml of the small-scale plasmid propagation growth was added to 250 ml of LB medium and allowed to grow under shaking at 180 rpm, at room temperature for 2 days. This was followed by plasmid extraction using the Promega Maxiprep kit (Promega, Langenfeld, Germany).

d. **Transfection to produce lentiviral particles for CD4 knockdown**

To produce lentiviral particles for CD4 knockdown, HEK-293T cells were transfected using PolyJet™ (Tebu-Bio) transfection reagent to 1,100 ng of the plentiCRISPRv2 plasmid with ligated sgRNA, 1,100 ng of psPax2, and 200 ng of the pMDG.VSV-G plasmid. Two days following transfection, the supernatant containing viral particles was collected, and short-centrifuged to pellet any floating cells. The supernatant was collected in new Eppendorf tubes and frozen at -80℃ for future use.

3.3 Cell culture techniques

Cell culture experiments were conducted using aseptic techniques in a controlled environment provided by a cell culture hood.

3.3.1 Culture and transmission of eukaryotic cells

We cultured both adherent and suspension cell types. Both were cultured in incubators, in culture flasks, at 37°C in a saturated, humid atmosphere with 5% CO_2.

For adherent cells, we began passaging when the cells were observed under a microscope to have reached 75% confluence. We first pipetted the medium using a sterile pipette, then washed the cells twice with PBS solution, added 2 ml of trypsin, and kept the flask in the incubator for 5 min. Finally, the cells were resuspended in 8 ml of complete DMEM medium, and 0.2–0.5 ml of the cell suspension was pipetted into a new flask with 10 ml of complete DMEM medium. The remaining cell suspension was used for further experiments.

For suspension cells, we passaged the cells every 4–6 days. First, all the media and cells in the culture preparation were transferred to a sterile centrifuge tube using a sterile pipette. The cells were then centrifuged, and the medium was discarded. We

added 10 ml of PBS solution to the centrifuge tube and resuspended the cells. The cells were centrifuged again to clean the cells. Finally, after discarding the PBS solution, the cells were resuspended in complete RPMI-1640 cell culture medium, and 2 ml of the suspension was added to a cell culture flask containing 8 ml of the medium.

3.3.2 Cell counting

Before cell counting, we dispersed and resuspended the cells (see Section 3.3.1). We took 10 μl of the cell suspension, applied it to a cell counting plate, and counted the cells located within the squares under a microscope.

To calculate the number of cells, the following formula was used:

Cell number = number of cells counted / large squares counted × dilution × chamber factor (10.000)

3.3.3 Cryoconservation

Cell lines can be stored in liquid nitrogen for long periods of time when not in use. Cell mixtures need to be prepared and counted before freezing the cells (as shown in Sections 3.3.1 and 3.3.2), and cell cryopreservation solutions must be prepared simultaneously. The cell cryopreservation solution consisted of 10% DMSO and 90% FBS. When the above steps are finished, 5-10*10^5 cells are resuspended in 1 ml of cell cryopreservation solution and placed in a 2-ml cell freezing tube. Because DMSO is toxic to cells, we need to work fast. After completing the above steps, we placed the cell cryotubes into a freezer container filled with isopropyl alcohol. The container was placed in a -80°C freezer for 24 h. The next day, we put the cell cryotubes into liquid nitrogen at -152℃ for long-term storage.

3.3.4 Thawing of cryoconserved eukaryotic cell lines

To recultivate cryoconserved eukaryotic cells, after removing the cells from the liquid nitrogen, in order to avoid the cell toxicity of DMSO, we need to thaw the cells quickly in a 37°C water bath.

After thawing the cells, we transferred them to 15-ml falcon tubes. We added 9 ml of culture medium to mix them thoroughly. Next, we proceeded to the 'washing step'. This is done by centrifugation at 500 rpm for 5 min, discarding the liquid in the tube, and resuspending the cells in 10 ml of the medium. Washing was repeated twice to eliminate the cell cryopreservative. Finally, the cells were resuspended in 10 ml of complete medium and transferred to cell culture flasks.

3.3.5 Transfection of eukaryotic cells with PolyJet™

To transfect the plasmid of interest into cells for expression, we used the transfection reagent, PolyJet™ (Tebu-Bio, Offenbach am Main, Germany).

First, grown HEK-293T cells were prepared and counted as described in Sections 3.3.1 and 3.3.2. A quantity of 6–8*10^5 cells were inoculated into individual wells of a 6-well plate and incubated overnight in an incubator. The next day, 2,000–2,500 ng of plasmid was transferred into a reaction tube containing 250 µl of normal DMEM solution. PolyJet™ reagent (3.5 µl) was added to the reaction tube and mixed thoroughly, then incubated for 20 min at room temperature in a dark environment. After the incubation was completed, the solution was mixed with 1.8 ml of complete DMEM to form a 2-ml solution. We replaced the medium in one well of the 6-well plate with this solution and gently shook the plate to spread the medium evenly over the cells. The 6-well plate was then placed in an incubator. After 2 days, the cells or supernatant can be used for further experiments.

3.3.6 Single cell cloning by serial dilution

This technique is widely used for the clonal isolation of hybridomas and other cell lines that are not attachment dependent.

In the preparatory phase, we need to prepare a 96-well plate. We labelled each well from top to bottom as A to H, and from left to right as 1 to 12. Then, we filled the reagent dispensing tray with 12 ml of the appropriate medium and used an 8-channel pipette to add 100 µl of medium to each well of the 96-well plate, except well A1, which was empty. Meanwhile, we designed the cells to be inoculated and counted them, as described in Sections 3.3.1 and 3.3.2.

Next, we added 200 µl of cell suspension (2,000 cells) to well A1. Then, using a single-channel pipette, we quickly transferred 100 µl from the first well to well B1 and mixed by gentle pipetting, avoiding bubbles. We repeated these 1:2 dilutions down the entire column, using the same tip, discarding 100 µl from well H1 so that it ended up with the same volume as the wells above it.

With an 8-channel pipette, we added 100 µl of medium to each well in column 1. Then, using the same pipette, we quickly transferred 100 µl from the wells in the first column (wells A1 through H1) to those in the second column (wells A2 through H2) and mixed by gentle pipetting, avoiding bubbles.

Using the same tip, we repeated these 1:2 dilutions across the entire plate, discarding 100 µl from each of the wells in the last column (A12 through H12), bringing the final volume of all the wells to 200 µl, by adding 100 µl of the medium to each well. Then, we labelled the plate with the date and cell type and placed it into the incubator.

3.4 Virological techniques

3.4.1 Preparation of reporter vectors using the three-plasmid system

The three-plasmid system was employed to generate HIV-1 reporter vectors that did not encompass the entire lentiviral genome of HIV-1. This system utilises three distinct plasmids. Importantly, these reporter viruses lack the accessory genes of HIV-1 and cannot replicate within host cells. These characteristics facilitate safe and manageable experimentation under Biosafety Level 2 conditions.

The following plasmids were utilised to produce HIV-1 reporter vectors: pMDLg/pRRE, pRSV*Rev*, and pSIN PPT. If the envelope plasmid of HIV-1 is then added to the three-plasmid system, an HIV-1 pseudovirus can be generated and used to infect cells.

The structural genes, *Gag* and *Pol*, are included in the expression plasmid pMDLg/pRRE. Additionally, this plasmid contains the Rev response element (RRE). As a protective mechanism, *Gag* and *Pol* encode cis-repressive sequences, which are only expressed when the Rev protein binds to the RRE. The *Rev* gene is encoded in the pRSV*Rev* plasmid. Without the Rev protein, viral mRNA cannot exit the nucleus in an unspliced form.

Furthermore, the pSIN PPT Luc ires GFP plasmid, which encodes firefly luciferase, was incorporated into this system. This plasmid allows for the quantification of viral entry into host cells. This plasmid could be omitted from the system if the quantification of luciferase activity was unnecessary. Additionally, it also contains the RRE.

Table 26. HIV-1 pseudovirus*

Plasmid	Amount (ng)	Features
pMDLg/pRRE	800	Expression vector, contains *Gag Pol* and RRE.
pRSV*Rev*	350	Expression vector contains *Rev*
pSIN PPT Luc ires GFP	800	Expression vector, contains the luciferase reporter gene and RRE.
HIV-1*Env*	600 or pMD.G: 250	Expression envelope.

*Transfection in one well of a 6-well plate.

To produce pseudotyped vectors, which are able to transduce almost every cell, the plasmid pMD.G, encoding the glycoprotein of the Vesicular Stomatitis Virus (VSV-G) is required.

HEK-293T cells were transfected using the specified expression plasmids, following the procedures outlined in Section 3.3.5. Subsequently, supernatants containing viral particles were collected 2 days post-transfection and subjected to centrifugation at 1,200 × g for 5 min to eliminate cellular debris. If not employed immediately, these supernatants were stored at -80°C.

3.4.2 Production of replication-competent HIV viruses

To produce replication-competent HIV-1 viruses, we transfected the full-length HIV-1 virus plasmid into HEK-293T cells. Or, if we wanted it to contain the luciferase reporter gene, we co-transfected the viral plasmid with the pSIN PPT plasmid. This work must be done under Biosafety Level 3 conditions. The supernatant containing the virus particles was collected 2 days later, also by centrifugation.

3.4.3 Stable transduction by Mouse Leukaemia Virus (MLV) vectors

To achieve stable integration of the DNA of interest into the target cell genome and ensure continuous expression, we employed the MLV-based vector, pHIT60 PMLVg/p. This vector encompasses the *Gag* and *Pol* genes driven by the CMV promoter. Transfection of HEK-293T cells was performed, as described previously. Transfected plasmids included 1,100 ng of pHIT60, 1,100 ng of plasmids containing specific antibiotic resistance genes and target genes, and 200 ng of pMD.G. VSV-G pseudo-reporter vectors can infect virtually all cell types.

Two days after completion of transfection, we collected the supernatant, as described

in Section 3.4.1. We used 1 ml of DMEM medium mixed with 1 ml of the resulting MLV vector, which was applied to the target cells, and incubated for 2 days. Successfully transduced cells were selected under antibiotic pressure.

3.4.4 Luciferase assay

The pseudoviruses/viruses generated in Sections 3.4.1 and 3.4.2 can be used to infect cells. Due to the presence of the *pSIN* plasmid, the pseudoviruses/viruses generated carry the firefly luciferase gene. If the infection is successful, the firefly luciferase gene is able to integrate into the cell genome and express firefly luciferase. At this point, we can measure and quantify its expression using a luminometer.

First, we seeded the cells to be injected in a 96-well plate. We put 6,000 cells/well into the incubator for 24 h. On the second day, the prepared viral solution was applied to the cells, and the culture was continued. After another 2–3 days, we removed the medium, and a mixture of 50 µl of fresh medium and 50 µl of luciferase substrate and lysis buffer was applied to the cells. The cells were blown using sterile tips to mix the cells and allow the cells to lyse. Incubation was continued for 30 min in a dark environment. Finally, 90 ml of the mixture was transferred 1:1 to a new, black 96-well plate.

Subsequently, we introduced the samples to the luminometer for analysis. In the sample wells, the emitted light was quantified by the luminometer. The substrate used in this assay contained beetle luciferin, catalysed to oxyluciferin by firefly luciferase. This chemical reaction results in the formation of oxyluciferin in an electronically excited state, leading to the emission of light. The reaction releases a photon of light as oxyluciferin returns to its ground state, allowing for luminescence measurements. Light emission is directly proportional to the quantity of luciferase produced by the target cells. This, in turn, reflects the number of reporter vectors that have been successfully transduced and are present within the cells.

3.4.5 Spinoculation

During the infection process, to increase the efficiency of the infection, we used a method called spinoculation [112]. This method improves the efficiency of infection and does not affect cellular characteristics or gene expression. This was accomplished by placing the plates in a centrifuge and centrifuging them at 1,000 × g for 1 h after inoculation with the virus.

3.5 Immune biological techniques

3.5.1 Flow cytometry

When analysing cell membrane proteins, western blotting is a relatively complex method. However, flow cytometry is much easier and can validate cell membrane proteins while sorting cells based on the results. Therefore, in our experiments, the choice of flow cytometry was more appropriate for the analysis of the cell surface receptors CD4 and CXCR4.

First, we digested and counted 5*10^5 cells of interest and transferred them to a reaction tube. The cells were precipitated by centrifugation at 500 × g for 5 min, and the culture was discarded. Afterwards, we used 100 μl of FACs buffer to resuspend the cells. Next, the cells were incubated with the antibody by adding the appropriate antibody to the reaction tube (the volume of the antibody and the reaction conditions were used according to the manufacturer's instructions). After incubation, the cells were washed three times with PBS. Cells determined to express the protein of interest were used as positive controls, and cells not expressing the protein were used as negative controls. Finally, the cells were resuspended in 500 μl of FACs buffer and analysed on the flow cytometer BD FACSCanto (BD Bioscience). When we wanted to sort the cells, we used the BD FACSDiva™ (BD-FACSAria™ Fusion). The results were analysed using FlowJo 10.4.0.

3.5.2 Production of protein lysates

In order to isolate the viral proteins present in the culture medium, the cellular debris in the supernatant was first precipitated by centrifugation at 5,000 × g for 5 min. The remaining supernatant was added to a tube containing 2 ml of 20% sucrose-PBS. Next, ultracentrifugation was performed at 4°C and 37,000 rpm for 2 h. The supernatant was discarded, and the virus was lysed using 40 µl of RIPA buffer on ice for 20 min. Finally, using the same centrifugation conditions for 30 min, the supernatant was discarded and the viral protein remained in the tube. It was stored at -20°C until further use.

The procedure for disaggregating the cellular proteins was essentially the same as that for disaggregating the viral proteins, beginning with centrifugation at 5,000 × g for 5 min to obtain a cell pellet. The amount of RIPA buffer required was 100 µl.

3.5.3 Immunoblotting

Immunoblotting was conducted to examine protein expression in cells and viral supernatants.

The proteins were initially separated using SDS-PAGE, as described in Section 3.2.4. Subsequently, they were transferred onto a PVDF membrane, a procedure that took 35 min at 20 V using Bio-Rad’s semi-dry blotting device.

Following the transfer, the PVDF membrane was immersed in a 5% non-fat milk solution for 1 h to block nonspecific binding sites. After this blocking step, the membrane was incubated with the primary antibody at 4°C overnight with gentle shaking.

The following day, the membrane was subjected to three washes with TBST buffer for 15 min each to remove the unbound primary antibody. Subsequently, the secondary antibody, diluted in milk, was applied to the membrane and incubated for 1 h at room temperature. Once again, the membrane underwent three washes with TBST buffer for 15 min each to eliminate any excess secondary antibody.

Finally, the ECL Western Blotting Detection System (Amersham Bioscience Europe GmbH, Freiburg, Germany) was employed to visualise the proteins following the manufacturer's protocol.

4. Results

As mentioned earlier, to use domestic cats as an animal model for HIV-1 infections, at least two issues have to be addressed: The entry of HIV-1 into feline cells and the resistance to feline APOBEC3 proteins.

4.1 Experimental tool preparation

To generate a replication competent HIV-1 that can infect feline cells:

4.1.1 Construction of HIV-1 m7NDK plasmid

The HIV-1 m7NDK (NDK, group M, subtype D) virus produces a specific type of HIV-1 virus with seven specific mutations in its envelope gene. This virus infects cells without relying on human CD4 and using only human CXCR4 [58]. Feline CXCR4 is highly similar to human CXCR4. The identity on amino acid level is 94.7% (Figure 2). Thus, we predict, m7NDK may infect cat cells by using cat CXCR4.

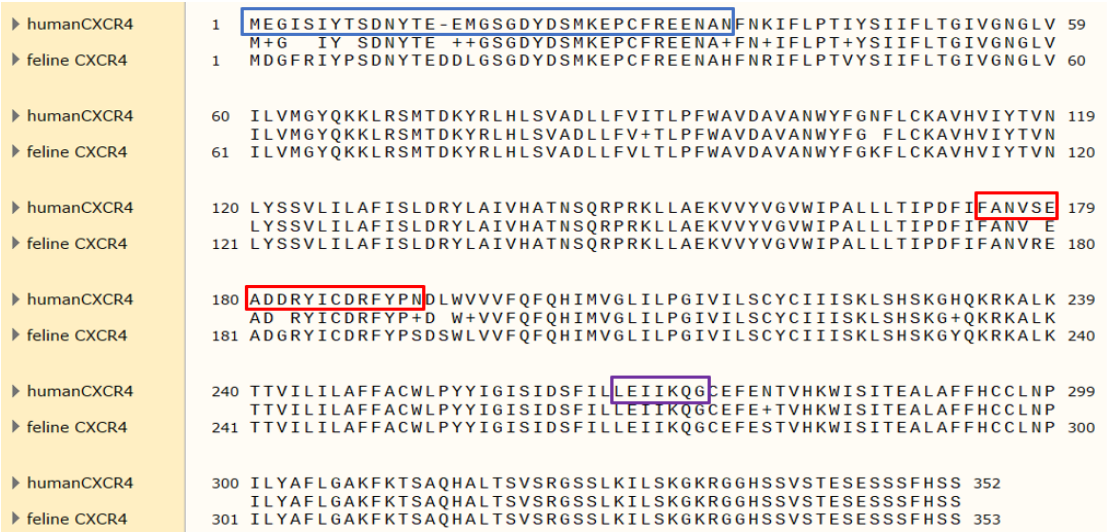

Figure 2. Comparison of the protein sequence of human and feline CXCR4.

Human CXCR4 (gene ID 7852) is 94.7% identical to feline CXCR4 (gene ID 493676). Predicted domains and the key sites where CXCR4 binds to the HIV-1 envelope are indicated: N-terminal (1-34) residues boxed in blue, ESL2 (residues 174-192) boxed in red, and ESL3 (residues 267-273) boxed in purple [113]. The middle lane of the alignment shows residues identical for both proteins and (+) indicates similar amino acids.

The goal was to produce a CD4-independent replication-competent HIV-1 virus. The plasmid expressing the envelope of m7NDK was kept in our laboratory; however, there was no replication-competent HIV-1 m7NDK virus published. Therefore, the properties of the m7NDK replication-competent virus have yet to be studied. To test a replication-competent HIV-1 with the m7NDK Env, the seven amino acid variants of the envelope M7NDK needed to be changed in the HIV-1 NDK WT viral genome. The method used was overlapping PCR. In addition, we also introduced these seven mutations into the corresponding positions of the *env* gene of HIV-1 NL4-3, called m7NL4-3.

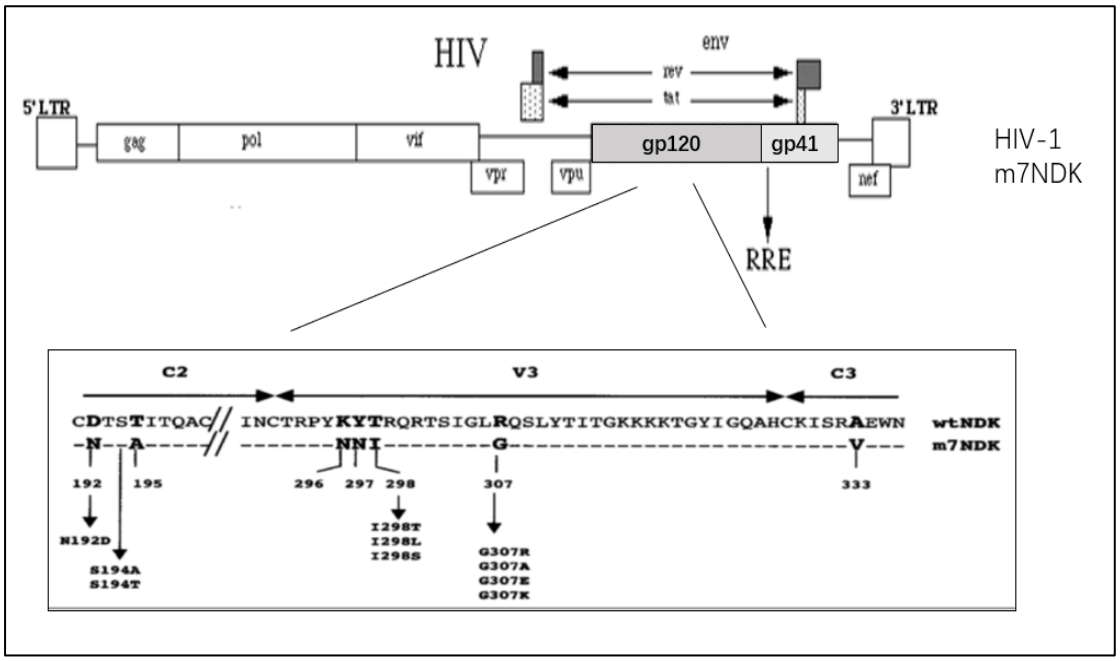

Figure 3. Schematic representation of the seven variants of the m7NDK *Env* gene compared to wild-type NDK. These variants are all located in the gp120 section of the envelope, specifically at the C2 (residues 192, 195), V3 (residues 296, 297, 298, and 307), and C3 (residue 333) region. (The schematic in the box below was taken from Dumonceaux et al. [58]. Authorized by the relevant copyright holder)

We cloned the m7NDK and m7NL4-3 replication competent viruses according to the diagram above (Fig. 3).

After cloning the virus plasmids, the next step was to determine whether the viral plasmid were able to produce infectious HIV after the introduction of these mutations. We transfected the plasmid into HEK-293T cells, and after two days, we analyzed the P24 protein in the supernatant and the cell lysates to find out whether there was HIV

produced (Fig. 4).

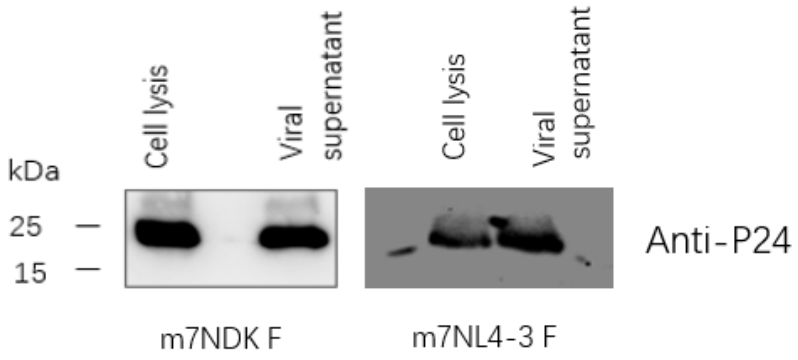

Figure 4. Expression of m7NDK and m7NL4-3 replication-competent virus.
Immunoblotting of HEK-293T cells transfected with the expression plasmid for m7NDK (left side) or m7NL4-3 (right side) replication competent virus. Viral P24 protein (capsid protein) was expressed in both cell lysates and supernatant of the transfected cells. P24 was detected by anti-P24 antibody. “F” is for replication-competent virus.

From the above results, we can see that the viral plasmids (m7NDK and m7NL4-3) can produce the P24 protein after the env mutations, indicating that the plasmid can make the virus. Therefore, we can use the new viral plasmid to conduct infection experiments.

4.1.2 Preparation of cells for experiments

Construction of lentiviral CRISPR/Cas9 expression vectors with single guide RNA (sgRNA) for CD4 knockdown

To verify whether the HIV-1 with the m7 env mutations (both pseudotyped HIV vectors and replication-competent viruses) can exhibit the ability to enter cells independently of human CD4, we need to establish cell lines that express only human CXCR4 but not human CD4. The CRISPR-Cas9 knockout method was used to knock out CD4 in THP-1 cells, a human leukaemia monocytic cell line that expresses CD4 and CXCR4 on its surface. This method is based on lentiviral delivery of a genome-scale CRISPR-Cas9 knockout (GeCKO) library [114].

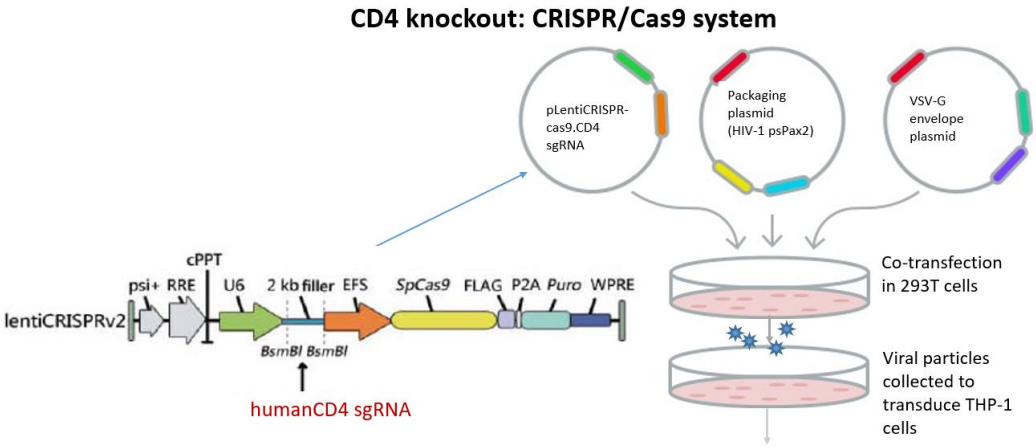

Figure 5. Schematic representation of the method for knocking down the cell surface CD4 receptor using the CRISPR/cas9 system. Cas9 and sgRNA is expressed together on a lentiviral vector. HIV-based pseudotyped viral vector particles were used to transduce THP-1 cells.

THP-1 cell transduction for CD4 knockdown

For CD4 knockout, THP-1 cells were transduced with plentiCRISPRv2-based viral particles, produced by the transfection of HEK-293T cells (Fig. 5). Cells were selected against puromycin. Untransduced wild-type cells were also included in the selection as controls. Selected cells were checked for CD4 depletion using FACS with the monoclonal mouse anti-human CD4/FITC antibody, in comparison with untransduced cells (Fig. 6). As the FACS results showed a CD4 knockdown population of cells, single cells were selected in 96-well plates using FACS and were allowed to grow into single clones. When the wells were full, the cells were transferred to the wells of a 24- and then a 6-well plate. Then we extracted 10,000 cells for FACS, CD4 knockout was confirmed and selected for subsequent infection experiments.

In the first step, after knocking out the CD4 receptor in the THP-1 cells using CRISPR/Cas9 technology, we needed to test expression of CXCR4. We used CD4 or CXCR4 antibodies (anti-CD4 or anti-X4) to bind CD4 or CXCR4 on the surface of THP-1 cells as an experimental group. Isotype control antibody-treated cells were used as a negative control group. We used the FACS approach to detect receptors (Fig. 6).

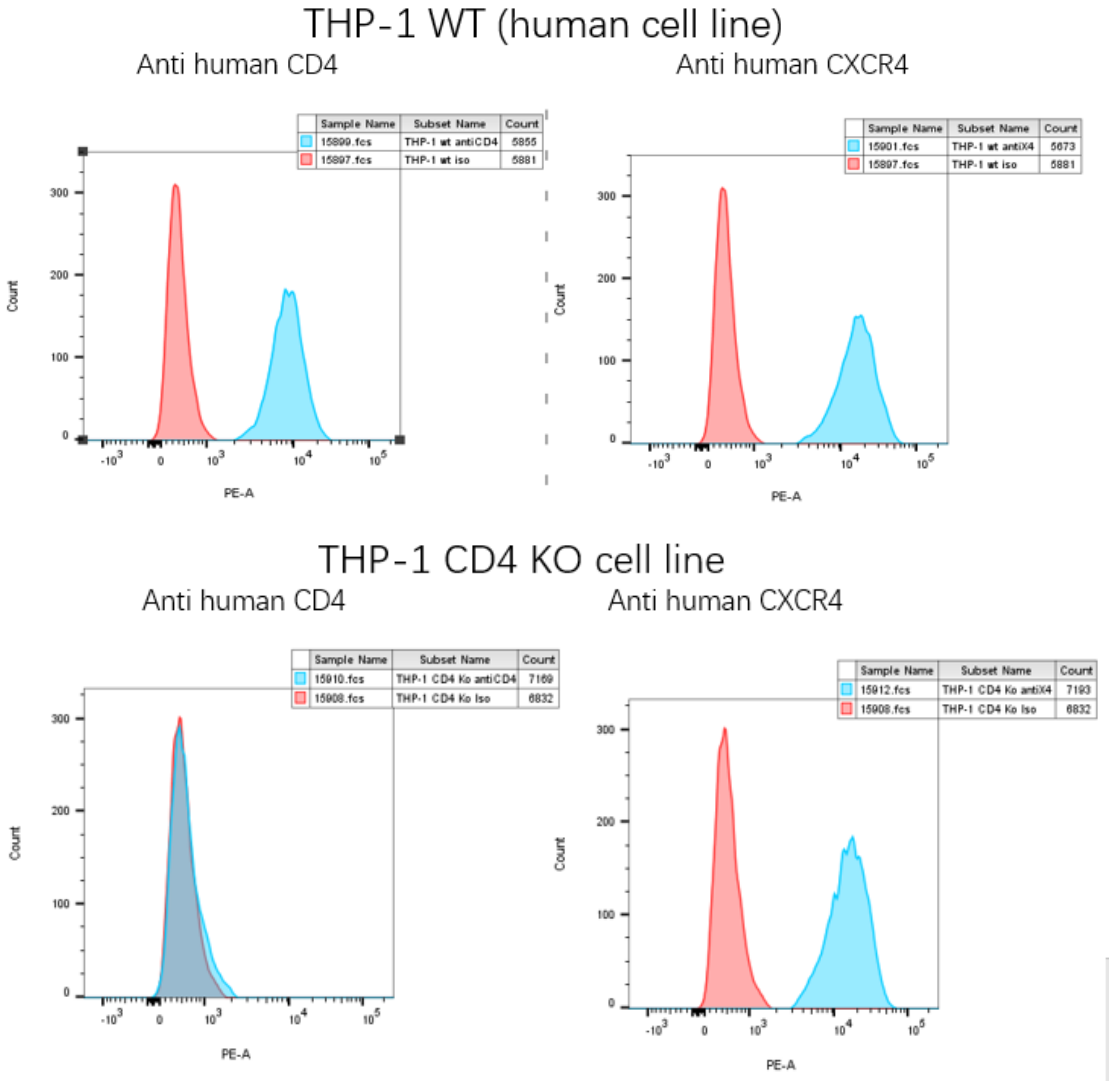

Figure 6. Flow cytometry analysis of CD4 knockout in human THP-1 cells.
We used the CRISPR/Cas9 approach to knock out the CD4 gene in the THP-1 cell line. The THP-1 cell line (both wild-type and CD4 KO cell line), were incubated with an antibody against human CD4 or human CXCR4 as well as isotype control as a negative control. In the above illustration, all red elements represent the isotype control, the negative control group. The blue elements represent the corresponding antibody signal for anti-CD4 or anti-CXCR4. The CD4 knockdown was successful according to flow cytometry. The CXCR4 receptor was not affected, and THP-1 CD4 KO cells still expressed CXCR4.

Then, we also need to prepare HOS cells (human cell line) expressing the CXCR4 receptor (called here HOS+X4) and CRFK cells (cat cell line) expressing CXCR4 (called here CRFK+X4). Cells were generated using retroviral vectors expressing CXCR4. In addition, HOS cells or CRFK cells that can express both CD4 and CXCR4 were required. Such cells we took out from the stock. Expression of CXCR4 and CD4

was confirmed by specific antibodies and FACS (Fig. 7 and 8).

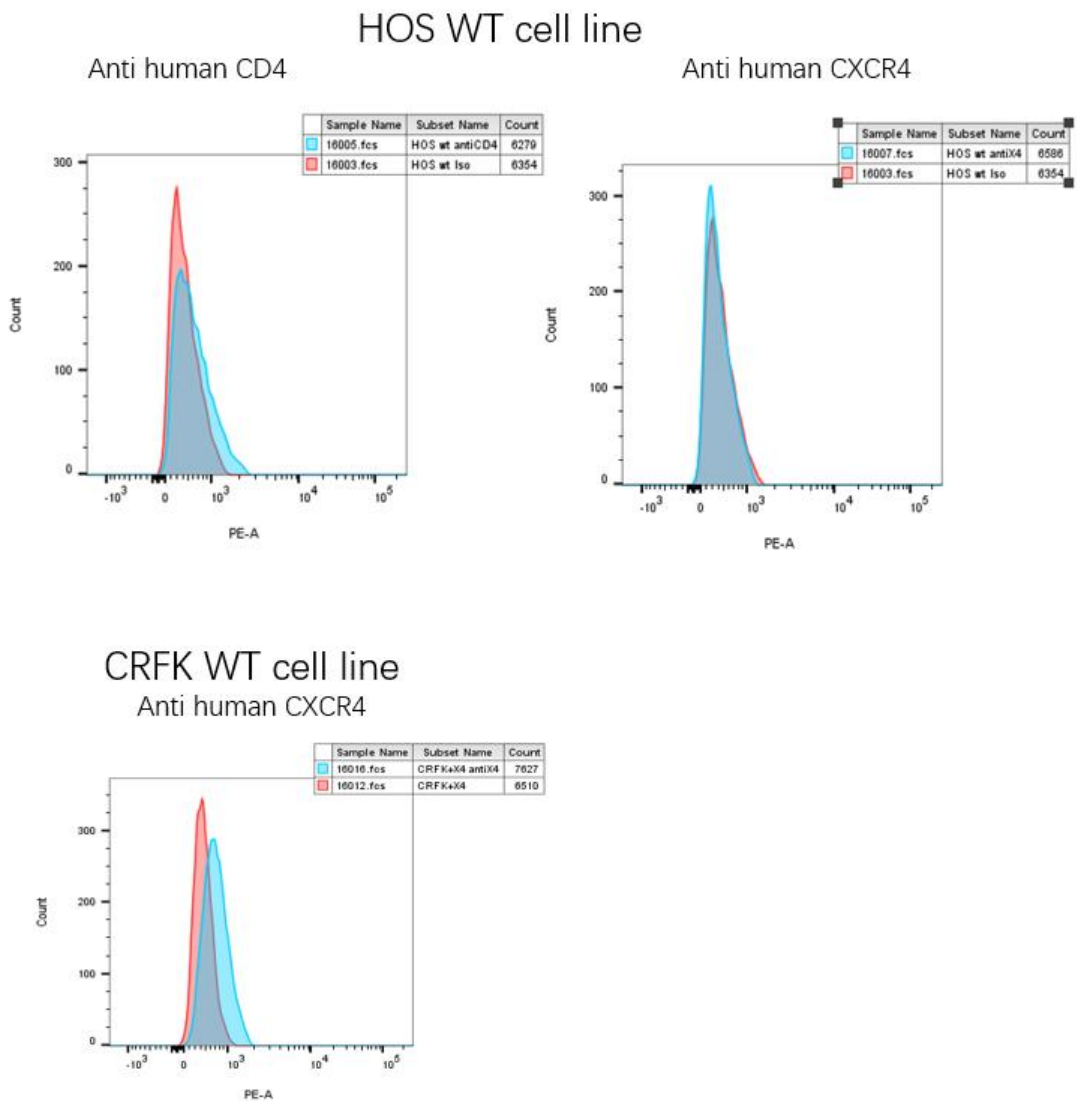

Figure 7. Flow cytometry analysis of HOS and CRFK WT cell line.
Red elements represent negative controls and blue elements represent anti-CD4 or anti-CXCR4. The HOS WT cell membrane surface does not express human CD4 or human CXCR4. Also, CRFK cell express low levels of CXCR4 receptors.

After determining that neither wild-type HOS cells nor wild-type CRFK cells express significant amounts of the CXCR4 receptor, we set out to modify them to express CXCR4 (Fig. 7 and 8).

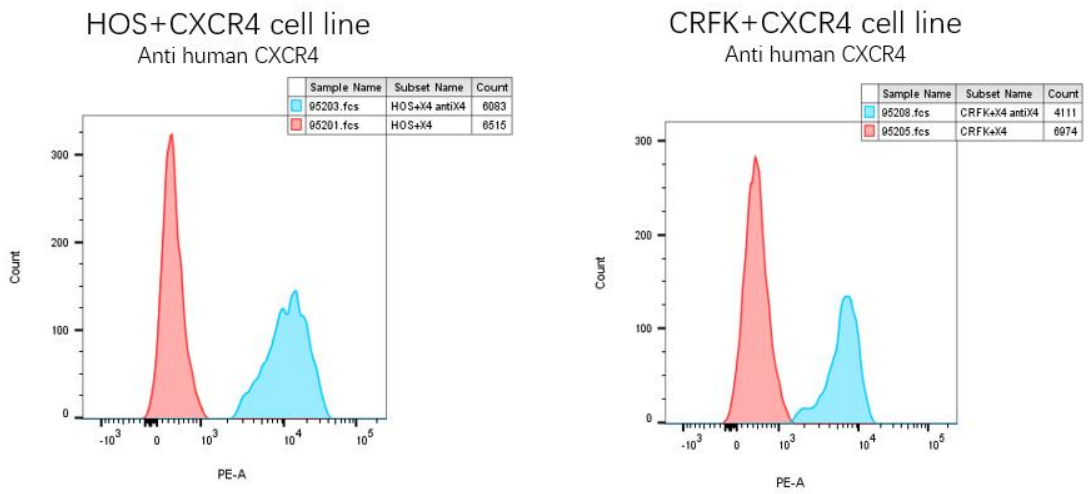

Figure 8. Flow cytometry analysis of the CXCR4 receptor on HOS or CRFK cell line.
Red elements represent negative controls and blue elements represent anti-CXCR4. New HOS cells (HOS+X4) and CRFK cells (CRFK+X4) could express CXCR4 receptor now.

4.2 Infection results

In the experiments of others, cell infections of pseudoviruses with the envelope of m7NDK was described [115]. Non-dependence on CD4 was observed. It was also noted that they could infect cells using human CD4 as a typical receptor-like HIV-1 virus, and that the infection efficiency decreased when utilising CXCR4 alone. We wanted to confirm these findings in our cell systems and test the newly generated replication-competent viruses.

For the cell infection experiments we prepared first pseudoviral vector particles encapsidating a luciferase transfer vector. Luciferase activity in the transduced target cells above the background shows infection (Section 3.4.4). We start infection by trying an HIV-1 pseudovirus containing the m7NDK envelope (Fig. 9).

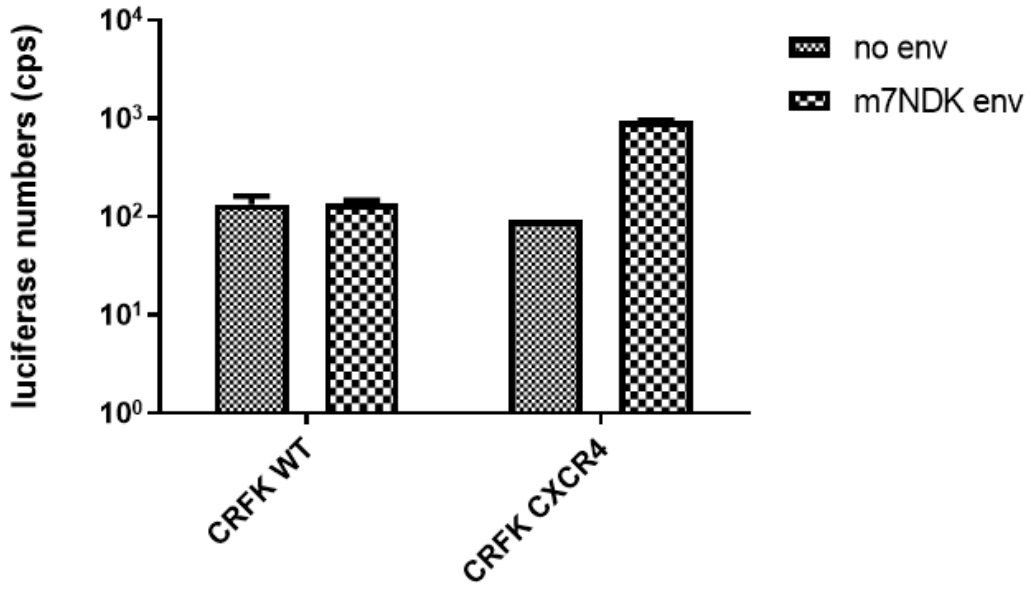

Figure 9. Test of HIV luciferase reporter vectors pseudotyped with m7NDK envelope. The reporter vector without any envelope protein was used as a negative control group. When infected with CRFK cells wild-type, the m7NDK pseudovirus showed little difference from negative controls expressing no envelope. However, when infected with CRFK cells expressing only the CXCR4 receptor, the luciferase activity measured in the cells was nearly 10-fold higher compared to negative controls.

The analysis of the luciferase activity showed that m7NDK pseudotyped vectors were not able to transduce CRFK wild-type cells (Fig. 9). The measured luciferase activities showed similar values than the empty vector (no envelope). However, when CXCR4 was present, the measured luciferase activities showed 10-fold higher values than the empty vector. This suggests that the m7NDK pseudovirus infects CRFK cells that express only the CXCR4 receptor.

Next, we wanted to test if the replication-competent viruses of HIV with the m7 env mutations. We use different HIV viruses to infect first HOS cells (Fig. 10). The viruses were: an “empty” vector that did not express any envelope, which served as a negative control; NL4-3 replicate-competent virus (NL4-3 F: “F” for replication-competent virus), a regular HIV-1 that requires both CD4 and CXCR4; m7NL4-3 F, an NL4-3 that has been edited to contains the m7 env mutations; m7NDK F, m7NDK replicate-competent virus (three different virus preparations with and without concentration were tested) (Fig. 10).

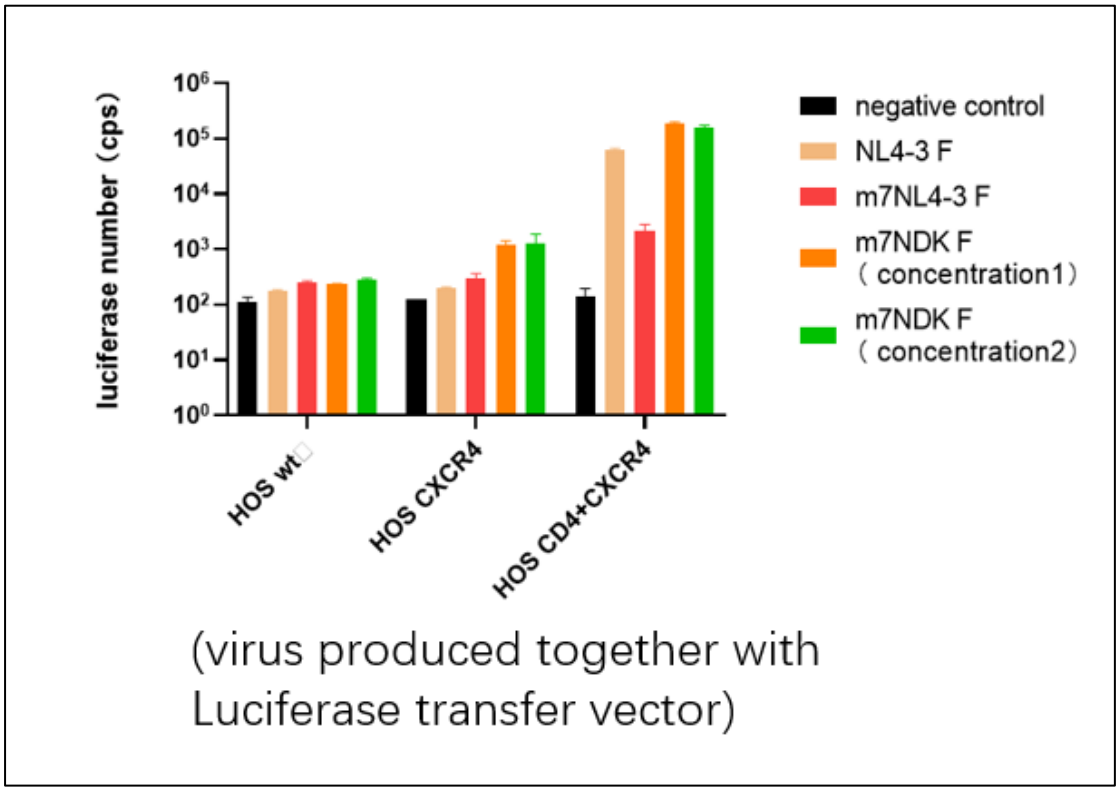

Figure 10. Luciferase results for HIV-1 infection analysis.

We used different full-length HIV-1 viruses to infect different HOS cell lines. The negative control group were uninfected cells. When HOS wild-type cells were infected with selected HIV viruses, the luciferase values measured in all groups were comparable to those of the negative control group. We then used virally infected HOS cell lines expressing only the CXCR4 receptor, and the luciferase values measured in the m7NDK F group rose more than 10-fold compared to the empty vector group. However, the NL4-3 and m7NL4-3 groups were almost comparable to the empty vector group. Next, upon infection of HOS cells expressing both human CD4 receptor and human CXC4 receptor, there was a more than 1,000-fold increase in luciferase values measured in both the NL4-3 F group and the m7NDK F group compared to the empty vector group, as well as a more than 10-fold increase in the m7NL4-3 group. We also compared the m7NDK F group infected with HOS CXCR4 with the m7NDK F group infected with HOS CD4+CXCR4. In the presence of CD4 receptors, luciferase values were elevated 100-fold.

After analysing the luciferase activity, we conclude that no HIV virus tested could infect the wild-type HOS cells, because this cell line does not express CD4 or CXCR4. When infecting HOS cells that expressed only the CXCR4 receptor, HIV-1 NL4-3 F was unable to enter, because NL4-3 requires the help of CD4 for infection. Thus, there was a significant increase in luciferase number when infecting HOS cells expressing both CD4 (T4) and CXCR4 (X4) receptors.

In contrast, our target virus, m7NDK replication-competent virus, had a >10-fold increase in its luciferase count in HOS cells expressing only CXCR4 compared to the negative control. This may indicate that m7NDK virus enters HOS cells by utilising the CXCR4 (X4) receptor alone. The luciferase number further increased in infections of HOS cells expressing CD4+CXCR4, indicating that m7NDK virus also typically utilised the CD4 receptor, further increasing their infectious ability. There was no significant difference in the amount of luciferase between the two independent concentrated viruses (Fig. 10).

When the HOS cells expressing only CXCR4 were infected with a m7NL4-3 virus, the luciferase activity values were almost comparable to those of negative controls, suggesting that this virus cannot infect HOS cells expressing only the CXCR4 receptor; Therefore, these seven variants on m7NDK are critical in helping it produce CD4-independent capacity. However, these seven mutations do not help all HIV-1 viruses to produce a CD4-independent capacity. Then we also used m7NDK replication-competent virus to infect other cells as well.

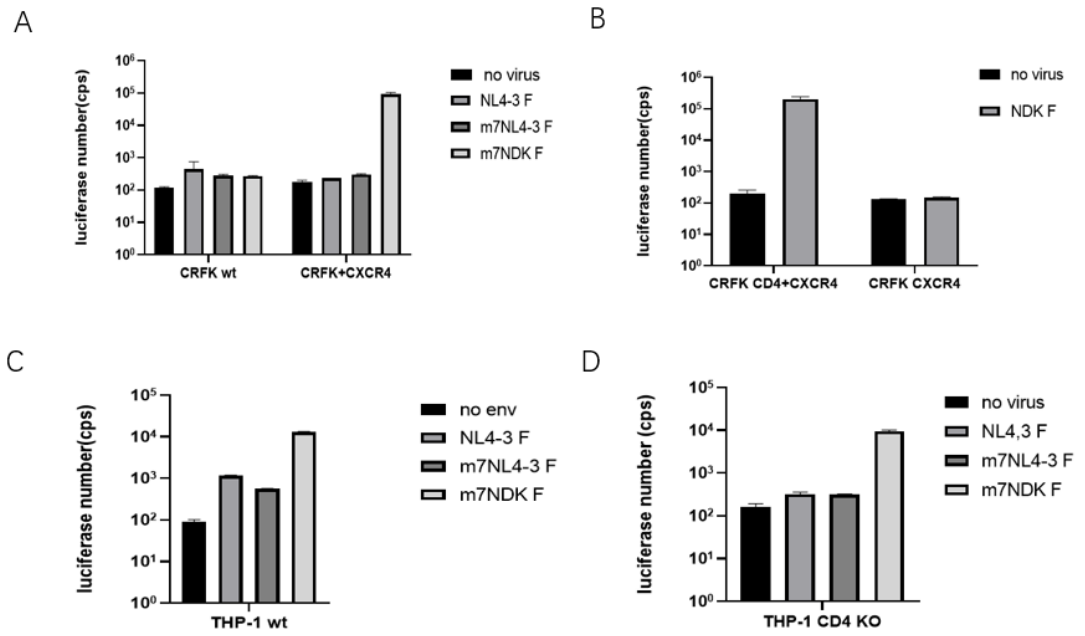

Figure 11. Luciferase results for HIV-1 infection analysis.

(A) When infecting CRFK+CXCR4 cells, only the luciferase activity of the m7NDK F group is 1000 times that of the control group. Others have no significant difference from the control group. (B) After we learned that the HIV-1 m7NDK virus could infect CRFK cells expressing only CXCR4, we also tested the original HIV-1 NDK virus. The results showed that the virus did not have this property and could only infect CRFK cells expressing CD4 and CXCR4. (C) In contrast to the control group, luciferase values rose more than 10-fold in the NL4-3 F group and more than 100-fold in the m7NDK F group. It was suggested that both viruses can enter THP-1 cells. (D) When infected with THP-1 CD4 KO cells, the luciferase values measured in the m7NDK F group remained more than 100-fold higher than in the control group, indicating that this group was infected. Whereas the other two groups had values that were only about twice as high compared to the negative control group.

The results shown in Fig. 9 and 10 demonstrate that the HIV-1 m7NDK is a virus that can infect cells using classical pathways, like typical HIV-1. It can also infect cells without CD4 and in the presence of CXCR4 alone, but the original NDK cannot (Fig 11). So, to further determine whether HIV-1 m7NDK utilises the CXCR4 receptor for its CD4-independent ability, we blocked the cell surface receptor with inhibitors (AMD3100 and fostemsavir) (Fig. 12). AMD3100 is used to interfere infection with the CXCR4 receptor on the cell surface, while fostemsavir is a CD4 receptor inhibitor.

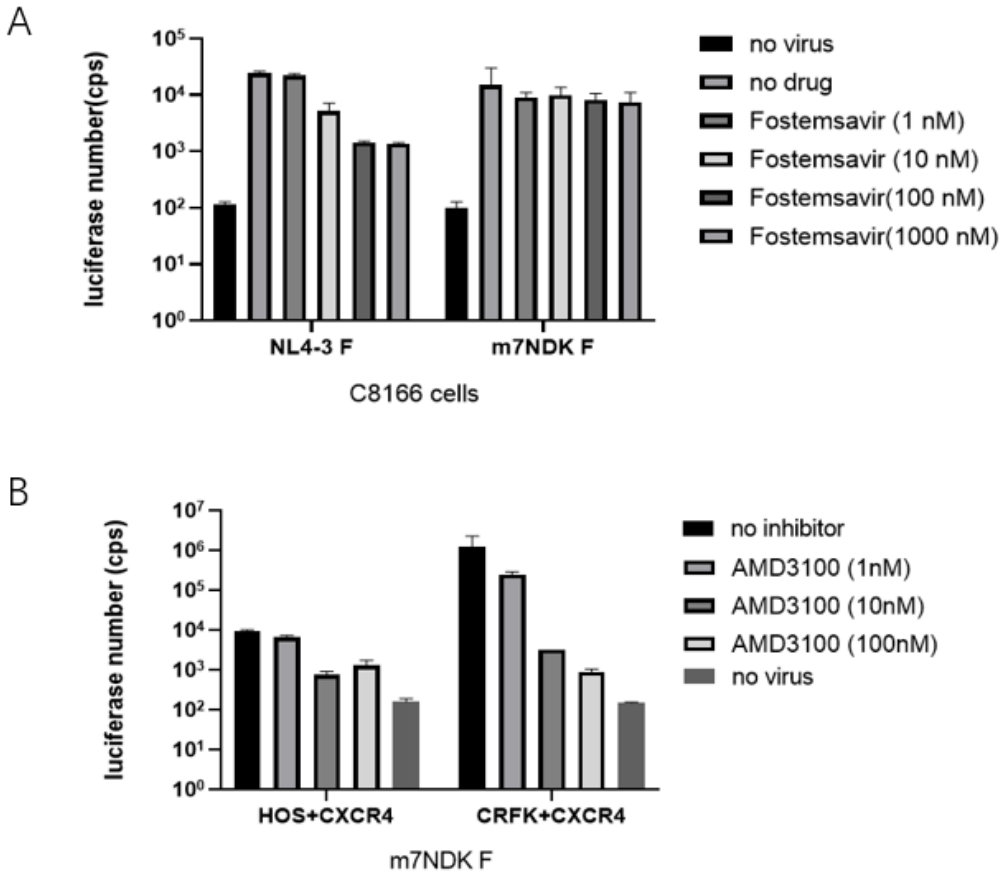

Figure 12 Luciferase results for HIV-1 infection analysis after using inhibitors.

C8166 is a human T-cell leukaemia cell line expressing human CD4 and CXCR4. AMD3100 is a CXCR4 inhibitor. Fostemsavir was used to block the CD4 receptor. We treated cells with either drug for ≥12 h before infecting them with the virus. (A) The ability of NL4-3 F to infect C8166 cells is affected by increased, concentrations of the CD4 inhibitors, whereas the infection of m7NDK F is not affected by the CD4 inhibitor. (B) When only CXCR4 receptors are expressed on the cell surface, the infectious ability of m7NDK F is impaired by increased concentrations of the CXCR4 inhibitor.

After we blocked the CD4 receptor on C8166 cells using different concentrations of the CD4 inhibitor, we found that fostemsavir did not significantly change the ability of m7NDK F to infect these cells. However, infection by the wild-type HIV-1 NL4-3 F virus was reduced considerably. In contrast, after treatment of cells expressing only CXCR4 with different concentration gradients of the CXCR4 inhibitor, AMD3100, the infectivity of HIV-1 m7NDK F on these cells was significantly reduced. These findings are in agreement with the model that m7NDK can infect cells CD4-independent but CXCR4 dependent.

From the above experimental results, we can say that the HIV m7NDK replication-competent virus is a new virus obtained from HIV-1 NDK by seven specific mutations in the envelope gene. It can not only infect cells expressing both human CD4 and human CXCR4 like normal HIV-1 viruses, but it can also enter cells using the CXCR4 receptor alone. At the same time, this infectious ability is affected by inhibitors of the CXCR4 receptor.

4.3 Establishment of new HIV-1 chimeras resistant to feline APOBEC3

It is clear that at least two crucial reasons prevent domestic cats from being an animal model of HIV-1. The first obstacle is the question of how HIV-1 enters cat cells, and we have shown through previous findings in this thesis that HIV-1 m7NDK can be used to bypass this restriction. The next hurdle was to address the inhibition of HIV-1 by APOBEC3 proteins in cat cells. We already know that the weapon the FIV virus uses to fight the feline APOBEC3 protein is the *Vif* gene. To protect HIV-1 in cat cells against antiviral APOBEC3 proteins we thought to integrate the *Vif* gene of FIV into HIV-1 m7NDK to form a new chimeric “FHIV-1”. Previously, it has been demonstrated that the *Vif* gene of FIV clone 34TF10 can achieve protection against feline APOBEC3 if integrated into HIV-1 NL-Bal (using CCR5 as a co-receptor) and can replicate in cat cells [116]. However, this chimeric virus was unable to infect unmodified cat cells because of its dependency for human CD4 and human CCR5. Therefore, the next step in this experiment was to establish a suitable chimeric m7NDK F, which could be used to tackle feline APOBEC3.

4.3.1 Generation of chimeric m7NDK F with *Vif* of FIV

The aim was to use HIV-1 m7NDK full-length virus as the backbone structure to integrate FIV *Vif* into it and finally form a new chimera to realise the function of FIV *Vif*.

However, which FIV *Vif* to choose, where to start replacing HIV *Vif*, how much of the HIV *Vif* gene should be retained, and whether the start codon of FIV *Vif* should be retained are all issues we need to consider. After discussion and some preliminary experiments, the following three approaches are the most likely scenarios to achieve this purpose. As options for *Vif* there were from two molecular FIV clones FIV34TF10*Vif* and FIVc36*Vif* available. The chimeric m7NDK virus structures are shown in the figure 13. The method used to clone these constructs was overlap PCR and the oligos used have been described in subsection 2.5.2.

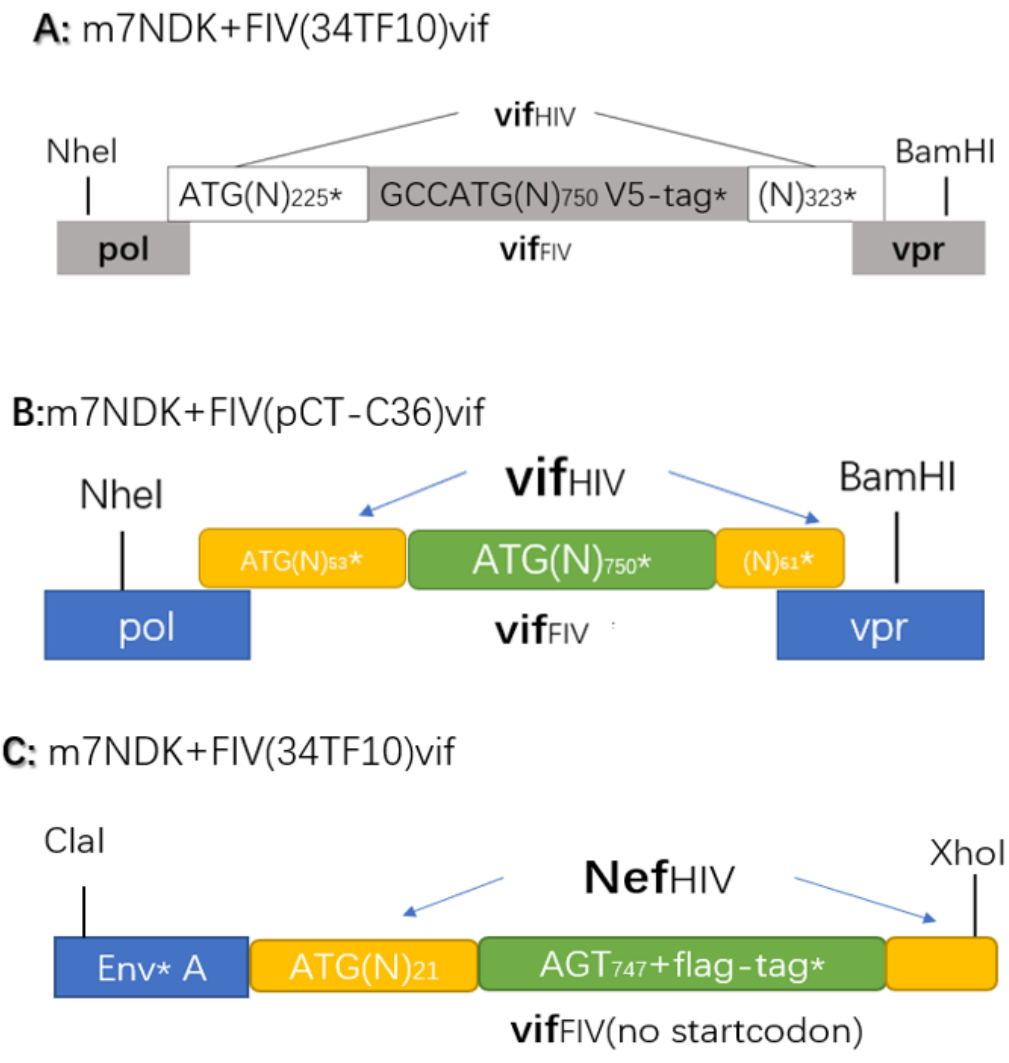

Figure 13. Chimeric FHIV-1 structural schematic.

The '*' sign in the figure stands for the stop codon. "N" represents adenine (A), cytosine (C), guanine (G), or thymidine (T). (N) number represents the number of Ns., and the restriction endonucleases used in the construction process are shown on both sides.

Schematic diagrams of the three chimeric structures to be selected, where ‘A’ and ‘C’ are chosen from FIV34TF10*Vif*, and ‘B’ is selected from FIVC36*Vif* (Fig. 13). The structure of ‘A’ is the same as that described in the paper of NL-Bal.*vif*FIV [116]. FIVpC36 is a highly pathogenic FIV that has been previously described [117]. In ‘A’, ‘B’, and ‘C’, we wanted the newly constructed chimeric viruses to express the new FIV *Vif*. The difference is that, because the HIV *Vif* function is not required, we eliminated the HIV *Vif* function by embedding FIV *Vif* in the middle of the HIV *Vif* gene in ‘A’ and ‘B’. The FIV *Vif* gene will be translated after a deleted section of the HIV *Vif* gene. Instead, the FIV *Vif* gene would be translated after a segment of the mutated HIV *Vif* gene. Hopefully, this will express the function that enables the chimeric virus to express the full FIV *Vif* for ‘C’, on the other hand, it was designed to determine whether the FIV *Vif* embedded in the *Nef* gene could also be translated afterward. As the published paper suggested [116], chimera A seems most likely to exert the effect. Therefore, we modified it in several ways (A1–A3) in addition (Fig. 14). ‘A1-A3’ are as variants of ‘chimera A’. These variants differ from chimera A in that A1 deletes the termination codon in the first part of the m7NDKVif that is retained so that this part of the HIV Vif can be co-translated with FIV Vif; A2 reduces the length of the retained HIV Vif; and A3 deletes the V5-tag to exclude the effect of the V5-tag on FIV Vif.

4.3.2 Degradation of feline APOBEC3

In order to verify whether the several chimeric HIV-1s we established have a degrading effect on feline APOBEC3, we co-transfected various plasmids for chimeric HIV-1 (400 ng) with feline APOBEC3Z3-HA (1600 ng). The results were examined 2 days later by western blotting.

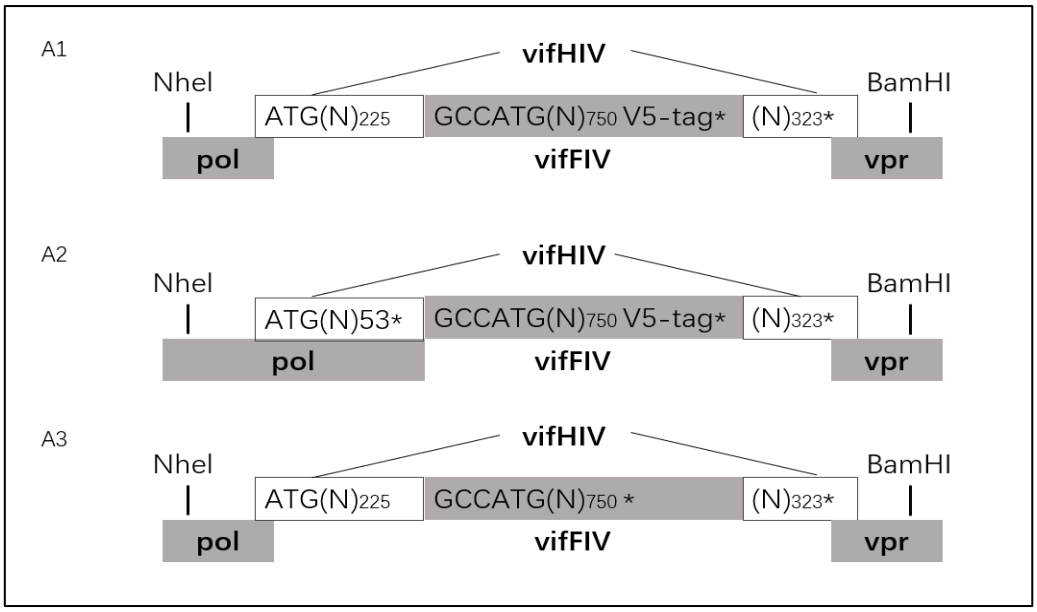

Figure 14. Schematic representation of three variants of chimeric m7NDK+FIV34TF10*Vif*. Variant of 'chimera A', A1-A3. The symbol '*' represents a stop codon. "N" represents adenine (A), cytosine (C), guanine (G), or thymidine (T). (N) number represents the number of Ns., and the restriction endonucleases used in the construction process (NheI and BamHI) are shown on both sides.

After preparing all chimeric HIVs, we used the Western blot method to test their ability to degrade feline APOBEC3 (Fig. 15).

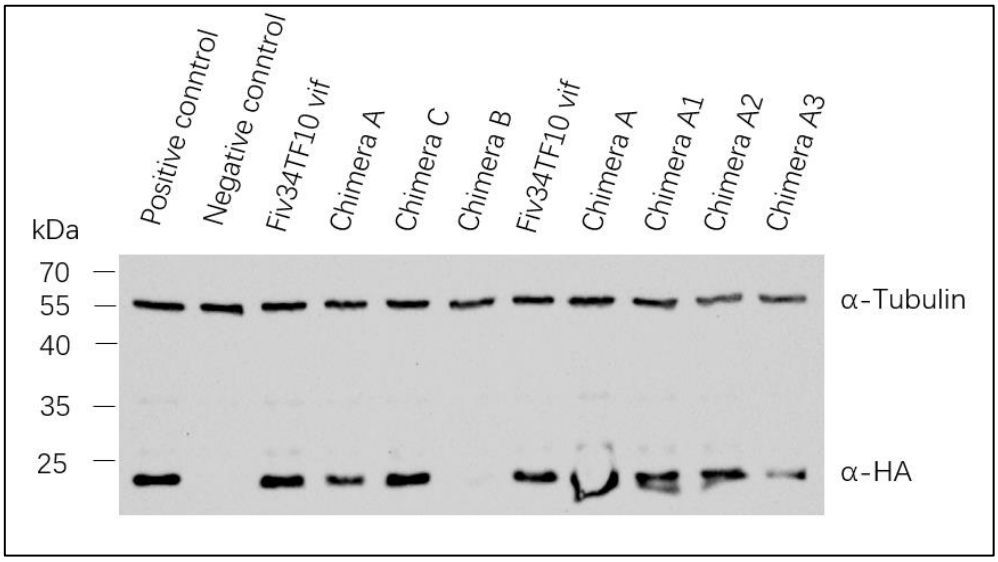

Figure 15. The degradation result for feline A3Z3-HA.

Protein lysates from HEK-293T cells. The lysis products were immunoblotted with the indicated antibodies. The empty vector (pcDNA3.1) was co-transfected with feline A3Z3-HA (feline APOBEC3Z3 with an HA tag) as a positive control, and FIV *Vif*, which is known to be helpful, was co-transfected with feline A3Z3-HA as a negative control. The test groups were cell lysis products after two days of co-transfection with different chimeras and feline APOBEC3Z3-HA. For unknown reason was no degradation of the A3Z3 observed when the FIV Vif expression plasmid was tested.

Based on the above results, we found that none of the plasmids containing FIV34TF10*Vif*, which we thought would be most likely to be effective in the early stages, successfully degraded feline A3Z3-HA, including chimera A and its variants, as well as chimera C. Only construct A3 induced a partial depletion of the A3Z3 (Fig. 15). Fortunately, the plasmid containing FIVpCT-C36*Vif*, chimera B, was successful in degrading feline A3Z3-HA.

Next, we again co-transfected chimera B with other plasmids in a certain ratio into HEK-293T cells. 2 days later, we immunoblotted the cell lysis products of the 293T cells using the indicated antibodies. The P24 antibody was used to detect the expression of the P24 protein in the HIV, and the HA antibody was used to detect the expression of the co-transfected APOBEC3s, as all of the used APOBEC3s contained the HA tag. α-Tubulin was used to detect intracellular Tubulin protein. The results obtained are shown in Fig. 16.

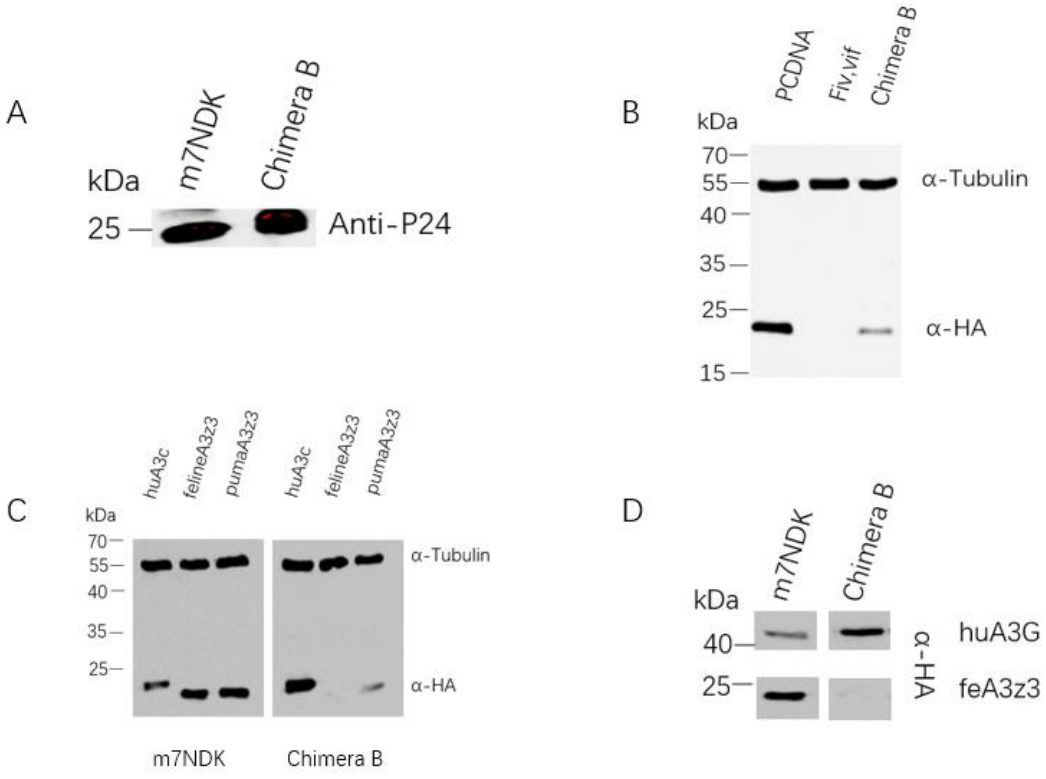

Figure 16. Protein immunoblotting results of m7NDK chimera B.

Protein lysis from HEK-293T cells. The "hu" in (C) and (D) means human. (A) Both HIV m7NDK and chimera B can produce P24 proteins. (B) The empty vector, FIV,*vif*, and chimera B were co-transfected with feline APOBEC3Z3-HA into HEK-293T cells, respectively, and HA protein was detected two days later. The results showed that the HA-tag protein was down-regulated; (C) Co-transfection of m7NDK or chimera B with the corresponding APOBEC3s-HA. Chimera B degraded feline A3Z3 and puma A3Z3 in cells but not human A3C; however, HIV m7NDK could degraded human A3C but unable to degrade feline or puma A3Z3; (D) Chimera B degraded feline A3Z3, but not human A3G; HIV-1 m7NDK degraded human A3G, but not feline A3Z3.

The results of immunoblotting revealed that chimera B formed after insertion of FIV pC36 *Vif* into m7NDK could produce P24 protein. And FIV p36 *Vif* exerted its function in this chimera because chimera B could down-regulate feline APOBEC3Z3. However, it could not down-regulate human APOBEC3s because it destroyed HIV,*vif*. However, the original HIV m7NDK virus could down-regulate human APOBEC3s proteins.

5. Discussion

HIV/AIDS currently does not have a perfectly matched small animal model. However, an animal model would help for drug and vaccine development against HIV/AIDS and several unknown in vivo question, e.g. such as cellular reservoirs. The domestic cat is a likely candidate; however, because of the species-dependent nature of the HIV-1 virus, we need to figure out a way to free HIV-1 from its limitations. In this study, we synthesised a novel chimeric HIV-1 virus. This virus can infect cat cells and antagonise the feline APOBEC3 proteins. It brings the domestic cat one step closer to becoming a new model of HIV-1 infection.

5.1 Establishment of a CD4-independent HIV-1 replication-competent virus

We have already learned that the first limitation of the HIV-1 virus in entering cat cells is the limitation of cell surface receptors, and in general, the HIV-1 virus requires human CD4 and a co-receptor (human CCR5 or CXCR4). However, some time ago, a team of researchers discovered/created HIV envelopes in the laboratory that did not rely on human CD4 receptors. They described a population of CD4+ CEM cells chronically infected using HIV-1 NDK and long-term infection, and identified a class of virus that could be stably infected with CD4 negative cells and could replicate within human CD4 negative cells. At the same time, they described the exact location of this mutant virus and named it m7NDK [118]. In addition, other HIV viruses that are not CD4-dependent have been discovered [119], [120]. They can enter cells using only a co-receptor, some using CCR5, some using CXCR4, and not only in HIV-1 but also in HIV-2 envelopes. However, no virus has been reported in nature that can stabilise the complete replication of the HIV-1 CD4-independent virus that causes epidemics. We hypothesize that this may have something to do with natural selection. HIV-1 typically relies on two receptors for cell entry. Gp120, present on HIV-1 virions, initially binds to CD4 on the cell surface, prompting structural changes that reveal a binding site for a

7-transmembrane co-receptor [118]. Whereas under natural conditions, when HIV infects animal cells, the virus can utilize CD4 as well as co-receptors without being forced to evolve the ability to use only co-receptors. There is also a possibility that after CD4 binding the env goes through conformational changes and opens a surface that can bind the coreceptor. This may help the virus to escape neutralizing antibodies that target the coreceptor binding site. And, based on our current experimental results, the efficiency decreases when infecting cells utilizing only the co-receptors.

The reason we picked m7NDK among the many different CD4-independent HIV envelope is simple. Firstly, both HIV-1 and HIV-2 have viruses that can enter cells independently of CD4. However, we could directly exclude HIV-2 envelopes because there is a gap in the genetic structure between HIV-2 and HIV-1, and it would be simpler to use HIV-1 viruses. Virus types that use CCR5 as a receptor can then be excluded. This is because feline CCR5 is very different from human CCR5. The only remaining suitable type is the HIV-1 envelope using the CXCR4 type. The cat CXCR4 receptor is >94% genetically identical to the human CXCR4 receptor and can be utilised by HIV-1 in a very suitable manner [121].

Following the results of the Dumonceaux study [58], seven site-specific variants were introduced into the HIV-1 NDK replication competent virus to create a replication competent HIV-1 m7NDK virus. Therefore, the infection experiments could be continued to the next step. We used HIV-1 m7NDK to infect the cells that only expressed human CXCR4: THP-1 (human cell line), HOS (human cell line), and CRFK cells (cat cell line). As a result, all of these cells could be infected, but not if the regular HIV-1 NL4-3 virus was used. As previously described, the Envelope of regular HIV binds to the CD4 receptor and produces deformation before it can further bind to the co-receptor and thus enter the cell. m7NDK can bind to the co-receptor CXCR4 alone and therefore infect the cell, demonstrating that its envelope does not need to be deformed to have some affinity for the CXCR4 receptor. NL4-3, on the other hand, as

a CD4-requiring virus, does not exactly resemble the 3D structure of m7NDK, which may be the reason why it still does not produce the ability to directly utilize the CXCR4 receptor even after acquiring these seven specific mutations.

After treating the cell line with a CXCR4 inhibitor (AMD3100), we found that the inhibitor blocked the m7NDK infection. When we used a human CD4 inhibitor (fostemsavir) to treat cells that expressed both CD4 and CXCR4, the entry of m7NDK was not stopped. This indicates that the full-length m7NDK virus can indeed enter cells by using the CXCR4 receptor alone and that it is successful in both human and cat cell lines.

However, we also found some things that could have been improved in our experiments. m7NDK is CD4-independent. But, it can utilise CD4 just like other common HIV-1 viruses, and the infection efficiency of m7NDK is significantly higher when the cells express both human CD4 and human CXCR4 than when the cells express human CXCR4 alone. So, can the efficiency of HIV-1 infection of cells expressing feline CXCR4 be increased? Of course, first, we need to understand which one expresses CXCR4 receptor at a higher level in the natural state, feline cells or human cells, which has an impact on the efficiency of m7NDK infection. Indeed, direct transduction of the human CD4 receptor into cat cells to turn cat cells into cells that express CD4 and the CXCR4 receptor is a predictable and quickly successful approach. However, whether such an approach suits all cat cells is still being determined. One other thing is worth noting. As we've mentioned before, HIV researchers around the world have observed more than one type of non-CD4-dependent HIV, including HIV-1 and HIV-2, and there is also a preference for the CXCR4 receptor or a preference for the CCR5 receptor. Perhaps we can try other CXCR4-preferring CD4-independent HIV viruses to achieve satisfactory infection efficiency. There is also another method that is worth trying in the following experiments. Since FIV can utilize feline CD134, is it possible to form a chimera of a portion of FIV's envelope with HIV's envelope so that the new virus can utilize both feline CD134 and

CXCR4? In addition, the replication competent FHIV can be used for adaptation experiment and virus isolates that replicate and spread faster may be selectable which either bind better to CXCR4 or evolve the use of a new main receptor.

In conclusion, the replication-competent HIV-1 m7NDK virus has the ability to infect cat cells. In future experiments, we need to focus on improving its infection efficiency. For example, isn't it possible to modify the feline CD4 on the surface of cat cells to make m7NDK bind to it? This is because there have been attempts in our lab by experimentalists to modify feline CD4, allowing the modified receptor to gain the ability to bind weakly to the HIV virus [120]. A knock-on of the “missing” HIV env binding domain into one allele of the feline CD4 may have advantages of natural expression levels in compared to CD4 transgenic cats with a modified promoter.

5.2 A New chimeric HIV-1 virus degrades cat APOBEC3

The feline APOBEC3 protein is crucial for blocking HIV-1 in cat cells. We now know that m7NDK can infect cat cells. However, as an HIV-1 virus, there is no way for m7NDK to fight the A3 protein inside cat cells as FIV does. As the FIV virus can use its own Vif protein to counteract the antiviral effects of A3 inside cat cells, can we also replace the *Vif* gene of HIV-1 with FIV *Vif* to generate new viruses that can counteract feline A3? Other researchers have already used this approach, and Zielonka et al. developed a new virus by replacing some of the CCR5-tropic HIV NL-Bal *Vif* gene with FIV34TF10*Vif* [116]. The virus antagonised the feline A3 protein and replicated in cat cells. Using this experiment as inspiration, we considered whether m7NDK could be remolded to achieve such a function.

We selected the *Vif* of FIVc36 (a highly pathogenic FIV virus) as the target gene to replace a segment of the *Vif* of HIV-1 m7NDK replication-competent virus. The newly established virus was called Fc36*Vif*-m7NDK. The new chimeric virus plasmid and feline APOBEC3Z3 were then co-transfected into HEK-293T cells. The expression of feline A3 was detected after 2 days, and it was found that the expression of feline A3

was suppressed compared with the positive control. This experiment demonstrated that the FIVc36 *Vif* gene exerted its biological efficacy in the new chimeric virus.

However, during our experiments, we found that it took work to establish chimeric viruses that met the requirements. First, we tried to use the FIV34TF10*Vif* mentioned in the literature to replace the HIV-1 m7NDK*Vif*, and we found that the newly established chimeric viruses could not antagonise the expression of the feline A3 protein as expected. Neither adjusting the size of the gene retained by m7NDK*Vif* nor changing the position of the FIV *Vif* start codon achieved this. It is difficult to explain why this is the case. However, we can only speculate that the *Vif* gene, when integrated into different HIV-1 plasmids, either fails to translate the protein or, more likely, the translated protein domains are altered not to fulfil their original biological function. Thus, there is room for optimization of the expression of the FIV Vif in the chimeric FHIV viruses. Cell culture spreading infection experiments could be used to let the virus make the genetic adaptation it self.

5.3 Conceptualization of further possibilities for experimentation

The starting point of this experiment was to create a small animal model of HIV-1 infection. Inspiration came from the discovery of the CD4-independent HIV-1 envelope and an experiment by Zielonka et al. [116]. At the beginning of the experiment, we considered alternatives, such as replacing the HIV-1 envelope with FIV Env or using genetic engineering to modify feline hematopoietic stem cells and transplant them into cats so that the cats' immune cells could express human CD4. Still, we chose the current protocol because it is relatively simple and more operational. However, the results of the present protocol inevitably still have defects, such as low infection efficiency. If further optimisation is needed, we can go back to the original idea and combine multiple protocols to develop a perfect chimeric HIV and finally obtain a realistic and entirely usable animal model of HIV-1 infected cat cells.

6. References

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