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DOUTORAMENTO EM FARMÁCIA

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The Acquired Immune Deficiency Syndrome (AIDS) is the final stage of a chronic infection with the human immunodeficiency virus (HIV) [1,2]. The natural history of HIV infection is characterized by an insidious deterioration of the cellular immune system. The quantity and proportion of plasma $CD4^+$ T-cells decrease steadily over a period of years to decades, and this progressive loss of $CD4^+$ T-cells is associated with the development of AIDS in infected individuals [3-8]. There are currently 40 million people living with AIDS worldwide and it is estimated that 68 million will die of AIDS by 2020 [9]. In the absence of an effective vaccine against HIV, a worldwide search has been made in the past two decades to develop small-molecule inhibitors to target essential steps in the viral cycle [10].

Highly Active Antiretroviral therapy (HAART) is one of the most used treatment regimen in our days. The regimen employs a combination of therapeutic agents that target the viral reverse transcriptase (RT) and protease enzymes (PR) [10-12]. In the developed world, access to HAART has led to significant reductions in the morbidity and mortality attributed to HIV/AIDS. However, the emergence of drug-resistant virus isolates is causing an increasingly detrimental impact on treatment options and the disease outcome [13-15]. As a result, there is a urgent need to identify and develop new drugs that can be effective against these highly resistant virus isolates. Ideally, these new drugs should target steps in the HIV-1 replication cycle that are not blocked by the antiretroviral drugs currently in widespread use [10].

Integrase (IN), a third enzyme encoded in HIV-1, is currently the focus of an intense research effort to develop new anti-HIV-1 drugs [16-18]. This enzyme catalyses the integration of HIV genome into the chromosome of the host cell, arguably the most insidious step in the infection process [19]. Another HIV-1 protein that seems to be a potential target for HIV treatment is Vif [20,21]. This protein overcomes the innate antiviral activity of a cytidine deaminase APOBEC3G that induces G to A hypermutation in the viral genome, resulting in enhancement of viral replication infectivity [22,23].

In addition for developing new drugs that are effective against HIV-1, gene therapy has been highly regarded as a new form of molecular medicine in treatment of HIV/AIDS, either as an alternative or as a supplement to anti-retroviral chemotherapy [24-26]. Within this context, intracellular antibodies (intrabodies) represent a new class of neutralizing molecules with potential use in gene therapy approaches [27-29]. An intrabody consists of an antibody designed to be expressed intracellularly and directed to different subcellular compartments where they can exert their function more effectively [30]. The binding of an intrabody to an intracellular target protein has the potential to block, suppress, alter or even enhance the process mediated by that molecule [31-33]. The strong inhibiting potential of intracellular antibodies in the context of HIV gene therapy will be the focus of this dissertation thesis.

The thesis will be divided in 5 Chapters:

Chapter 1 will present a general introduction to HIV-1 virus biology and HIV/AIDS treatment. This part of the thesis will be used to describe in more detail the intracellular immunization (intrabodies) approach. In the end, HIV-1 IN protein will be presented as a potential target for HIV-1 inhibition. A brief description of Vif protein as a candidate for antiviral therapy will be also presented is this last section.

Chapter 2 will describe the selection and characterization of 5 rabbit anti-IN single-chain antibody fragments (scFv) that present high binding activity and specificity against HIV-1 IN protein. Results will be presented showing that scFv intrabodies can be efficiently designed to block integrase function and consequently inhibit early and late stages of HIV-1 replication.

Chapter 3 will present a study to show that rabbit VL single-domain can also be use as intrabodies. The VL single-domain analysed here was derived from an anti-Vif scFv that was recently developed from immunized rabbits to HIV-1 Vif protein.

Chapter 4 presents a study to generate lentiviral-derived particles with specificity of gene delivery for CCR5-expressing cells. To achieved this aim, we developed a novel Sindbis pseudotyped lentiviral vector where the Sindbis receptor binding envelope protein was modified to directly encode a scFv against the CCR5 chemokine receptor.

Chapter 5 will present a general conclusion.

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Abbreviations

Reagents

ABTS	2,2'-Azino-Bis (3-eThylenzthiazoline-6-Sulfonic acid)
Amp	Ampicillin
BSA	Bovine Serum Albumin
DMEM	Dulbeco's Modified Eagle Medium
ELISA	Enzyme-Linked ImmunoSorbent Assay
HRP	HorseRadish Peroxidase
IPTG	IsoPropyl-ß-D-ThioGalactopyranoside
Kan	Kanamycin
PAGE	PolyAcrylamida Gel Electrophoresis
PBS	Phosphate Buffer Saline
SDS-PAGE	Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis;

General

AIDS	Acquire ImmunoDeficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA-Editing enzyme Catalytic polypeptide-like 3G
CA	Capsid
CDR	Complementarity Determinating Region
CCD	Catalytic Core Domain
CTD	C-Cerminal Domain – CTD
CD4	Cluster Designation 4
DNA	Deoxyribonucleic Acid
E. coli	Escherichia coli
Gag	Group specific antigen polyprotein
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus type 2
Ig	Immunoglobulin
IgG	Immunoglobulin G
IN	Integrase
LTR	Long Terminal Repeats

mAb	Monoclonal Antibody
MA	Matrix
mRNA	messenger RiboNucleic Acid
NTD	N-terminal domain
NC	NuCleocapsid
Nef	Negative regulator factor
NLS	Nuclear Localization Signal
OD	Optical Density
ORF	Open Reading Frame
PBMC	Peripheral Blood Mononuclear cell
PCR	Polimerase Chain Reaction
PIC	Pre-Integration Complex
Pol	Polimerase
PR	Protease
RNA	RiboNucleic Acid
RT	RetroTranscriptase
SIV	Simian Immunodeficiency Virus
scFv	Single-Chain Antibodt Fragment
siRNA	Small interfering RNA
Tat	Transcriptional transactivator protein
Vif	Viral infectivity factor
VH	Heavy-chain Variable region
VL	Light-chain Variable region
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Glycoprotein G from Vesicular Stomatitis Virus

Amino Acids

A - Alanine	M -Methionine
C - Cysteine	N - Asparagine
D - Aspartic Acid	P - Proline
E - Glutamic Acid	Q - Glutamine
F - Phenylalanine	R - Argine
G - Glycine	S - Serine
H - Histidine	T - Threonine
I - Isoleucine	Y - Tyrosine
K - Lysine	V - Valine
L - Leucine	W -Tryptophan

Table of Contents

Abstract

The spread of HIV-1 has been dramatic since the early eighties, when the virus was discovered as the causative agent of AIDS. In the absence of an effective vaccine against HIV, a worldwide search has been made in the past two decades to develop small-molecule inhibitors to target essential steps in the viral cycle. Over the recent years, gene therapy has been highly regarded as a new form of molecular medicine in treatment of HIV/AIDS, either as an alternative or as a complement to anti-retroviral chemotherapy. An intrabody consists of an antibody designed to be expressed intracellularly and directed to different subcellular compartments where they can exert their function more effectively. The binding of an intrabody to its molecular target has the potential to block, suppress, alter or even enhance the process mediated by that molecule. Within this context, intracellular antibodies (intrabodies) represent a new class of neutralizing molecules with potential use in gene therapy approaches.

The HIV-1 integrase (IN) protein is currently the focus of an intense research effort to develop new anti-HIV-1 drugs. This enzyme catalyses the integration of HIV genome into the chromosome of the host cell, arguably the most insidious step in the infection process. In the first project of this thesis (Chapter 2), we explored the intracellular immunization approach by developing rabbit intrabodies against the HIV-1 IN protein. We immunized rabbits with HIV-1 IN and developed a combinatorial scFv library against IN. We were able to identify 5 different scFv's antibodies with high binding activity and specificity to IN. These scFv´s bound simultaneously to the

catalytic and C-terminus domains of IN. In addition, these antibodies have the ability to inhibit the strand transfer processing. Intrabody-expressing cells, either in their cytoplasm or nuclear compartments, were highly resistant to HIV-1 infection. Importantly, when HIV-1 particles where produced in the presence of anti-IN scFv, the expression of intrabodies did not affect virion production significantly. However, it markedly reduced the infectivity of progeny virions due to the incorporation of anti-IN scFv into the viral particles. These findings provide proof-in-principle that rabbit anti-IN intrabodies can be designed to block early and late stages of HIV-1 replication. As a result, our intrabodies might be useful agents for "intracellular immunization"- and used as new tools to study the structure and function of HIV-1 IN due to their epitope binding characteristics.

Another potential target for HIV-1 treatment is Vif. This viral protein overcomes the innate antiviral activity of a cytidine deaminase APOBEC3G that induces G to A hypermutation in the viral genome, resulting in enhancement of viral replication infectivity. We previously demonstrated that anti-Vif scFv and camelized VH intrabodies are an effective approach to inhibit this crucial step of the viral replication cycle. In the second project of this thesis (Chapter 3), we showed that the rabbit VL domain can also be very potentially used as an intrabody. Our results demonstrate that the anti-Vif VL single-domain preserve the antigen-binding activity and specificity in the absence of the parent VH domain. In addition, the VL single-domain was highly expressed in microbial cell culture and show favourable biophysical properties. The expression of the VL intrabody in eukaryotic cells also showed that the rabbit VL was correctly folded as soluble protein

in the reducing environment and could strongly neutralize HIV-1 infectivity. Therefore, the present study suggests that rabbit VL single-domains have also an enormous value as intracellular antigen recognition units.

Lentiviral vectors are among the most efficient tools for gene delivery into mammalian cells. A major goal of lentiviral gene delivery systems is to develop vectors that can efficiently target specific cell types. In the last project of this thesis (Chapter 4), we attempted to generate viral particles for targeting gene delivery. To achieve this goal we have used CCR5-positive cells as the target for our strategy. We designed a novel Sindbis pseudotyped vector where the Sindbis E2 receptor binding envelope protein was modified to directly encode a scFv against the CCR5 chemokine receptor. Targeting into specific cells was mediated by the anti-CCR5 scFv display, and viral titers were close to 10^6 EGFP transduction units/ml. Our data demonstrate that the length of the peptide linker that connects the heavy chain and light chain of anti-CCR5 scFv significantly affects the efficiency of infection. Infection levels obtained with Sindbis envelope displaying a scFv with a longer linker was consistently higher than that with Sindbis envelope displaying a scFv with a short linker. The results presented show that chimeric scFv-Sindbis pseudotyped lentiviral vectors have the potential to become an efficient and broadly applicable approach for targeting gene delivery to specific cells. Furthermore, this strategy has the potential to become a powerful approach for targeting gene delivery in anti-HIV gene therapy due to the important role of CCR5 expression in disease progression.

Resumo

O Síndrome da Imunodeficiência Adquirida (SIDA) representa hoje um dos principais problemas de Saúde Pública a nível mundial. Os agentes etiológicos responsáveis pela SIDA são os Vírus da Imunodeficiência Humana tipo-1 e tipo-2 (VIH-1 e VIH-2), ambos retrovírus pertencentes à família dos lentivírus. Segundo dados da Organização Mundial de Saúde (OMS), estima-se que cerca de 40 milhões de pessoas se encontrem infectadas com o VIH e que aproximadamente 20 milhões já morreram devido à doença, originando um número, com tendência crescente, de 68 milhões de pessoas até 2020. As drogas disponíveis actualmente como fármacos anti-VIH actuam na inibição de duas proteínas virais – a transcriptase reversa (RT) e a protease (PR). A aplicação de combinações destes agentes antiretrovirais (HAART) mudou dramaticamente o decorrer da infecção em muitos indivíduos infectados, levando a um declínio substancial na incidência da SIDA e da mortalidade associada nos países desenvolvidos. No entanto, um dos principais problemas relacionados com a aplicação deste tipo de terapia é o desenvolvimento de resistências virais aos fármacos utilizados. Este aspecto da resistência viral é muito importante na medida em que a susceptibilidade às drogas diminui, reduzindo, progressivamente, a eficácia das combinações terapêuticas usadas. A percentagem de novos indivíduos infectados com o VIH, que possuem vírus resistentes a pelo menos uma medicação antiretroviral tem vindo a aumentar. Deste modo, a irradicação total do VIH parece impossível com as actuais estratégias terapêuticas. Novas terapias e outros alvos mais

promissores precisam surgir rapidamente, com o objectivo de combater as formas mais resistentes do VIH que já circulam pelo mundo.

A terapia génica é uma nova estratégia de medicina molecular com enormes potencialidades na terapêutica de doenças infecciosas e cancerígenas. Esta forma de terapia pode ser complementar e adicionar eficácia à terapêutica farmacológica. A terapia génica apresenta-se de enorme importância no tratamento ao VIH e SIDA, captando nos últimos anos o interesse de muitos investigadores e empresas biotecnológicas. Neste contexto, os anticorpos intracelulares (intrabodies) representam uma nova classe de moléculas neutralizantes com um enorme potencial na terapia génica. Os anticorpos intracelulares consistem normalmente em fragmentos Fv de cadeia única (scFv) que têm como objectivo serem expressos dentro de células eucariontes. Estes anticorpos são desenvolvidos a partir de bibliotecas de genes de anticorpos contra determinado antigénio. Como tal, ao serem expressos apresentam a capacidade de neutralizar determinada proteína intracelular e/ou interferir com interacções proteína-proteína.

A proteína integrase (IN) do VIH-1 é actualmente considerada um alvo de enorme interesse na pesquisa de novas moléculas terapêuticas na inibição da replicação do VIH-1. A IN é responsável por uma das etapas mais críticas e importantes da replicação do VIH-1 – a integração do genoma do VIH no genoma da célula hospedeira. Neste contexto, o primeiro projecto desta dissertação de Doutoramento (Capítulo 2), teve como objectivo explorar a imunização intracelular através da construção de anticorpos intracelulares contra a IN do VIH-1. Para tal, imunizaram-se dois coelhos com IN e

construísse uma biblioteca combinatória de scFvs. A partir desta biblioteca identificaram-se 5 scFv diferentes com uma elevada capacidade de reconhecimento e especificidade contra a IN. Estes scFv interagem simultaneamente com o domínio catalítico e C-terminal da IN e demonstraram ter a capacidade de inibir a reacção de *strand transfer da IN in vitro*. Os resultados apresentados demonstraram ainda que células que expressem intracelularmente scFv anti-IN localizados no citoplasma ou núcleo, têm a capacidade de ser resistentes à infecção pelo VHI-1. Os resultados obtidos permitiram ainda verificar que quando partículas VIH-1 são produzidas na presença de anti-IN scFv, a expressão dos intrabodies não afecta a produção do virião. No entanto, a sua infeciosidade diminui drasticamente devido à incorporação dos scFv na partícula viral. Estes resultados sugerem assim que os scFv anti-IN têm a capacidade de inibir a replicação viral do VIH-1 durante a sua fase inicial e tardia. O presente estudo sugere assim que estes intrabodies anti-IN poderão ser moléculas bastantes promissoras a nível da terapia génica do VIH. Por outro lado, estes scFv poderão ainda ser usados como novas ferramentas para estudar a estrutura e função da proteína IN do VIH-1.

Uma outra proteína do VIH-1 que é considerada um excelente alvo terapêutico na inibição da replicação do VIH-1 é o Vif (Factor de Infecciosidade Viral). O Vif é uma proteína acessória do VIH cuja função principal é bloquear a acção do Apobec3G, uma citidina deaminase que tem a capacidade de tornar o VIH-1 não infeccioso através da indução de hipermutações G para A durante a síntese do ADN viral. Recentemente demonstramos que intrabodies anti-Vif derivados de coelho e no formato

scFv ou VH camelizados são uma estratégia bastante eficaz na neutralização da proteína Vif e inibição da replicação do VIH-1. O segundo projecto desta dissertação (Capítulo 3), teve como objectivo demonstrar que o domínio VL anti-Vif pode também ser uma molécula eficaz como anticorpo intracelular. Os resultados obtidos permitiram demonstrar que na ausência do domínio parental VH, o VL apresentou do mesmo modo um reconhecimento específico da proteína Vif. O domínio VL apresentou ainda altos níveis de expressão em *E. coli* e demonstrou ter propriedades biofísicas óptimas, nomeadamente solubilidades e estabilidades elevadas. Os resultados de expressão em células animais permitiram verificar que o domínio VL anti-Vif apresentou um *folding* correcto no ambiente redutor do citoplasma o que lhe permitiu deste modo inibir eficientemente a infeciosidade do HIV-1. O presente estudo sugere assim que domínios VL derivados de coelho poderão vir a ser uma abordagem bastante promissora para o desenvolvimento anticorpos intracelulares cada vez mais pequenos e robustos para futuras aplicações a nível da terapia génica.

Actualmente os vectores lentivirais são um dos vectores mais utilizados na inserção de genes em células eucariontes. Um dos grandes obstáculos destes vectores é não permitir um direcionamento específico para células alvo. O último projecto desta dissertação (Capitulo 4), teve por objectivo a construção de partículas lentivirais que permitam um direcionamento específico para células CCR5+. Para tal, construímos um vector quimérico Sindbis/lentiviral onde foi inserido um scFv anti-CCR5 na região da proteína E2 do envelope do vírus Sindbis. Os resultados obtidos

demonstraram que o direcionamento obtido pelas partículas quiméricas foi mediado pelo scFv anti-CCR5 e que a sua transdução permitiu obter títulos de 10^6 EGFP TU/ml. O tamanho do *linker* que liga o domínio VH com o domínio VL demonstrou influenciar a eficiência de transdução. Estes níveis demonstraram ser superiores nas partículas que apresentam um scFv com *linker* grande. Os resultados obtidos permitiram assim demonstrar que este tipo de partículas quiméricas poderão tornar-se numa estratégia bastante promissora para desenvolver vectores lentivirais que permitam um direcionamento específico para células alvo. Por outro lado, a estratégia desenvolvida neste estudo poderá ainda ser bastante promissora para proceder à inserção de genes anti-VIH em células alvo no âmbito da terapia génica do VIH-1 devido ao papel importante da expressão CCR5 na progressão da doença.

CHAPTER 1

Introduction

1.0 Human Immunodeficiency Virus

Great progress has been made with respect to our understanding of the immunopathogenesis of AIDS and the infectious agent, HIV, which causes the disease [1,2]. The natural history of HIV infection is characterized by an insidious deterioration of the cellular immune system. The quantity and proportion of plasma $CD4^+$ T-cells decreases steadily over a period of years to decades, and this progressive loss of $CD4^+$ T-cells is associated with the development of AIDS in infected individuals [3-6]. There are two types of human immunodeficiency virus: HIV-1 and HIV-2 [1,2,7]. Both virus are associated with the same mode of transmission and cause similar opportunistic infections and AIDS. However, HIV-2 often is less infectious early in the course of infection and, compared to HIV-1, the duration of increased infectiousness is shorter [7]. In addition, while HIV-2 is prevalent in West Africa and some countries in Western Europe, HIV-1 is prevalent worldwide [7,8].

1.1 HIV Structure and Genome

HIV is a member of the genus Lentivirus, part of the family of *Retroviridae*. HIV genome is encoded by RNA, which is reverse-transcribed to viral DNA by the viral RT upon entering a new host cell [34]. The general features of the mature HIV virion and the structurally characterized viral proteins are shown in Figure 1.1.

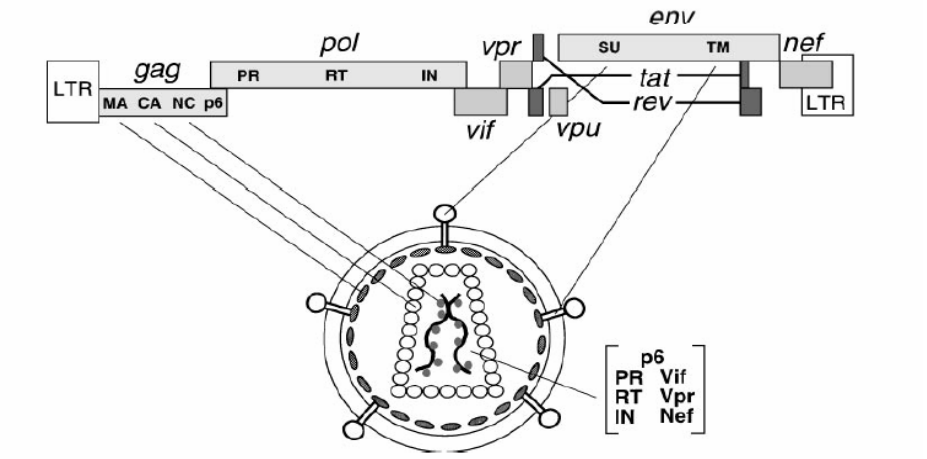

Figure 1.1 - Schematic diagram of the HIV-1 virion particle and genome (adapted from [35]).

All lentiviruses are enveloped by a lipid bilayer that is derived from the membrane of the host cell. Exposed surface glycoproteins (SU,gp120) are anchored to the virus via interactions with the transmembrane protein (TM, gp41) [35]. The lipid bilayer also contains several cellular membrane proteins derived from the host cell, including major histocompatibility antigens, actin and ubiquitin [36]. A matrix shell comprising approximately 2000 copies of the matrix protein (MA, p17) lines the inner surface of the viral membrane, and a conical capsid core particle comprising about 2000 copies of the capsid protein (CA, p24) is located in the center of the virus [34,35]. The capsid particle encapsidates two copies of the unspliced viral genome, which is stabilized as a ribonucleoprotein complex with about 2000 copies of the nucleocapsid protein (NC, p7), and also contains three essential virally encoded enzymes: protease, reverse transcriptase and integrase. Virus particles also package the accessory proteins, Nef, Vif and Vpr. Three additional accessory proteins that function in the host cell, Rev, Tat and Vpu, do not seem to be packaged [34,35].

The HIV-1 genome is encoded by a two single-stranded RNA molecule approximately 9 kb (9,000 nucleotide bases) in length, and contains 9 open reading frames (ORFs) [34]. The ends of each strand of HIV RNA contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as "switches" to control production of new viruses and can be triggered by proteins from either HIV or the host cell. The largest three reading frames transcribe the Gag, Pol, and Env polyproteins, which are proteolytically processed into proteins common to all members of the retrovirus family [34,35]. Gag is processed into matrix (MA), capsid (CA), nucleocapsid (NC), and p6 - proteins that make up the inner core of the viral particle. Derived from the Env polyprotein, gp120 (surface, SU) and gp41 (transmembrane, TM) make up the virus's outer membrane proteins [35]. The polyprotein Pol encodes all three viral enzymes – PR, RT and PR – which are translated together as a precursor and subsequently cleaved by the action of the HIV-1 PR. The six remaining genes, *tat*, *rev*, *vif*, *vpu, vpr*, and *nef* are regulatory genes for proteins that control the ability of HIV to infect cells, produce new copies of virus (replicate), or cause disease [35]. Tat and Rev regulate viral gene transcription and are essential for HIV replication [37-40]. The Vif protein overcomes the innate antiviral activity of a cytidine deaminase APOBEC3G (CEM15) that induces G to A hypermutation in the viral genome, resulting in enhancement of viral replication infectivity [20-23]. Vpu intervenes in the assembly process[41] and Vpr in the nuclear transport of the viral genome [42,43]. Nef might have multiple functions, including acceleration of clinical disease, enhancement of virion infectivity, downregulation of surface CD4 receptor and major histocompatibility class I molecules (MHC), modulation of signal

transduction, and the favoring of HIV entry into target cells by the CD4 and chemokine-dependent route [44].

1.2 HIV Tropism

HIV enters T-lymphocytes and monocytes through cognate binding of the viral glycoprotein gp120 with the cell surface CD4 molecule and a chemokine receptor [45-48]. Although a number of chemokine receptors have been associated with viral entry *in vitro*, CCR5 and CXCR4 appear to be the most relevant coreceptors used *in vivo* [48-50]. The interaction of these proteins induces the binding of the viral gp41 to heparan sulfate on the host plasma membrane; trigering the fusion of the viral envelope and the release of the capsid into the cytoplasm [34,51]. On the basis of cell tropism, HIV strains can be broadly divided into 2 categories, macrophage-tropic (M-tropic) and T-cell tropic (T-tropic) [48]. M-tropic strains use CCR5 as a coreceptor and are referred as R5 viruses. They primarily infect macrophages and primary T-cells and infect poorly $CD4^+$ T-cell lines [52]. T-tropic strains use the CXCR4 coreceptor, which is most expressed in $CD4^+$ T cells. Also referred as *X4* viruses, they induce the formation of syncytia in the infected cells [53,54]. Early in the course of HIV infection, the R5 strain viruses predominate, but eventually both X4 and R5 strains are recovered [54,55,56].

1.3 HIV-1 Replication Cycle

The defining characteristic of retroviruses is their ability to generate a double-stranded DNA copy of their double single-stranded RNA genome, the provirus, through the action of the viral RT [57,58]. The provirus is subsequently

integrated into the host chromosome through the action of the viral IN [16-18]. From this point on, proviral DNA is part of the host cell and cannot be eliminated unless the cell dies. A unique feature of lentiviruses is their ability to infect and integrate not just in actively dividing cells, but also into nondividing cells such as resting T cells and macrophages [58]. Creation of latent reservoirs occurs early in the course of HIV infection in humans, so that by the time of occurrence of an acute retroviral syndrome, HIV has disseminated widely and established a chronic infection in the host [59]. The HIV replication proceeds in a series of events that can be divided into two overall phases: "early" and "late" [34,58]. The **early phase** begins with the recognition of the target cell by the mature virion and involves all processes leading to and including integration of the genomic DNA into the chromosome of the host cell. The **late phase** begins with the regulated expression of the integrated proviral genome, and involves all processes up to and including virus budding and maturation (Figure 2.1).

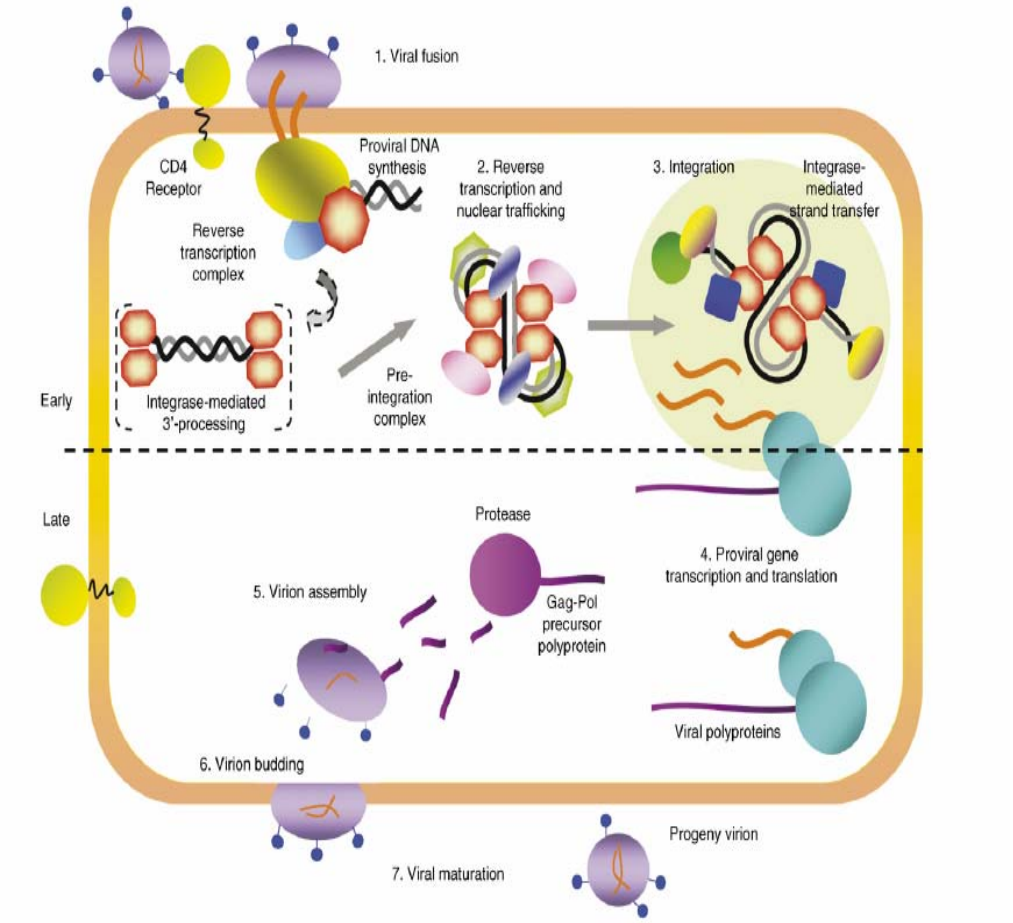

Figure 2.1 HIV-1 replication cycle. The viral replication cycle can be divided into two main stages: early and late. The major steps of the early stage include viral fusion, reverse transcription, nuclear trafficking and, lastly, integration. The late stage of viral replication begins with host-cell-mediated proviral gene transcription and translation followed by virion assembly, virion budding and, finally, viral maturation into infectious progeny virion capable of another round of infection with a new target immune cell (adapted from [60]).

1.3.1 Early phase

1.3.1.1 Virus Entry

HIV-1 enters the target cell through fusion of the virus envelope with the target cell membrane. The initial step of entry is believed to involve a high affinity binding between the surface envelope glycoprotein gp120 and the cellular receptor, CD4, which is present on the surface of a subset of T-lymphocytes and monocytes [45-49]. Binding of the surface subunit gp120 to CD4 and a coreceptor on the T-cell surface triggers conformational changes in the Env complex, leading to the insertion of the hydrophobic N-terminal fusion peptide (FP) of gp41 into the target cell membrane [61-64].

1.3.1.2 Reverse Transcription

After virus entry, the viral capsid is disrupted and the viral RT enzyme is fully activated. During reverse transcription, the two RNA molecules in the virion are converted to a linear double-stranded DNA [65,66]. The process of reverse transcription requires priming provided by the $tRNA^{lys3}$, which is annealed to the primer-binding-site (PBS) at the 5´end of the viral genome during particle formation. $tRNA^{lys3}$ is selectively incorporated in the HIV-1 virion, which contains approximately eight molecules of $tRNA^{lys3}$ per two copies of genomic RNA [67,68].

1.3.1.3 Nuclear Transport and Integration

After reverse transcription, the newly synthesized HIV-1 cDNA is transported

to the nucleus as part of a preintegration complex (PIC) [58]. This complex contains linear viral DNA and several viral proteins including MA, RT, IN, Vpr and NC [69]. Functional cellular proteins have also been identified functional in PICs [69]. Vpr protein is thought to enhance the HIV-1 preintegration complex transport to the nucleus [42,43]. Once near the nuclear membrane, peptide sequences from Vpr and MA provide a nuclear localization signal to actively enter the nucleus. The proviral DNA is then integrated at random sites throughout the cellular host genome by the action of the viral enzyme IN [19]. The integration process is arguably one of the most insidious steps in the infection process [17,70-72]. As a result, HIV-1 IN is currently the focus of an intense research effort to develop new anti-HIV-1 drugs [73]. In the present dissertation, IN was chosen as a potential target for HIV-1 gene therapy. Therefore, this HIV-1 enzyme will be described in more detail in a later section.

1.3.2 Late Stage

Following integration into the host chromosome, the integrated provirus serves as the template for the synthesis of the viral RNAs that ultimately encode the full complement of structural, regulatory, and accessory proteins used to direct virus replication [34].

1.3.2.1 HIV Transcription

The integrated provirus flanked by the tandem LTRs is organized as a eukaryotic transcriptional unit [34]. The 5' LTR contains a strong

enhancer/promoter and the 3' LTR contains an efficient polyadenylation site. The HIV-1 promoter is highly regulated by both viral and cellular factors [74]. Its activity varies greatly depending on the cell status. In many infected cells in HIV-positive individuals, virus expression is undetectable. Thus, a state of viral latency exists in individual cells, although the infection is chronically active in a fraction of cells due to continue HIV expression [58].

Transcription from the HIV-1 LTR leads to the generation of a large number (more than 30) of viral RNAs [75]. These fall into three major classes: 1) unspliced RNAs, which function as the mRNAs for the Gag and Gag-Pol polyprotein precursors, and are packaged into progeny virions as genomic RNA, 2) partially spliced mRNAs, which are around 5 kb in size and encode the Env, Vif, Vpu, and Vpr proteins, and 3) small (1.7 to 2.0 kb), multiple spliced mRNAs, which are translated into Rev, Tat, and Nef [58,75,76]. After HIV mRNA is processed in the cell's nucleus, it is transported to the cytoplasm.

1.3.2.2 HIV Translation, Assembly and Budding

To assemble a virus, the structural proteins must be produced first [77]. For HIV-1, the genomic RNA is also the mRNA that codes for the Gag precursor (Pr 55^{Gag}) and the Gag-Pol fusion protein (PR $160^{Gag\text{-}Pol}$). Pr 55^{Gag} is as polyprotein precursor that can be processed into MA (p17), CA (p24), NC (p7) and p6 [58]. The gene encoding the viral enzymes PR, RT and IN are translated as part of the fusion Gag-Pol polyprotein [78].

The Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum (ER) using the spliced *env* mRNA gene as the message [77,78]. Env is

posttranslationally modified in the ER and Golgi apparatus and is cleaved to produce the two envelope glycoproteins gp41 and gp120 [58]. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag and Gag-Pol polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell [79].

1.3.2.3 Maturation

As the particle buds and is released from the cell surface coated with gp120 and gp41, the virion undergoes a morphologic change known as maturation. This step involves proteolytic processing of the Gag and Gag-Pol polyproteins by PR. The mature virion is then ready to infect the next cell and initiate a new infection cycle [58].

1.4 HIV/AIDS Treatments

The spread of HIV-1 has been dramatic since the early eighties, when the virus was discovered as the causative agent of AIDS. In the absence of an effective vaccine against HIV, a worldwide search has been made in the past two decades to develop small-molecule inhibitors to target essential steps in the viral cycle [10]. Currently, nearly 25 antiretroviral drugs (ARV) have been licensed for the treatment of HIV-1: nine nucleoside reverse transcriptase inhibitors (NRTI), four nonnucleoside reverse transcriptase inhibitors (NNRTI), nine protease inhibitors (PI), one fusion inhibitor, one CCR5 inhibitor, and one integrase inhibitor [80-87]. The first CCR5 and integrase inhibitors were approved in the end of 2007, increasing the number of ARV

classes from four to six [86,87].

Anti-HIV drugs are most effective when taken in a combination. This treatment regimen is called Highly Active Antiretroviral Therapy (HAART). Current HAART options use combinations consisting of at least three drugs belonging to at least two types, or "classes," of antiretroviral agents. Typically, these classes are two nucleoside analogue reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitor (PI) or a non-nucleoside reverse transcriptase inhibitor (NNRTI) (Table 1.1). New classes of drugs such as Entry and Integrase inhibitors provide treatment options for patients who are infected with viruses already resistant to common therapies, although they are not widely available and not typically accessible in resource-limited settings [11].

Table 1.1 – Current Licensed antiretroviral drugs (adapted from 10)

Name	Trade name	Company	Launched
Nucleoside or nucleotide reverse-transcriptase inhibitors			
Zidovudine	Retrovir	GlaxoSmithKline	1987
Didanosine	Videx	Bristol–Myers Squibb	1991
Zalcitabine	HIVID	Roche	1992
Stavudine	Zerit	Bristol–Myers Squibb	1995
Lamivudine	Epivir	GlaxoSmithKline, Shire Pharmaceuticals	1998
Abacavir	Ziagen	GlaxoSmithKline	1999
Tenofovir disoproxil fumarate	Viread	Gilead	2001
Emtricitabine	Emtriva	Gilead	2003
Non-nucleoside reverse-transcriptase inhibitors			
Nevirapine	Viramune	Boehringer Ingelheim	1996
Efavirenz	Sustiva, Stocrin	Bristol–Myers Squibb, Merck	1998
Delavirdine	Rescriptor	Pharmacia & Upjohn, Agouron, Pfizer	1999
Protease inhibitors			
Saquinavir	Invirase	Hoffmann-La Roche	1995
Indinavir	Crixivan	Merck	1996
Ritonavir	Norvir	Abbott, GlaxoSmithKline	1996
Nelfinavir	Viracept	Agouron, Pfizer	1997
Amprenavir	Agenerase, Prozei	Vertex	1999
Lopinavir + ritonavir	Kaletra, Aluvia	Abbott	2000
Atazanavir	Reyataz, Zrivada	Bristol–Myers Squibb, Novartis	2003
Fosamprenavir	Lexiva, Telzir	Vertex, GlaxoSmithKline	2003
Tipranavir	Aptivus	Boehringer Ingelheim	2005
Darunavir	Prezista	Tibotec	2006
Entry inhibitors			
Enfuvirtide	Fuzeon	Trimeris, Roche	2003
Maraviroc	Celsentri, Selzentry	Pfizer	2007
Entry inhibitors			
Enfuvirtide	Fuzeon	Trimeris, Roche	2003
Maraviroc	Celsentri, Selzentry	Pfizer	2007
integrase *inhibitors*			
Raltegravir	Raltegravir	Merck	2007

1.4.1 HIV-1 Drug Resistance and Emergence of new Approaches for AIDS Treatment

In the developed world, access to multi-drug combination regimens have reduced the viral count to undetectable levels in infected patients; which resulted in significant reductions in the morbidity and mortality attributed to

HIV/AIDS [10,11,13]. However, full eradication of the infection has failed due to the persistence of latent HIV-1 in resting memory $CD4^+$ T-cells. Furthermore, the emergence of multi-drug-resistant viral strains in patients exposed to HAART regimens and the rapid escalation of infections in developing countries pose an overwhelming challenge to the treatment strategies of HIV/AIDS [13-15].

The emergence of HIV strains resistant to antiretroviral drugs aimed at any viral target is an inevitable phenomenon following the initiation of treatment in infected patients. The viral drug resistance occurs by at least two mechanisms. First, the lack of an inherent proofreading mechanism in RT naturally causes a high rate of mutations in each replication cycle [88]. Second, the pressure induced by an antiviral agent accelerates specific mutations in its target, particularly in the region where it binds, eventually leading to a permanent loss in potency [12,14]. The emergence of drug-resistant viral strains in combination with several other critical factors, such as a patient's inability to adhere to long-term treatment regimens, severe side effects associated with existing drugs, and the high cost of medication have require a high demand for the development of new therapeutic approaches for AIDS treatment [10,14]. Gene therapy is one such forward-looking strategy.

1.5 Gene Therapy for HIV-1

At the most basic level, gene therapy can be described as the intracellular delivery of genetic material to generate a therapeutic effect by correcting an existing abnormality or providing cells with a new function. Initially inherited

genetic disorders were the main focus of gene therapy but now a wide range of diseases, including cancer, vascular diseases, neurodegenerative disorders and other acquired diseases are being considered as targets. The ultimate goal of gene therapy is the amelioration of disease upon a single administration of an appropriate therapeutic gene. The genetic material considered for use intended to replace a defective or missing gene, augment the functions of the genes present, and instill a specified sensitivity to a normally inert prodrug or to interfere with the life cycle of infectious agents [89].

Gene therapy has gained special interest among AIDS researchers, since conventional therapies have shown limited success [24-26]. Alteration of the host cell could potentially confer permanent suppression of viral replication, after infection, or could provide protection against viral infection. Over the past 10 years several different anti-HIV-1 gene therapy approaches have been developed and tested [26]. These approaches can be classified into two main categories: RNA-based agents and protein-based agents.

RNA Strategies

RNA approaches include antisense oligonucleotides, ribozymes, RNA aptamers and decoys, and RNA interference (RNAi).

(1) Antisense oligonucleotides

Antisense oligonucleotides are short sequences of nucleic acids complementary to a given messenger nucleic acid (sense sequence) [89]. Antisense oligonucleotides were shown by several groups to inhibit gene expression in HIV-1 infection *in vitro* when targeted to such critical HIV-1

genes such as tat, rev, and integrase [90]. The problems associated with this type of therapy essentially derive from the short half-life of antisense oligonucleotides and the difficulty involved in accessing the inside of the cell [89].

(2) Ribozymes

Ribozymes are antisense enzymatic RNAs molecules that can specifically recognize and cleave target RNAs, and prevent or significantly reduce translation of proteins encoded by the sequences against which they are targeted [89]. The cleavage reaction is catalytic and no energy source is required. Since the first demonstration that ribozymes can inhibit HIV replication, several studies have demonstrated related ribozyme-based strategies for the treatment of HIV infection [91]. Three separate clinical trials have used ribozymes targeting HIV genes, including *tat, rev* and the viral U5 region [92]. Although these trials have not shown significant anti-HIV efficacy, they demonstrated that it is safe to mobilize stem cells or to collect peripheral blood mononuclear cells from persons with HIV, genetically modify the cells with retroviral-ribozyme vectors and reinfuse them into patients [25,26,].

(3) RNA aptamers and decoys

Another group of RNA molecules, RNA aptamers, have been evolved *in vitro* to bind targeted ligands with high affinity [93]. Although aptamers against HIV show promise, thus far there have been no clinical trials using anti-HIV aptamers [25]. One potential problem is that aptamers selected *in vitro* may not form the required tertiary structure in cells to effectively bind target proteins.

On the other hand, expressed RNA decoys have been more amenable to gene therapy [94]. RNA decoys employ short RNA oligonucleotides which mimic critical regulatory sequences in HIV. The majority of the RNA decoys have targeted the HIV regulatory gene products *tat* and *rev* [24-26,94].

(4) RNA interference

RNA interference (RNAi) was originally discovered in *Caenorhabditis elegans* as a biological response to exogenous double-stranded RNA molecules (dsRNA) which induce sequence-specific gene silencing [95]. Subsequently, it was reported that RNAi could take place in a wide variety of organisms, such as fungi, plants, invertebrates and vertebrates. In fact, RNAi serves as a safeguard for the preservation of genomic integrity. It protects the host from viral infections and invasion by mobile genetic elements by degrading the exogenous genomic material (e.g. viral RNAs). In mammalian cells, it had been known that introduction of dsRNA activated the IFN pathway and RNA binding protein kinase and induced non-specific degradation of RNA, translation inhibition and cell death [89]. However, Elbashir *et al* succeeded in inducing RNAi machinery in mammalian cells by using small dsRNA (called small interfering RNA; siRNA) without a non-specific response against the dsRNA [96]. This finding was a major breakthrough for the application of RNAi. Thereafter, siRNA became a widespread tool for specific gene silencing. Small interfering RNAs (siRNA) are double-stranded RNA sequences, approximately 22 base pairs in length that bind cellular mRNAs in a sequence-specific manner and cleave them at the center of the complementary region [97,98]. They achieve this through a series of steps involving the

recruitment of RNAi factors, formation of an RNA induced silencing complex (RISC), unwinding of the siRNA, and activation of RISC. RNAi triggers can also be produced by expressing short hairpin (shRNA) precursors that partly resemble endogenous microRNA precursors, allowing them to be exported to the cytoplasm and processed by the RNAi machinery [89]. Expressing short hairpin precursors encoding siRNAs targeting viral or cellular sequences can be readily accomplished from the backbone of viral vectors used in gene therapy. HIV-1 was one of the first infectious agents targeted by RNAi as a result of the virus' well-understood life cycle and pattern of gene expression [97]. To date, small interfering RNAs have been used *in vitro* to target viral genes like *tat* and *rev* and cellular genes like CCR5 with great success [97,98].

Protein-based strategies

Similar to the RNA-based inhibitors of HIV, proteins can be directed to inhibit either cellular or viral targets. The most relevant protein-based approaches include suicide genes, transdominant negative proteins, zinc finger transcription factors and intracellular intrabodies.

(1) Introduction of suicide genes

Generally, suicide genes code for enzymes that convert an inactive drug to a toxic form, allowing for the potential killing of the modified cells. The idea of using suicide genes was made popular as a potential approach for treating cancer [89]. As an anti-HIV strategy this method was tried *in vitro* as proof-of-concept in a study that used a retrovirus to transduce autologous CD8+ cells

from HIV-infected patients; the suicide gene was thymidine kinase expressed as a fusion protein with hygromycin phosphotransferase [99]. As with other gene therapy approaches, specific targeting of desired HIV-infected cell populations would present a challenge for this approach to be successful *in vivo.*

(2) Transdominant negative proteins

Mutations in a specific gene expressed in a dominant fashion where the mutant protein can interfere with the function normally carried out by the parent gene product. If the protein is multimeric, the nonfunctional protein can multimerize with the normal protein and the resultant complex is functionally inactive [89]. Thus, the dominant negative protein can have a strong inhibitory effect on the normal protein formation or function. Examples of this are HIV-1 Gag mutants and Rev M10 [100,101]. Rev M10 was the first transdominant negative protein used in clinical trials; it is a dominant negative mutant capable of binding the Rev Responsive Element (RRE) and has the capability of forming multimers [101].

(3) Polydactyl Zinc Finger Transcription Factors

Recent advances in the design, selection, and engineering of DNA binding proteins have led to the emerging field of designer transcription factors (TFs) [102]. Modular DNA-binding protein domains can be assembled to recognize a given sequence of a DNA in a regulatory region of a targeted gene. TFs can be readily prepared by linking the DNA-binding protein to a variety of effector domains that mediate transcriptional activation or repression. Furthermore, the interaction between the TF and the genomic DNA can be regulated by several

approaches, including chemical regulation by a variety of small molecules. Genome-wide single target specificity has been demonstrated using sequence-specific zinc finger (ZF) domains [102]. Any laboratory today can easily construct polydactyl ZF proteins by linkage of predefined ZF units that recognize specific triplets of DNA. Several laboratories are currently developing artificial transcription factors to inhibit HIV- replication [103]. For instance, we have recently in collaboration with Dr. Carlos Barbas III inhibited the HIV-1 replication with artificial transcription factors targeting the highly conserved primer-binding site [104].

(4) Intracellular Intrabodies

Intracellular antibodies (intrabodies) represent a new class of neutralizing molecules with potential use in gene therapy approaches [27-29]. An intrabody consists of an antibody designed to be expressed intracellularly and directed to different subcellular compartments where it can exert its function more effectively [30]. The binding of an intrabody to an intracellular target protein has the potential to block, suppress, alter or even enhance the process mediated by that molecule [31-33]. The strong inhibiting potential of intracellular antibodies in the context of HIV gene therapy will be the focus of this thesis. This new gene therapy approach will be described in detail in the next section.

1.6 Intracellular Antibodies as Specific Molecules for Gene Therapy

Antibodies are prime recognition molecules: they possess a high specificity and affinity for their target, and can be easily raised against virtually any

protein [105]. For these reasons, antibodies have been extensively studied and subjected to more modifications than any other type of molecule: antibodies are being fragmented, remodeled, made part of chimeric molecules, or covalently linked to other compounds. Modified antibodies have now been used for decades as tools for molecular biology, and as therapeutic agents [106,107].

One of the latest antibody applications involves intracellular antibodies [27-29]. Early studies showed that the cDNA of an antibody (heavy and light chains) against yeast alcohol dehydrogenase I (ADH I) could be expressed in the cytoplasm of *Saccharomyces cerevisiae* and could neutralize the enzyme *in vivo* [108]. Further studies verified that the assembly of an antibody could take place in the reducing environment of a mammalian cytoplasm [109]. Protein engineering of antibody binding sites demonstrated the feasibility of joining heavy chain and light-chain variable domains through a synthetic linker, maintaining binding specificity and affinity of the parent molecule [110,111]. With these advances in the field and further improvements, intrabodies were shown to inhibit functions in mammalian cells and therefore represent now a new class of neutralizing molecules with a potential use in gene therapy [27-33].

1.6.1 Antibody Structure and Function

In vertebrates, there are five different classes of antibodies known as IgD, IgA, IgM, IgE and IgG, which differ in their function in the immune system [105]. IgGs are the most abundant immunoglobulins in the blood and currently are the intact format almost exclusively used as therapeutic antibody. This typical antibody consists of two antigen-binding fragments (Fabs), which are linked

via a flexible region (hinge) to a constant Fc region (Figure 1.3A). IgG antibodies are usually "Y" shaped molecules comprising two identical light chains (L) and two identical heavy chains (H) linked together by disulfide bonds. The heavy chains contain a variable domain (VH domain) and three constant domains (CH1, CH2 and CH3). By contrast, the light chains contain a variable domain (VL domain) and a single constant domain (CL domain). The variable domains show three regions of hypervariability in sequence called the complementarity determining regions (CDRs) [105]. They differ in length and sequence between different antibodies and are responsible for the specificity (recognition) and affinity (binding) of the antibodies to the antigen. The remaining V region amino acids act as a scaffold to support the CDRs loops and are referred as framework residues (FR). On the other hand, the Fc constant region recruits cytotoxic effector functions through complement and/or through interactions with γFc receptors. In addition, this non-antigen binding part of an antibody is also associated with serum half-life of antibodies. In this case, the Fc region binds to FcRn receptors on endothelial cells and rescues antibody molecules from intracellular degradation [106,107].

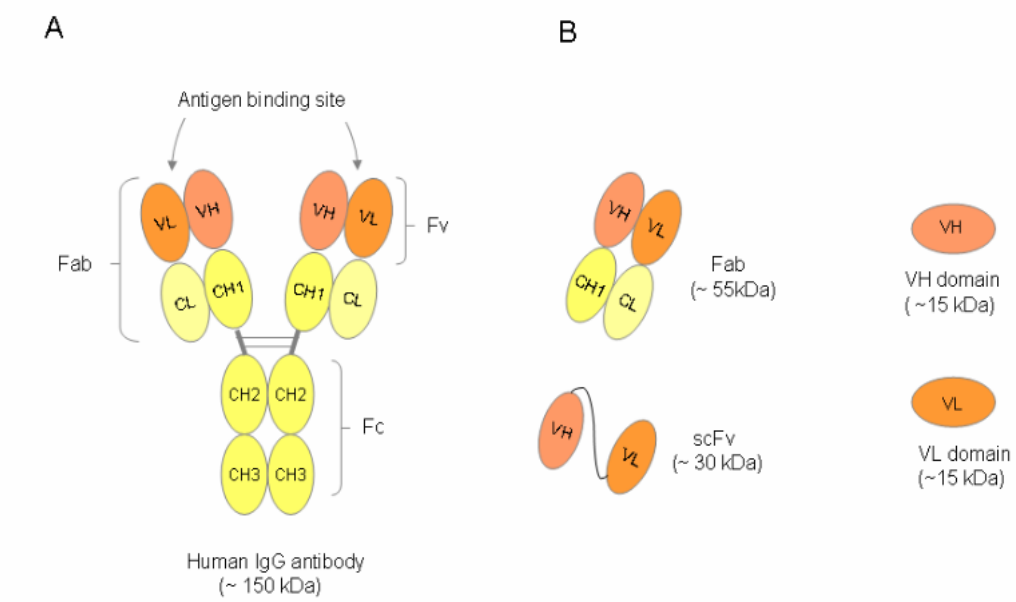

Figure 1.3 - A - Schematic representation of the structure of a conventional IgG antibody. IgG antibodies comprise a pair of identical heavy and light chains linked by disulphide bonds. Light chains contain one constant domain (CL) and one variable domain (VL), while heavy chains contain three constant domains (CH1, CH2 and CH3) and one variable domain (VH). The variable domains of both the heavy and light chains are responsible for the antigen-binding site of the molecule (Fv). The Fc constant region recruits effector functions of the immune system. Constant light (CL) and heavy (CH) chain domains are represented in yellow. Variable light (VL) and heavy (VH) chain domains are represented in orange. **B -** Schematic representation of antibody constructs. The engineering of antibody fragments that can be generated from an intact conventional IgG: antigen-binding fragment (Fab), single-chain Fv fragment (scFv) and heavy and light chains only (V domains) (adapted from [112]).

1.6.2 Intrabody Formats

The most commonly used form of intrabody consists of engineered scFv in which the variable domain of the heavy chain (VH) is connected to the light chain (VL) through a peptide linker, preserving the specificity and affinity of the parent antibody (Figure 1.3B) [110,111]. The most common peptide linker is a

flexible $(Gly_4Ser)_3$, but many other linker designs have been successfully employed. A scFv antibody can have either VH–linker–VL or VL-linker-VH configuration [29].

Single-chain Fv fragments are usually viewed as the smallest antibody units, which form complete antigen binding sites. However, early observations in the late 1960s indicated that sometimes VH domains alone retained a significant part of the original binding activity [113-115]. Based on this concept, in 1989 Greg Winter's group at Medical Research Council, Cambridge-UK, isolated heavy chain variable domains with antigen affinity against lysozyme derived from an immunized murine VH library [116]. Despite such promising results, the efficient expression of VH fragments is usually confronted with folding problems, low solubility and high tendency for aggregation caused by the exposure of the hydrophobic VH/VL interface upon removal of the VL domain. Nevertheless, these problems seem to have been overcome, or at least greatly reduced for some mouse and rabbit VH domains by the identification and design of mutations that minimize the hydrophobic interface and by direct selection of highly stable single-domains from phage display libraries [117-126]. In contrast, another promising alternative is the naturally heavy chain antibodies devoid of light chain that were recently discovered in camelids (camels and llamas) [127]. These heavy-chain antibodies recognize the antigen by one single variable domain and can be obtained from either immunized or non-immunized animals. In camelids, the variable heavy chains are referred to VHH (~15 kDa) and consist of four framework regions and three CDRs [128]. In VHH the third antigen-binding loop (CDR3) is often stabilized by disulfide bonds and is, on average, longer than a human or mouse VH-CDR3 loop. These smaller

antibody molecules might, therefore, reach targets not easily accessible by currently marketed mAbs therapies, such as enzyme active sites and canyons in viral and infectious disease biomarkers [129].

Thus an intrabody can also be reduced in size to a single functional variable domain. For this designed format, a single VH domain, known as an IDab, possesses excellent solubility, stability and expression within eukaryotic cells. An ideal IDab should exhibit specific antigen recognition and neutralizing activity. IDabs isolated for RAS protein have been shown to inhibit RAS-dependent oncogenic transformation of NIH3T3 cells [130]. We have also recently developed a minimal rabbit scaffold VH fragment with intrabody properties derived from anti-Vif scFv that was engineered to mimic camelid antibody domains [121,131]. These camelized VH single-domain intrabodies were highly expressed in reducing environments and exhibited a strong neutralization of HIV infectivity [121]. Natural VHH antibodies have also been validated as intrabodies. In this case, several Bax-specific VHH intrabodies were stably expressed in mammalian cells and prevented mitochondrial membrane potential collapse and apoptosis after oxidative stress in the host cells [132]. Another format is the Fd fragment (VH-CH1) which has been shown to inhibit glucose-6-phosphate dehydrogenase in the cytoplasm of mammalian cells [133]. Recently, a VL-Dab and its disulfide bond-free derivative were also successfully utilized as an anti-huntingtin intrabody in Huntington's disease [134,135]. The development of rabbit VL single domains as potential intrabodies will be also evaluated in the present dissertation.

1.6.3 Molecular Mechanism of Intrabodies

Intrabodies have been used to alter the functions of the target antigens by modifying related cellular pathways or by redirecting the antigen to a new cellular compartment [29]. The unique molecular characteristics of intrabodies allow them to affect protein functions by:

(1) Sequestration of a target protein from its normal subcellular compartment of action.
(2) Mediating enzyme function through blocking of the active site or modulation of its conformation.
(3) Disrupting biological or signal pathways via interfering normal protein– protein or protein–DNA interactions.
(4) Inducing cell death via activation of the caspase-3-mediated apoptotic pathway.
(5) Selective degradation via the ubiquitin–proteosome pathway.

Firstly, intrabodies can be designed to be expressed in different subcellular compartments such as cytoplasm, nucleus, endoplasmic reticulum (ER), Golgi, mitochondria, peroxisomes, plasma membrane and other locations [30]. DNA recombinant techniques allow classical intracellular trafficking signals to be genetically fused to the N- or C-termini of antibodies to direct the intrabodies to specific subcellular localizations in order to block or interfere target antigen function [30]. For instance, ER-retained intrabodies are designed with a signal leader peptide sequence at the N-terminus and a retention peptide, KDEL, at the C-terminus to tether intrabodies within the lumen of the ER. The engineered intrabody can then interact with targeted secretory-pathway proteins, sequestering them within the ER and inhibiting their natural

expression. Retention of antibodies in the ER can effectively down regulate its target receptors or signaling molecules, such as reduction of cell surface expression of VEGF-R2 and epidermal growth factor receptor (EGFR) [136,137]. Similar target protein retention in the trans-Golgi has been reported for intrabodies containing a trans-Golgi retention signal [138]. For expression of cytoplasmic intrabodies, the signal sequences are removed and cytosolic intrabodies are translated on free polysomes. Cytoplasmic intrabodies with an intrinsically stable sequence that can fold properly in the absence of disulfide bond formation are required to function appropriately [30].

Secondly, intrabodies can modulate enzymatic function by blocking an enzyme active site, by sequestering substrate, or by modulating the conformation of an enzyme catalytic site. It has been shown that cytoplasm-expressed single-domain intrabodies targeting the protein kinase Etk could inhibit its autophosphorylation and ability to phosphorylate its substrate. This activity resulted in a partial inhibition of cellular transformation in Src-transformed cells [139].

Thirdly, intrabodies can be used to disrupt biological signaling pathways of target proteins by interfering with normal protein-protein or protein-DNA interactions. For example, a nuclear-targeted intrabody has been used to bind to cyclin-E resulting in the inhibition growth of a breast cancer cell line [140]. Fourthly, when genetically fused to caspase-3, intrabodies can be used to promote death of target cells (e.g. cancer cells) by activating the caspase-3-mediated apoptosis pathway [141]. It is also possible to design intrabodies that can promote selective degradation of cellular protein targets via the ubiquitin-

proteasome pathway by fusion with F-box [142,143]. In addition, intrabodies also have the potential to cause gain-of-function after binding to their target proteins. For example, it has been demonstrated that certain anti-p53 scFv antibodies restored the transactivating activity of mutant p53 in p53 knockout human tumor cells [144].

The strong inhibiting potential of intracellular antibodies is well known in the field of cancer, HIV, transplantation, autoimmune and neurodegenerative diseases [121,131,133-140,144-147]. Despite such successful results obtained, the efficient cytoplasmic expression of intrabodies is generally confronted with folding problems, low solubility, short protein half-life and high tendency for aggregation [29]. These problems are most likely caused by the reducing environment of the cell cytoplasm. The preserved intrachain disulfide bridges of heavy and light chains is not formed in scFv's expressed in the cytoplasm, thus resulting in unstable intrabodies that are non-functional inside the cell [30]. Therefore, only intrinsically very soluble and stable scFv fragments will be able to fold correctly in sufficient amounts to be active as intrabodies [150]. At this time, no rules or consistent predictions can be established about intrabodies that will tolerate the reducing cellular environments. Within this context, there is a strong interest in the ability to express functional antibodies in this environment and only recently several different approaches started to emerge with such requirements [151,152]. These approaches include *in vivo* screening for intrabody-antigen interaction based on two hybrid screening [152-154] and construction of antibody libraries using randomized CDRs on scFv frameworks that have been selected for high solubility and stability in an intracellular environment [154-157]. Several other strategies have also been

developed to address these same concerns. For example, stable and functional cytoplasmic cysteine-free scFv have been generated by using DNA shuffling and phage display [150,151]. VH and VL single domain antibody libraries are becoming a major strategy to be used to avoid disulfide bond requirement [130,132,133,134]. In addition, camelization strategies have also been used to improve the solubility and stability of rabbit VH domains [121]. Finally, fusion of intrabodies with *E. coli* maltose binding protein were shown to enhance their solubility and stability in bacteria and mammalian cell cytoplasm [158].

1.6.4 Intrabody Gene Sources

To obtain the variable domain sequences to construct an intrabody, two sources have commonly been used: hybridomas and antibody libraries. Hybridomas producing either murine monoclonal [159-161] or human monoclonal antibodies [162] have been the source of VH and VL cDNA for those particular antibodies. Specific primer sequences can be used to amplify the immunoglobulin chains that will be used to construct scFv's. Two disadvantages of hybridoma technology are the length of time required to develop antibody candidates and the need to restart the antibody development process when unwanted characteristics such as cross reactivities are discovered [163]. Currently antibody libraries are the main source to select intrabodies. In general, three types of antibody libraries may be used: immune, naïve and synthetic [163,164].

Immune libraries are derived from the IgG mRNA of B-cells of an immune source, such as immunized animals or in some instances human B-cells. These

libraries result in more and sometimes higher affinity antibodies than those rescued from hybridomas [163,165,166].

The second type of libraries is obtained from large naïve repertoires of antibody fragments which can be recovered from non-immunized donors. In this case, mRNA from B-cells is isolated from haematopoietic organs and IgM variable regions are then amplified by PCR using degenerate oligonucleotide primer sets and cloned directly into phage display vectors [167-170.]

Synthetic antibody libraries are constructed entirely *in vitro* using oligonucleotides that introduce areas of complete or tailored degeneracy into the CDRs of one or more V-genes [171]. By introducing degeneracy in specific position codons of synthetic oligonucleotides, the degree of randomization can be precisely controlled. There are examples of semisynthetic antibodies libraries, which incorporate a balance of natural and synthetic repertoires and are often created to increase natural diversity while maintaining a certain level of functional diversity [172].

1.6.5 Antibody Selection Methods

Many methods can be used to obtain antibody genes for the construction of intrabodies. The most relevant selection platforms will be briefly described below.

1.6.5.1 Phage Display Technology

Phage display technology had its beginnings in 1985 when George Smith cloned a fragment of the gene encoding the *Eco RI* endonuclease into the gene

III insertion site of filamentous phage f1 and created a fusion protein, which was displayed on the virion surface [173]. Following these studies, describing the display of peptides on the surface of filamentous phage, several laboratories simultaneously developed phage display systems for antibody fragments in the early 1990s [174,175]. In these systems, antibody genes are linked to the amino terminus region of the phage minor coat protein pIII. When expressed, the encoded fusion product is incorporated into the mature phage particle during normal phage biogenesis. The resulting phage particle expresses antibodies on its surface and contains the antibody encoding gene. This linkage between antibody genotype and phenotype allows the enrichment of antigen-specific phage antibodies, using immobilized or labelled antigen [176,177]. The selection process by phage display can be divided into four main steps: (1) coating of antigen; (2) incubation of phage repertoire with antigen; (3) washing to remove non-specific phages; and (4) elution and reamplification of antigen-specific phages. Usually, three to six rounds of binding, elution and amplification are sufficient to generate antibodies with high affinity and specificity. Still, this selection success hinges on the combination of this display and enrichment method, with the generation of large and highly diverse repertoires of phage antibody libraries [178-180]. Phage display technology is currently one of the most well-established platforms used to identify intrabodies.

1.6.5.2 Ribosome Display Technology

Ribosome display, originally described by Mattheakis and co-workers [181], is a cell-free system for the *in vitro* selection of peptides and proteins from large libraries. It uses the principle of coupling individual nascent proteins

(phenotypes) to their corresponding mRNA (genotypes), through the formation of stable protein-ribosome-mRNA (PRM) complexes. This permits the simultaneous isolation of a functional nascent protein, through affinity for a ligand, together with the encoding mRNA, which is then converted and amplified as DNA for further manipulation. Ribosome display has recently been adapted to the screening of antibody libraries by Pluckthun[182] and Taussig [183].

Ribosome display has several potential advantages over phage display. Firstly, larger libraries (10^{12}–10^{14} members) can be constructed with ribosome display compared to phage display. Secondly, ribosome display avoids transformation and cloning steps that can be laborious with large phage display libraries and may result in the loss of diversity. Finally, libraries can be further diversified during PCR steps in ribosome display using low-fidelity polymerases. Thus, high-affinity antibodies initially not present in libraries can be generated and selected against a large array of antigens [184].

1.6.5.3 Cell Display Technology

Before the advent of phage display systems, antibodies were displayed in bacterial cells, although isolation of specific clones was difficult due to inefficient screening methods. The recent development of high-speed flow cytometers has re-activated the efforts in cell surface display, and several high-affinity antibodies are now starting to be isolated by this method. For screening purposes, a library of cells (e.g. *E. coli* or *Saccharomyces cerevisiae*), each displaying multiple copies of a different antibody variant, is incubated with a fluorescently tagged antigen in buffer [185,186]. Cells displaying antibodies that

specifically bind the ligand become fluorescently labeled and are easily isolated by fluorescence-activated cell sorting (FACS). With flow cytometry, the binding of each clone in the library to a particular ligand is easily determined quantitatively. Therefore, the brighter the fluorescent signal for antigen binding, the higher the affinity of the clone for the antigen. In addition, parameters such as ligand concentration or time for the dissociation of antibody-antigen complexes can be easily optimized. These features are particularly significant for antibody affinity maturation, and in fact, the limited data reported so far indicate that cell surface display may be a superior screening technology for that purpose [187,188].

1.6.5.4 Yeast Two-Hybrid System

Intracellular interaction of antibody with target antigen in yeast could be evaluated by providing conditional cell growth advantage through controlled expression of selected reporter genes [152,155]. In such a system, the antigen is usually cloned in frame at C-terminus of the DNA binding domain of the *E. coli* protein LexA and scFv antibody fused at the N-terminus of the activation domain of the herpes virus 1 VP16 transcription factor. After cotransfection of these plasmids into the yeast or mammalian cells and upon interaction, the antibody-antigen complex binds to the promoter of reporter genes containing relevant DNA binding sites and activates their transcription. For example, activation of *HIS3* gene controlled by a minimal transcription promoter with LexA binding sites upon antigen-antibody interaction enables the host yeast to grow on plate without histidine and with 3-amino-triazole (3-AT) for selection [155]. In addition, Auf der Maur *et al*. (2001) developed a related

procedure in which stable intrabodies could be selected independent of their antigens based on strong correlation between the degree of reporter gene activation and the stability/solubility of the fused antibody [156]. It should be noted that a library size of 10^7 clones can be screened per assay in these yeast systems, which is at disadvantage as compared to $>10^{10}$ clones that can be handled with relative ease using phage display.

1.6.5.5 Protein Fragment Complementation Selection (PCA)

Protein fragment complementation assay (PCA) has been adapted to screen for antibody binding by reconstituting the activity of dihydrofolate reductase (DHFR) that confers a survival advantage on transformed *E. coli* [190]. In this method, antibodies and antigens are linked with dissected portions of mouse DHFR (mDHFR). The interaction of antibody and antigen brings the two halves of dissected mDHFR together, thus restoring its enzyme activity and allowing transformed *E. coli* to grow on minimal medium in the presence of antibiotic trimethoprim [190]. Four different target antigens were tested by this system, it was shown that there was about seven orders of magnitude more colonies in antigen pool containing specific antigen as compared to few colonies found in pool with only non-specific antigen [190]. The procedure is relatively simple and fast and only involves the transformation of plasmids, functional expression of the fusion proteins and analysis of the grown bacterial cells. Notably, it gives a very low background of false positive results. The antigen does not need to be purified and immobilized. However, since the screening is performed in the cytoplasm of *E. coli*, antibodies with inherent stable framework can be isolated under reducing conditions but they would be

lack of post-translational modification.

1.6.6 Intrabody Delivery

Currently, lentivirus vectors are the main gene delivery tool used to obtain high transduction efficiency and long-term expression of intrabodies. Lentiviral vectors were developed by investigators at the Salk Institute in the mid-1990s, using design strategies defined over the prior decade to optimize gamma-retroviral vectors for safety and effectiveness [191]. Like the lentiviral subclass of *Retroviridae* from which they are derived, lentiviral vectors can integrate in nondividing cells, making them more effective than retroviral vectors for gene transfer to postmitotic or slowly dividing cells, which may include hematopoietic stem cells and T cells [191-196]. Many of the lentivirus vectors used in gene therapy are based on the HIV-1. Typically, HIV vectors can accommodate fairly large gene inserts and can provide long-term expression through chromosomal integration [193]. Today, the limitations of using lentivirus vectors in clinical trials are mainly due to the lack of sufficient methods for production of high-titer virus stocks and the safety concerns related to their origin from HIV, despite the engineering of packaging cell lines and deletions of genes required for viral replication [193,197,198]. One approach to address safety issues has been to develop lentivirus vectors incapable of replication in human cells [193]. Another main challenge faced by lentiviral gene delivery systems is the development of vectors that can efficiently target specific cell types. The most common approach developed to date changes the tropism of envelope lentiviral receptor binding domain with a cell-specific ligand or a scFv that recognizes and binds to specific cell surface

molecules [199-208]. Some of these approaches have allowed some degree of cell-type-specific viral entry, however, the envelope alterations in most strategies also affect viron assembly and lead to low fusion activity and viral titers [193,194,209]. Within this context, there is strong interest in developing new and improved strategies to allow lentiviral cell-specific targeting. The present dissertation thesis will also focus the development of lentiviral-derived vector that can efficiently target specific-cells.

1.6.7 Comparison of intrabodies and other intracellular targeting strategies

Other strategies for the inactivation of specific intracellular molecules are also being developed. These techniques include the use of antisense RNA [210] and RNA interference (RNAi) [211,212]. Unlike intrabodies, which are targeted to specific protein domains, these methods act at the level of mRNA destruction or inhibition of protein synthesis, and can potentially entirely remove the protein target from the cell. The use of RNAi has proven to be highly effective in the removal of target RNA. Stably expressing vector systems for the small interfering RNA (siRNA) molecules have been developed, potentially allowing long-term expression in cells [213].

In general, the RNAi strategy follows a simple design with well-defined algorithms and is less technically challenging than intrabody techniques; however, it has non-specific effects [211,212]. This non-specificity takes the form of dsRNA-triggered responses mediated by interferon-associated pathways, which do not exist in invertebrates and plants [214-216]. A gene expression profiling study indicated that >1,000 genes involved in diverse cellular

functions are non-specifically stimulated or repressed in mammalian tissue-culture cells treated with conventional 21-bp RNAi [215,217]. Another limitation of the RNAi technique is the relatively short half-life of the desired knockdown effects unless stimulatory RNAs are expressed via transfected recombinant DNA (which delays observation of the knockdown effect). In contrast, it is useful to use intrabodies for a nearly instantaneous and durable effect. Intrabodies can block particular binding interactions of target molecules, by changing their structural conformation or by exerting positive functions including catalytic functions, stabilization of protein-protein or protein-DNA interactions.

1.6.8 Rabbit Antibodies as Intrabodies Source

The rabbit antibody repertoire has been used for decades in diagnostic applications in the form of polyclonal antibodies. Nowadays, it offers an attractive alternative for the generation of therapeutic monoclonal antibodies [218,219]. Humanized antibodies from immunized rabbits extend the accessible epitope repertoire of a given antigen [220]. Epitopes that are not immunogenic in human or mice might be immunogenic in rabbits. This is of particular interest for the development of therapeutic scFv or single-domain antibodies that need to be evaluated in disease mouse models, and are required to recognize both the human antigen and its mouse homologues. Moreover, as previously demonstrated, rabbit antibodies can be converted to humanized antibodies that retain both high specificity and affinity for the antigen [220]. In addition, we have already demonstrated that rabbit antibody fragments against viral proteins can be highly expressed in reducing environments of mammalian cells and

consequently block viral protein functions [121,131,221]. Within this context, rabbit antibodies are used as intrabody source in the present thesis.

1.7 HIV-1 IN protein as therapeutic target for intracellular immunization

Each step of the HIV life cycle can be a potential target for inhibition. RT and PR were the initial targets for inhibition and subsequently, viral entry and integration received considerable interest from the scientific community [82]. As describe above, IN makes an attractive target for inhibition since is absolutely required for a stable and productive infection by HIV-1 [222-224]. Furthermore, IN is highly conserved among HIV-1 clinical isolates and no human homologue known of IN exists [16,19,224,225]. During the last 10 years, several IN inhibitors have been designed to block this critical step in the HIV replication cycle [17,226]. However, progress in the development of IN inhibitors have been slow, given that most of these compounds have not met the minimum standards required to be defined as lead molecules in the search for clinically useful, or turned out to be toxic in cell cultures [17,18]. Within this context, intracellular antibodies against IN protein might be a strong alternative approach to identify new therapeutic molecules that can efficiently block the integration process. Therefore, in the present thesis, IN of HIV-1 was chosen as a potential therapeutic target for our intracellular immunization strategy. Structure and function of IN will be therefore described in detail in the following section.

1.7.1 HIV-1 IN

The HIV-1 IN protein belongs to a protein superfamily of nucleotidyl

transferases that include RNase H, Holliday junction resolvase RuvC, bacterial Mu and Tn5 transposases, the recombination-activating gene (RAG) 1 subunit of the RAG1/2 recombinase, and the Argonaute protein of the RNA-induced silencing complex (RISC) [227-229]. These enzymes break and/or join nucleic acids via their phosphodiester backbones. The enzyme active-site domains adopt a similar protein fold (referred to as the RNase H fold) and harbour conserved Asp and Glu amino acid residues that coordinate Mg^{2+} metal ions to effect bimolecular in-line nucleophilic substitution (Sn2) reactions at the scissile phosphoester bond [19].

1.7.2 HIV-1 DNA Integration

The process of retroviral integration has been divided as a two well-characterized catalytic steps referred to as 3' processing (the removal of two terminal nucleotides from the 3'ends of the viral double-stranded DNA) and strand transfer (transfer of viral DNA to the human chromosomal DNA) [19,69,230,231]. However, if HIV-1 integration is to be considered as possible drug target, it may be better described it as four unique processes that can be targeted and potentially inhibited: (1) assembly of a stable complex between IN and specific viral DNA sequences at the end of the HIV-1 long terminal repeat regions (LTRs), (2) 3'-end processing, (3) strand transfer, and (4) DNA gap repair and ligation (Figure 1.4).

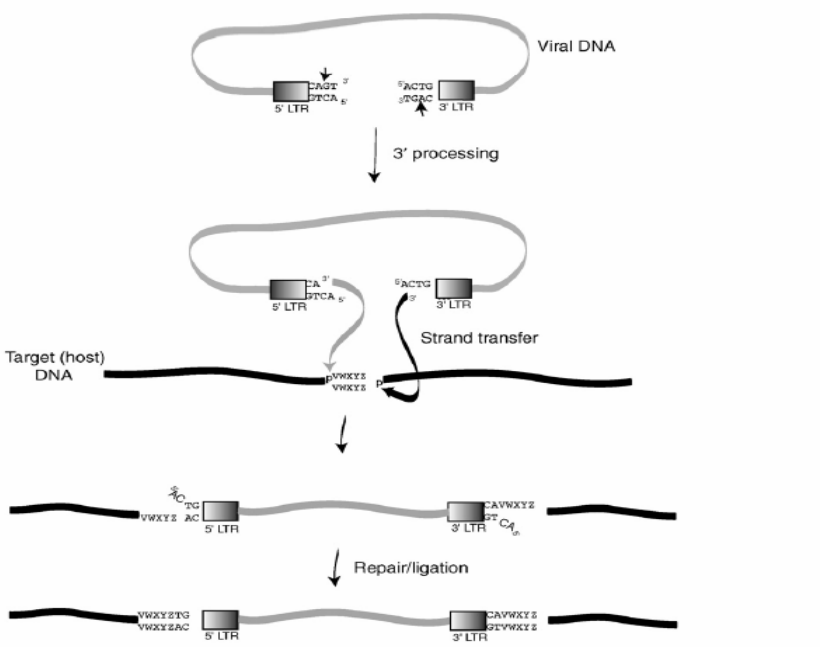

Figure 1.4 DNA cutting and joining steps in retroviral integration. The IN enzyme cleaves the viral DNA ends after a CA dinucleotide (3'end processing) and integration occurs in the nucleus (strand transfer). Viral DNA integration leads to a signature duplication of a short stretch of cellular DNA, represented as 'VWXYZ'. After integration, cellular enzymes repair and ligate the viral/cellular DNA junction (adapted from [12]).

(1) IN binds to viral DNA

After the viral RT enzyme creates a double-stranded DNA product, IN assemble at the ends of the viral DNA and binds to the HIV-1 LTR region. The viral LTR ends contain specific DNA sequences that are required for recognition by IN resulting in a stable viral DNA-IN binding complex [19,69,232]. This is a crucial initial step in the integration process. One class of INIs in preclinical development is the pyrano-dipyrimidines (PDPs). These compounds have been shown *in vitro* to prevent viral DNA binding to IN, thus

preventing proper assembly of viral DNA (step 1) and consequently any further steps of integration [233].

(2) First catalytic step - 3'end processing

During 3'-end processing, IN removes a pGT dinucleotide at each end of the viral DNA LTRs adjacent to a highly conserved CA dinucleotide from the 3' end extremity of the linear viral genome producing new 3' hydroxyl ends (CA-3-OH) [19,230,231,234]. This reaction takes place in the cytoplasm of the $CD4^+$ T-cell within the preintegration complex (PIC). This complex contains linear viral DNA and several viral proteins including MA, RT, IN, and NC [69,235]. The HIV-1 PIC is a cytoplasmic nucleoprotein structure derived from the core of the virion and is responsible for reverse transcription of viral RNA to DNA, transport to the nucleus, and integration of the viral DNA into the genome of the host cell [19,69]. Another class of INIs in preclinical development is the styrylquinolines. These agents have been described as potent *in vitro* 3'-end processing inhibitors by directly competing for HIV LTR substrates [236].

(3) Second catalytic step - strand transfer or "transesterification"

Strand transfer is temporally and spatially separated from 3' end processing and occurs after the transport of the PIC from the cytoplasm through a nuclear pore into the cell's nucleus [19,237,238]. After the PIC is transported, the processed viral DNA-IN complex is joined to the host DNA by IN. Both 3' ends of the viral DNA generated from the first reaction are processed simultaneously [239]. In the nucleus, the IN binds to the host's cellular DNA and mediates a concerted nucleophilic attack by the 3' hydroxyl residues of the viral DNA on

phosphodiester bridges located on either side of the major groove in the target DNA [19,239]. Next the processed CA-3-OH viral DNA ends are ligated to the 5'-O-phosphate ends of the target DNA, irreversibly binding the viral DNA to the cellular DNA [239,240]. IN strand transfer inhibitors (STIs) can prevent this catalytic reaction from taking place. The β diketo acids (DKAs) and their DKA derivatives are examples of strand transfer inhibitors that have been shown *in vitro* and *in vivo* to effectively stop this process [97,241]. Oxadiazoles are another class of INIs in early development that are described as nuclear import inhibitors [242]. These compounds have been shown *in vitro* to indirectly prevent the second catalytic step of integration by targeting and preventing the nuclear translocation of the HIV-1 PIC [242].

(4) Gap repair and ligation of viral DNA to host DNA

After the strand transfer reaction, the new product (viral DNA-cellular DNA combination) is a gapped intermediate product in which the 5-phosphate ends of the viral DNA are not attached to the 3-OH ends of the host DNA [230]. The integration reaction is completed by the removal of the two unpaired nucleotides at the 5' end of the viral DNA and the repair of the single-stranded gaps created between the viral and target DNA [243]. Although IN may be involved in these repair reactions, it is not necessary because the host cell already has the machinery to carry out such processes. So repair is probably accomplished by host cell DNA repair enzymes [230,243]. Staggered strand transfer and gap repair result in the duplication of host cell sequences immediately flanking the inserted proviral DNA. For the integration reaction, no source of energy is needed and only divalent cations such as Mn^{2+} or Mg^{2+}

are required for the catalytic activity [239].

1.7.3 HIV-1 IN structure

The HIV-1 IN consists of a 288 amino acid protein (32 kDa) encoded at the 3'end of the HIV *pol* gene, which contains three distinct domains: (1) N-terminal, (2) catalytic core, and the (3) C-terminal (Figure 1.5A) [19,230,244,245]. To date, the insolubility of HIV-1 IN has been the main barrier in obtaining a crystal structure of the full-length IN. However, the discovery of a single mutant, through site-directed mutagenesis, within the catalytic core of IN dramatically increased its solubility while retaining catalytic activity [246]. The substitution of lysine 185 for phenylalanine enabled IN50-212 to be crystallized and its structure to be solved for the first time by Dyda *et al* [227]. Since then other crystal structures of the isolated catalytic core and in complex with either the C-terminal or the N-terminal have been resolved (Figure 1.5B) [247-251].

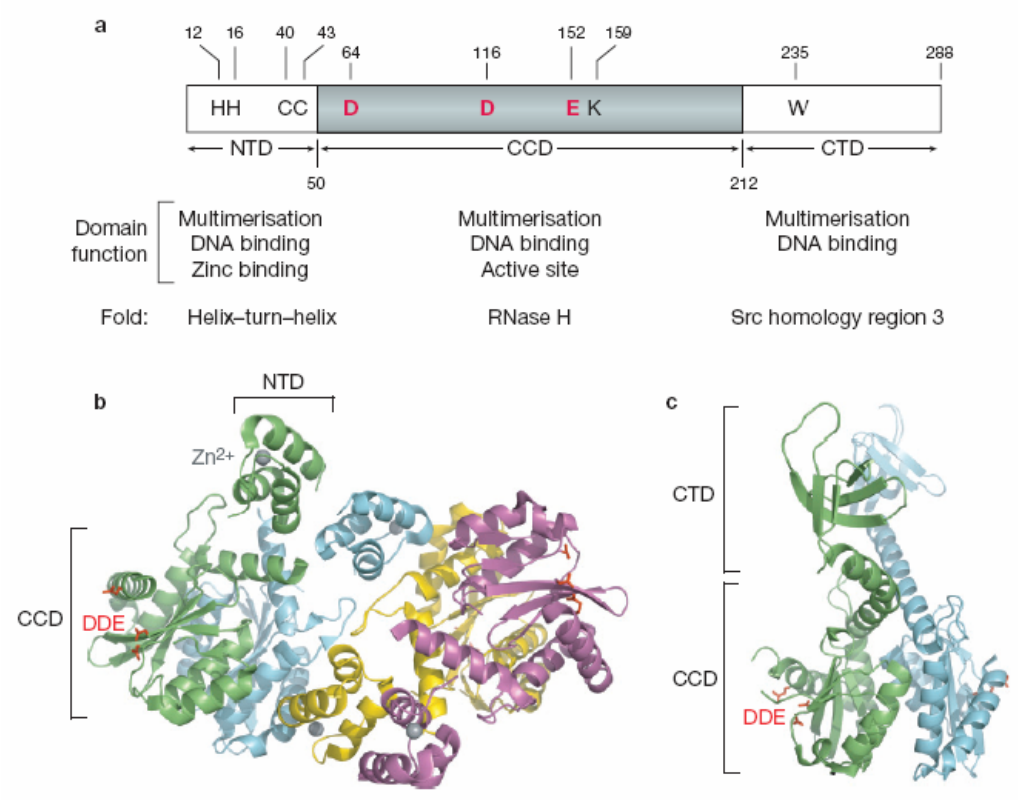

Figure 1.5. **Integrase protein domains and structures.** (a) Domain organisation and amino acid residues conserved among retroviral integrase proteins. The enzyme comprises the N-terminal domain (NTD, aa 1–49), catalytic core domain (CCD, aa 50–212) and C-terminal domain (CTD, aa 213–288). The H and C residues within the NTD are additionally conserved among retrotransposon integrase proteins. The D and E residues in the CCD form the DDE motif (red font). The K159 residue, which contributes to binding to the viral long terminal repeat (LTR), is found among a smaller subset of transposase proteins. The W 235 in the CTD is conserved only among the retroviral integrases. The function and fold of the protein domains are indicated below the domain organisation. (b) X-ray crystal structure of IN1–212 [protein database code (PDB) 1K6Y](Ref. [250]). Two NTD–CCD dimers (green/blue and yellow/magenta) are identified within the crystallographic asymmetric unit. Grey sphere, zinc atom; red side chains, DDE active-site residues. (c) X-ray structure of dimeric IN52 –288 (PDB code 1EX4). The CTD of the blue monomer may help to position the LTR DNA end to the DDE active site of the green monomer for catalysis (Ref [251]) (adapted from [72]).

(1) N-terminal domain - NTD

The N-terminal domain (amino acids 1–49) is believed to be involved in protein multimerization and contains a histidine-histidine-cystine-cystine (HHCC) motif that coordinates zinc binding [244,252-255]. The binding of zinc to the HHCC motif stabilizes the protein folding of IN and bound zinc is required for optimal enzymatic activity [252]. Point mutations of His or Cys residues abolish the zinc binding ability of IN and affect the 3'-processing and strand transfer activity but do not impair disintegration activity [256,257]. These results confirm that the active site is not located in the NTD and that this domain is not the single DNA binding domain in the protein [257-261]. Further, chimeric experiments with HIV-1 IN and visna virus IN indicated that the NTD also does not contribute to viral DNA specificity [262]. However, the NTD is proposed to interact with DNA in the context of the whole protein [257-260].

The structures of the NTD of both HIV-1 and HIV-2 IN have been solved by multidimensional heteronuclear magnetic resonance [249,263]. The HIV-1 IN N-terminal domain is composed of four α helices. A hydrophobic core stabilizes the lower region of the monomer, and coordination of zinc to the His and Cys residues of the HHCC motif stabilizes the upper part of the monomer structure [249]. The monomer of the N-terminal domain of HIV-2 integrase adopts the same structure [263]. The N-terminal domain of HIV-1 IN is a dimer with an interface composed predominantly of hydrophobic residues, whereas that of HIV-2 appears to be monomeric [19].

(2) Catalytic core domain - CCD

The central or catalytic core domain (amino acids 50–212), which is well conserved among retroviral IN and also shares significant sequence similarity with the transposase proteins of many mobile genetic elements, contains the well-know DDE motif [19]. These invariant residues, Asp64 (D64), Asp116 (D116), and Glu152 (E152) are key residues of the active site [257,264]. In the context of full-length IN, the CCD has been assigned a direct role in carrying out the 3'-processing and strand transfer reactions, in recognizing the conserved CA/TG base pairs near the viral DNA end and in recognizing target DNA [264-269]. Mutations of the conserved residues D116N, E152Q abolished detectable activity in all three processes while the mutations D64E and D64N retain barely detectable strand transfer and disintegration activity [244,264]. Mutational studies on other residues within the CCD identified K159 as an important residue for the recognition of the conserved CA dinucleotide of viral DNA [270] and excluded other residues initially thought to be important for activity [271]. The flexibility of the active site loop that consists of residues 141-148 is important for integration. When residues G140 and G149 that act as conformational hinges for the loop are replaced with alanines the flexibility of the loop is diminished. These mutants loose catalytic activity but retain DNA binding affinity. Based on this observation the loop is proposed to have a role in a reaction step after the DNA binding step [272]. The importance of the flexible loop 140-149 was also corroborated by molecular dynamics studies [273].

The structure of the catalytic domain of HIV-1 IN has been determined by x-

ray crystallography [227]. It is a dimer that is spherical in shape, with each monomer forming one hemisphere. The catalytic residues D64, D116, and E152 in the IN structure are in close proximity, coordinate divalent metal ion, and define the active site [227]. However, the residues comprising the active site region exhibit considerable flexibility, suggesting that binding of DNA substrate is required to impose the precise configuration of residues that is required for catalysis.

(3) C-terminal Domain – CTD

The C-terminal domain (amino acids residues 213-288) is the least conserved domain of IN [19]. The CTD is proposed to be involved in the multimerization of IN [274], in binding DNA nonspecifically and is required for 3'-processing and strand transfer activity [234,265,275]. Mutational analysis identified residues in the CTD critical for oligomerization and DNA binding [276]. Viral DNA specificity was mapped to both the CTD and CCD and CTD-DNA interactions were identified just inside the CAGT base pairs of the viral DNA. A similar function for site-specific DNA binding is exhibited by transposases [277]. Earlier studies attributed the interactions of the target DNA to the CTD however; the present studies indicate that all three domains interact with the target DNA [19]. Furthermore, experiments showed that CTD is involved in interactions with HIV-1 RT [278,279]. The structure of the C-terminal domain of HIV-1 IN was determined by two laboratories using multidimensional heteronuclear magnetic resonance [247,280]. Both structures are dimeric, with each monomer composed of five-stranded β barrel that is topologically very similar to solved structures of Src homology 3 (SH3) domains. The dimer is formed by stacking of β strands

2, 3, and 4 of each monomer, with the β sheets from the two subunits stacked in an antiparallel configuration to form the dimer interface [19,247,280].

1.7.4 Multimeric Organization of HIV IN

The active form of IN is believed to be a multimer although the degree of active multimer, has not been determined. The evidence for an active multimer is derived from domain deletion studies demonstrating that isolated domains of HIV-1 IN do not possess activity [19]. In contrast, independent domains of IN can complement each other and restore IN activity [281-284]. The *trans* interactions between the NTD and the CCD of HIV-1 IN mutant proteins indicated that an intact NTD and CCD must be part of different monomers within the multimer. On the other hand, the CTD could function in both *cis* and *trans* interactions with the CCD [285]. Based on gel filtration experiments full-length IN exists in a dimer-tetramer equilibrium in solution. The isolated CCD and CTD and the two domain CCD-NTD complex exist as a dimer [19]. On the other hand, the two domain CCD-CTD complex exists in a dimer-tetramer equilibrium. These results point to the CCD and CTD as the necessary domains for multimerization [274]. Nevertheless, Zn^{2+} was found to promote multimerization indicating that the NTD as well is important for protein-protein interactions [252,286]. Experimental data point towards a tetramer as the minimal active form of IN. Cross-linked tetramers could catalyze full-site integration *in vitro* while dimmers could integrate just one viral LTR end to the target DNA [287]. In detailed IN-DNA models DNA cross-links occurred in *trans* to the active site [288,289]. A reconstructed tetramer of HIV-1 IN was consistent with the cross-linking experiments and confirmed the observation that the

F185K mutation disrupts complex formation *in vivo* by preventing the tetramerization of IN [290].

1.7.5 Host Factors of Integration

The exact mechanism of HIV-1 integration has not been established so far and studies support the idea that host factors are required to accomplish integration of the viral DNA into the human genome *in vivo*. The disruption of key interactions between IN and direct cellular cofactors might provide a novel therapeutic approach for the design and development of new classes of anti-retroviral agents [291]. Therefore, a brief description of each IN-interacting cellular cofactor is shown below.

LEDGF/p75 is the most recently identified cellular partner of HIV-1 IN. LEDGF/p75 is a nuclear protein that may act as a chromatin docking factor or receptor for lentiviral pre-integration complexes. LEDGF/p75 tethers HIV-1 IN to chromatin, protects it from degradation, and strongly influences the genome-wide pattern of HIV integration [292].

BAF, a single polypeptide that consists of 89 amino acids was first identified as a host factor of MoMLV [293] and later as part of the PIC complex of HIV [294]. BAF prevented autointegration in the MoMLV virus and a similar role has been proposed for the HIV-1 virus [294].

HMG-I(Y) was identified in PICs isolated from HIV-1 infected cells. *In vitro* depletion of HMG-I(Y) from PICs diminished activity that was restored upon back-implementing the protein [295]. Mechanistic studies indicated that an

interaction between HMG-I(Y) and cDNA promotes the formation of active IN-DNA complexes, presumably by bringing together DNA segments [295].

INI1, identified in a yeast two-hybrid screen, is the first protein shown to directly interact with HIV-1 IN and stimulate its *in vitro* activity [296]. The role of full-length INI1 in HIV-1 replication is not yet clarified and the lack of strong experimental evidence makes it difficult to assign a function for INI1 during HIV-1 replication *in vivo* [296,297].

The human polycomb group EED protein is another host factor of HIV-1 IN and may have a functional role at the early steps of infection. EED contains two discreet binding domains located at its N-terminal domain that binds at the CTD of IN [298]. Another candidate co-factor is the yeast cellular protein HSP60 that interacts with HIV-1 IN *in vitro* and stimulates processing and strand transfer activities [299].

1.8 Vif protein as therapeutic target for intracellular immunization

The Vif protein of HIV-1 plays a dramatic importance in viral infectivity [20,21]. Vif is a basic protein of 23 kDa required in the virus-producing cells during the late stages of infection to enhance viral infectivity 10- to 1000-fold [300-303]. HIV-1 *vif*-defective virus can replicate in permissive cells such as Jurkat and SupT1 cells, but cannot replicate in non-permissive cells such as macrophages, primary human T cells, and some restrictive T cell lines [300-304]. Previous studies found that non-permissive cells contain an anti-viral cellular factor capable of suppress the HIV infectivity, recently identified as APOBEC3G, for which the antiviral action is overcome by Vif [22,23]. APOBEC3G is a virion-encapsidated

cellular protein that deaminates dC to dU in minus-strand viral cDNA during reverse transcription, preferentially at CCCA sequences [22,23,305,306]. The uracil-containing cDNA may activate a cellular uracil-DNA-glycosylase causing the failure of reverse transcription, characteristic of *vif*-defective virus [307] and impair the integration of the provirus in the host genome [308]. Furthermore, if the reverse transcription is completed at low efficiency and the resulting proviral double-stranded cDNA is integrated in the cell genome, the massive C-U conversion in the minus strand leads to a massive G to A hypermutation of the proviral plus-strand cDNA [305,306,309]. The exact mechanism how Vif counteracts APOBEC3G action is not clear. It has been shown that Vif inhibits APOBEC3G translation or intracellular half-life [310,311]. Recent evidences showed that Vif interacts with APOBEC3G as part of a Vif·Cul5·Sk-p1-Cullin-F-box complex resulting in the polyubiquitination and proteasomal degradation of APOBEC3G [312,313]. The increase degradation and/or reduced level of APOBEC3G expression by Vif reduces its incorporation into virions and consequent absence during reverse transcription in the target cell, allowing the virus to replicate [311,314].Thus, Vif is an excellent target for therapeutic intervention.

Recently we developed a specific scFv from immunized rabbits to HIV-1 Vif protein that was expressed intracellularly and inhibited reverse transcription and viral replication [131]. Moreover, we have also shown that anti-Vif camelized VH domains alone may be useful as intrabodies, with improved intracellular expression [121]. In the present dissertation, we will now show that rabbit VL domains can also be potentially used as intrabodies.

CHAPTER 2

Generation of recombinant rabbit single-chain antibodies that simultaneously bind to the catalytic and C-terminus domains of HIV-1 integrase protein and strongly inhibit HIV-1 infection

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Abstract

Since the discovery of the HIV-1 as the causative agent of AIDS its spread has been dramatic. Current therapies use a combination of drugs targeted at the viral RT and PR enzymes. The clinical benefits of these therapies are considerable, although many multiple drug-resistant viral strains have arisen. The addition of new anti-HIV drugs targeting a third step of the viral replication may help in preventing resistance development. The HIV-1 IN protein establishes infection during the early stages of the retroviral life cycle and therefore is an attractive target for therapeutic intervention. Based on this, our strategy is to make susceptible CD4+ cells resistant to infection by expressing scFv intrabodies against HIV-1 IN. In the present study, we have for the first time immunized rabbits with HIV-1 IN and developed a combinatorial scFv library against IN. We were able to identify 5 different scFv antibodies with high binding activity and specificity to IN. These scFv bound simultaneously to the catalytic and C-terminus domains of IN. Cells expressing anti-IN scFvs localized in either cytoplasmic or nuclear compartment were highly resistant to HIV-1 infection. Furthermore, when HIV-1 particles where produced in the presence of anti-IN scFv, the expression of intrabodies did not affect virion production significantly. However, it markedly reduced the infectivity of progeny virions due to the incorporation of anti-IN scFvs into the viral particles. These results provide proof-in-principle that anti-IN scFv intrabodies can be designed to block early and late stages of HIV-1 replication without causing cellular toxicity, and as a result, they may be useful agents for "intracellular immunization"-based on gene therapy strategies for HIV-1 infection.

2.1 Introduction

The spread of HIV-1 has been dramatic since the early eighties, when the virus was discovered as the causative agent of AIDS [1,2]. In the absence of an effective vaccine against HIV, a worldwide search has been made in the past two decades to develop small-molecule inhibitors to target essential steps in the viral cycle [10]. Highly Active Antiretroviral Therapy (HAART) is one of the most used treatment regimens in our days. The regimen employs a combination of therapeutic agents that target the viral reverse transcriptase (RT) and protease enzymes (PR) [10-12]. In the developed world, access to HAART has led to significant reductions in the morbidity and mortality attributed to HIV/AIDS. However, the emergence of drug-resistant virus isolates is causing an increasingly detrimental impact on treatment options and the disease outcome [13-15]. As a result, there is a pressing need to identify and develop new drugs that can be effective against these highly resistant virus isolates.

Integrase (IN) is currently the focus of an intense research effort to develop new anti-HIV-1 drugs [16-18]. This enzyme catalyses the integration of the HIV genome into the chromosome of the host cell, arguably the most insidious step in the infection process [19]. The HIV-1 IN consists of a 288-amino-acid protein (32 kDa) encoded at the 3'end of the HIV *pol* gene, which contains three distinct domains: N-terminal, catalytic core, and the C-terminal [19,230,244,245]. The N-terminal domain (amino acids 1–49) is believed to be involved in protein multimerization and contains a histidine-histidine-cystine-cystine (HHCC) motif that coordinates zinc binding [244,252-255]. The central or catalytic core domain (amino acids 50–212) contains a highly

conserved triad of acidic residues D64, D116, and E152 (DDE motif) that are involved in catalysis [19,257,264]. Mutation of any of these acidic residues has been shown to abolish HIV-1 IN activity [244,264]. The C-terminal domain (amino acids 213–288), the least conserved of the three protein domains, binds the viral DNA ends and might also contribute to the binding of chromosomal DNA during integration [234,265,274,275].

During the last 10 years, several IN inhibitors have been designed to block this critical process in the HIV replication cycle [17,226]. However, progress in the development of IN inhibitors has been slow, given that most of these compounds have not met the minimum standards required to be defined as lead molecules in the search for clinically useful application, or turned out to be toxic in cell cultures [17,18]. Only recently this situation has changed when the first IN inhibitor for clinical use, the MK-0518 (raltegravir), was validated and received the FDA approval [97]. Although a second compound, GS-9137 (JTK-303, elvitegravir) is in clinical trials, new agents and therapeutic approaches must continue to be identified and developed to block this crucial step in HIV-1 replication [98,99].

Over the recent years, gene therapy has been highly regarded as a new form of molecular medicine in treatment of HIV/AIDS, either as an alternative or as a complement to anti-retroviral chemotherapy [24-26]. Within this context, intracellular antibodies (intrabodies) represent a new class of neutralizing molecules with potential use in gene therapy approaches [27-29]. An intrabody consists of an antibody designed to be expressed intracellularly and directed to different subcellular compartments where they can exert their function more effectively [110,111]. Intrabodies can be expressed in several forms [30].

The most commonly used format consists of a single-chain antibody (scFv) in which the variable domain of the heavy chain (VH) is connected to the light chain (VL) through a peptide linker. The specificity and affinity of the parent antibody is preserved in this process [110,111]. The binding of an intrabody to its molecular target has the potential to block, suppress, alter or even enhance the process mediated by that molecule [31-33].

A number of murine monoclonal antibodies (mAbs) directed against HIV-1 IN has been raised and characterized by several groups [315-323]. Some of these mAbs have already been cloned into scFv fragments and tested as intrabodies [320,321]. Although some of these block HIV-1 replication, they are all derived from murine IgG molecules and were generated by hybridoma technology. The rabbit antibody repertoire has been used for decades in diagnostic applications in the form of polyclonal antibodies. Nowadays, it offers an attractive alternative to the murine antibody repertoire for the generation of therapeutic monoclonal antibodies [220]. Combinatorial rabbit scFv libraries have the potential to display the entire immunological record of an individual, allowing the detection and recovery of any antibody ever made, irrespective of whether it is currently being produced. In addition, we have recently demonstrated that rabbit antibody fragments against viral proteins can be highly expressed in reducing environments of mammalian cells and consequently block efficiently viral protein functions [121,131,221].

In the present study, we have for the first time immunized rabbits with HIV-1 IN and developed a combinatorial scFv library against IN. We were able to identify 5 different scFv antibodies with high binding activity and specificity to IN. These scFvs bound simultaneously to the catalytic and C-

terminus domains of IN. In addition, antibodies binding to these epitopes inhibited the strand transfer processing, whereas no effect was observed in the 3'end processing. Intrabody-expressing cells localized in either cytoplasm or nuclear compartments were highly resistant to HIV-1 infection. Moreover, when HIV-1 particles where produced in the presence of anti-IN scFv, the expression of intrabodies did not affect virion production significantly. However, it markedly reduced the infectivity of progeny virions due to the incorporation of anti-IN scFvs into the viral particles. These findings provide proof-in-principle that rabbit anti-IN scFv intrabodies can be designed to block early and late stages of HIV-1 replication. As a result, they might be useful agents for "intracellular immunization"- and used as new tools to study the structure and function of HIV-1 IN due to their epitope binding characteristics.

2.2 Material and Methods

2.2.1 Plasmids

Plasmid coding for HIV-1_{NL4-3} was obtained from the AIDS Research and Reference Reagent Program. Plasmid pComb3X is derived from pComb3H [324]. Plasmid pCEP-IN^s was kindly provided by Dr. Zeger Debyser and contains an HIV-1 IN synthetic gene that was designed to be highly expressed in mammalian cells [325]. The envelope plasmid pMD.G, was kindly provided by Dr. Didier Trono. Plasmid pcDNA3.1/Zeo(+) and pBR322 were obtained by Invitrogen. pMX-KRAB8FPBS2puro is a retroviral vector derived from pMX-puro, which comprises the 5' LTR and 3' LTR regions, lacks the complete *gag*, *env*, or *pol* genes and contains a puromycin selectable marker [326]. The pMX-KRAB8FPBS2puro vector expresses a single bicistronic message for the translation of the 8FPBS2 zinc finger protein and additionally appends an HA-tag [327]. Plasmid pNde675 has been previously described and is derived from pCR2.1 (Invitrogen) [328].

2.2.2 Antibodies and cell culture

The following antibodies were used for the present study: anti-M13-HRP mAb (Roche), anti-HA-HRP mAb (Roche), rhodamine-conjugated anti-HA mAb (Roche), Alexa-conjugated goat anti-rabbit immunoglobulin antibody (Pierce), anti-IN polyclonal antibody (#7375) and anti-p24 mAb (both from AIDS Research and Reference Reagent Program). 293T (American Type Culture Collection), Gag-Pol 293 cells (Clontech) and HeLa CD4 LTR-β-Gal cells (AIDS Research and Reference Reagent Program) were

maintained in DMEM and Jurkat cells were maintained in RPMI 1640 medium. Media was supplemented with 10% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and 2 mM L-glutamine. Stable Jurkat clones expressing anti-IN scFv were maintained in the presence of puromycin (1 μg/ml). All cell cultures were maintained at 37°C in 5% CO_2. Tissue culture media and reagents were from BioWhitaker.

2.2.3 Rabbit immunization

Two New Zealand White rabbits were treated with 4 subcutaneous injections containing 200 μg of purified HIV-1 IN protein in a 1 ml emulsion of adjuvant (Ribi Immunochem Research, Hamilton, MT). The injections were administered in a 2-3 week intervals. Antisera from the immune animals was analyzed for binding to HIV-1 IN protein by ELISA using HRP-conjugated goat anti-rabbit Fc polyclonal antibodies as secondary antibodies (Pierce). Five days after the final boost, spleen and bone marrow from one leg were harvested and used for total RNA preparation with TRI-REAGENT from Molecular Research Center (Cincinnati, OH) according to the manufacturer's protocol.

2.2.4 cDNA synthesis, antibody library construction and phage display

First-strand cDNA was synthesized using the Superscript Pre-amplification System with oligo(dT) priming (Life Technologies). Specific oligonucleotide

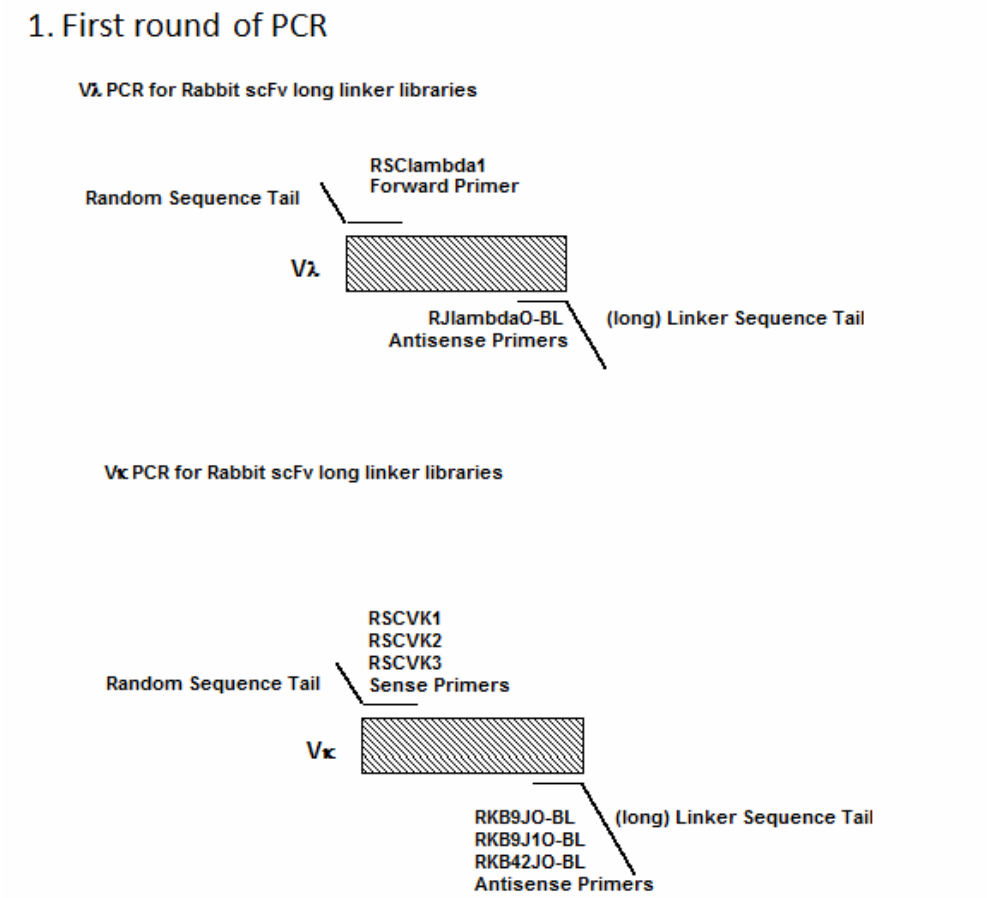
primers covering all known variable rabbit antibody family sequences were used to amplify separately VH and VL gene segments (Table 2.1) [324].

Table 2.1 – Primers used to amplify chimeric rabbit scFv libraries.

Primer	Sequence 5´to 3'
RSClambda1	GGG CCC AGG CGG CCG AGC TCG TGC TGA CTC AGT CGC CCT C
RJlambda0-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA GCC TGT GAC GGT CAG CTG GGT CCC
RSCVK1	GGG CCC AGG CGG CCG AGC TCG TGM TGA CCC AGA CTC CA
RSCVK2	GGG CCC AGG CGG CCG AGC TCG ATM TGA CCC AGA CTC CA
RSCVK3	GGG CCC AGG CGG CCG AGC TCG TGA TGA CCC AGA CTG AA
RKB9J0-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TAG GAT CTC CAG CTC GGT CCC
RKB9J10-BL	GGA AGA TCT AGA GGA ACC ACC CCC ACC ACC GCC CGA GCC ACC GCC ACC AGA GGA TAG GAT CTC CAG CTC GGT CCC
RSCVH1	GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG GAG GAG TCC RGG
RSCVH2	GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG AAG GAG TCC GAG
RSCVH3	GGT GGT TCC TCT AGA TCT TCC CAG TCG YTG GAG GAG TCC GGG
RSCVH4	GGT GGT TCC TCT AGA TCT TCC CAG SAG CAG CTG RTG GAG TCC GG
RSCG-B	CCT GGC CGG CCT GGC CAC TAG TGA CTG AYG GAG CCT TAG GTT GCC C
RSC-F	GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC
RSC-B	GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG

The purified variable regions products were then assembled into scFv format by overlapping PCR. An 18–amino acid linker fragment (SSGGGGSGGGGGGSSRSS) was used to connect the VL and VH fragments. The final DNA fragments encoding a library of scFv antibody fragments (VL–linker–VH) were gel-purified, digested with *Sfi*I, and cloned

into the appropriately cut phagemid vector pComb3X. A schematic representation of the PCR steps is shown in Figure 2.1. pComb3X contains a suppressor stop codon and sequences encoding peptide tags for purification (6-His) and detection (HA). The recombinant phagemid was introduced into competent *E. coli* ER2538 cells by electroporation. The phage library displaying the scFv fragments was panned against immobilized HIV-1 IN antigen using a solid-phase protocol. For each round of selection, 1 μg of antigen in PBS was coated on triplicate wells of a 96-well microtiter plate (Corning). The plates were coated overnight at 4°C and subsequently blocked with 3% BSA in PBS for 1 h at 37°C. The blocking reagent was then replaced by 50 μl of freshly prepared phage from the starting library. Plates were incubated at 37°C for 2 h. The unbound phages were discarded and the wells were washed with 0.5% Tween–PBS and incubated at room temperature for 5 min. The wells were washed 5 times in the first round of selection and 10, 15 and 15 times in the following 3 rounds of selection, respectively. The bound phage were eluted by applying 50 μl/well of 10 mg/ml trypsin and incubating at 37°C for 30 min. Eluted phage from triplicate wells were combined and directly added to fresh ER2538 bacterial culture for infection, phage amplification and titration. One hundred and fifty scFv phage-clones from the final output were randomly selected for ELISA to evaluate their binding activity against HIV-1 IN. HRP-conjugated anti-M13 mAb (Roche) was used for detection. All clones with a strong signal above background were further analyzed by DNA fingerprinting and sequencing.


1. First round of PCR
Vλ PCR for Rabbit scFv long linker libraries
RSClambda1
Forward Primer
Random Sequence Tail
Vλ
RJlambdaO-BL
Antisense Primers
(long) Linker Sequence Tail
Vκ PCR for Rabbit scFv long linker libraries
RSCVK1
RSCVK2
RSCVK3
Sense Primers
Random Sequence Tail
Vκ
RKB9JO-BL
RKB9J1O-BL
RKB42JO-BL
Antisense Primers
(long) Linker Sequence Tail

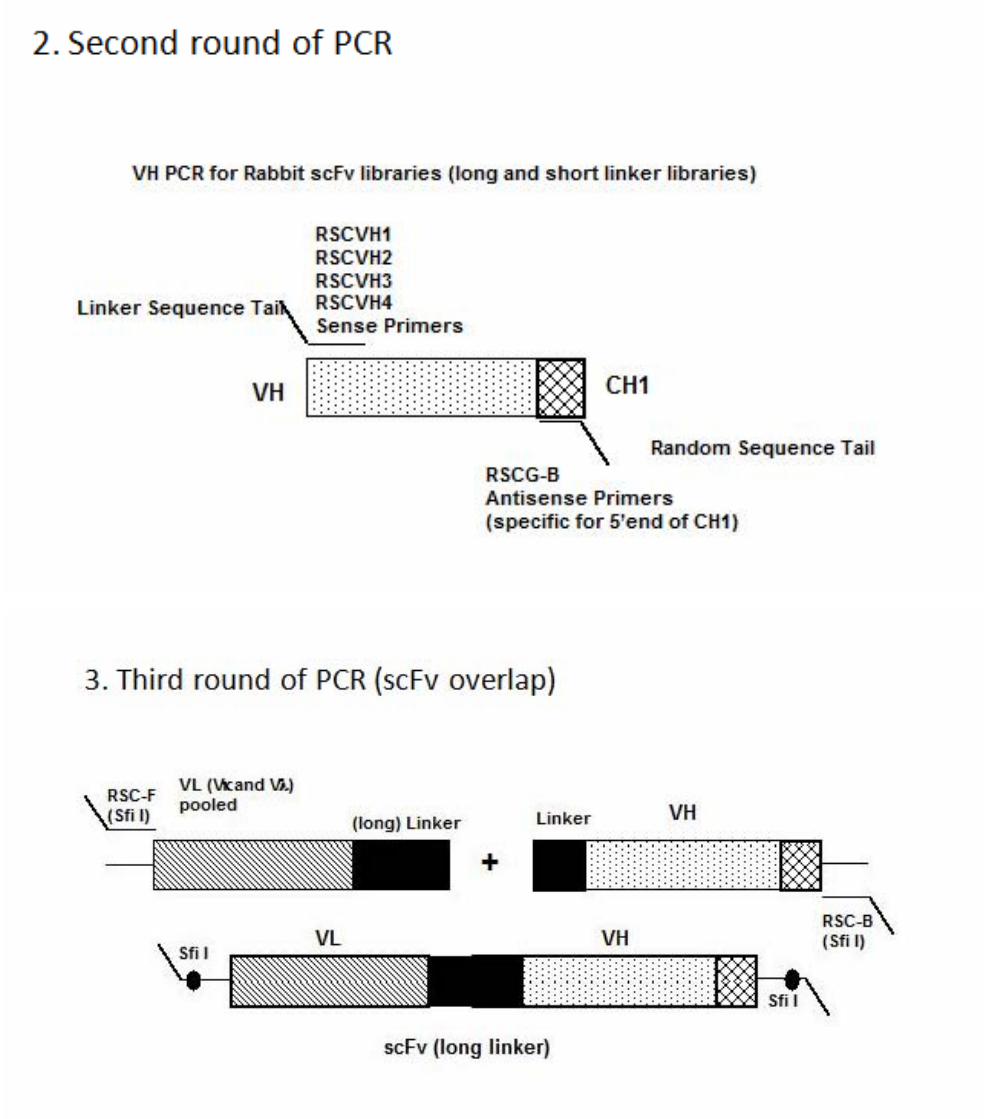


Figure 2.1 Generation of scFv antibody fragments by PCR overlap extension for cloning into the pComb3x. In the first PCR round, the variable light chain regions (Vλ and Vκ) were amplified from rabbit cDNA by using specific VL primers (Table 2.1). Each sence primer has a 5' sequence tail that contains an SfiI site and is recognized by the sense extension primer used in the third-round of PCR, each reverse primer has a linker sequence tail that is used in the overlap extension. In the second PCR round the variable heavy chain

regions (VH) are amplified from rabbit cDNA by using specific VH primers (Table 2.1). Each of the RCSVH sence primers is combined with the RCSG-B reverse primer, which recognizes the 5' end of the rabbit CH1. The sence primers have a sequence tail corresponding to the linker sequence that is used in the overlap extension PCR. The reverse primer has a sequence tail corresponding to the linker sequence that is used in the overlap extension PCR. The reverse primer has a sequence tail containing an *SfiI* site, this tail is recognized by the reverse extension primer used in the second-round PCR. In the third PCR round each purified variable regions products are assembled into scFv format by overlapping PCR using the RSC-F and RSC-B. These primers recognize the sequence tails that were generated during the first and second round of PCR. The 800 bp PCR products have asymmetric SfiI sites on the 5' and 3' ends that are used for cloning into the pCom3x vector (Adapted from [324]).

2.2.5 Expression and purification of anti-IN antibody fragments

To express and purify selected anti-IN scFvs, phagemid DNA was transformed into non-suppressor *E. coli* strain TOP10F. A fresh colony of each clone was grown at 37º C overnight in SB medium containing 100 μg/ml of ampicillin. A 10 ml sample of cells was used to inoculate 1 liter of SB medium containing 100 μg/ml of ampicillin. Cells were grown at 37º C until A_{600nm}= 0.9, induced by the addition of 0.5 mM IPTG and growth was continued for 18 hours. After induction, bacteria were harvested by centrifugation (4,000 × *g*, 4°C, and 15 min), resuspended in 50 ml equilibration buffer (20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole (pH 7.4)), supplemented with protease inhibitors (Roche), and lysed by sonication. Cell debris were removed by centrifugation (40,000 × *g*, 4° C, 30 min), and the supernatant was filtered through a 0.2-μm syringe filter. All chromatographic steps were performed at 4 ºC. First, scFv extracts were

purified by nickel chelate affinity chromatography using the C-terminal His-tag. Bound proteins were eluted with a linear imidazole gradient (0-300 mM imidazole in 20 mM NaH_2PO_4, 0.5 M NaCl (pH 7.4)). The appropriate fractions from IMAC were pooled, dialyzed against 20 mM Tris (pH 8.0), 50 mM NaCl, 1 mM EDTA and loaded onto a mono Q anion exchange column (Pharmacia), equilibrated in the same buffer. Elution from the anion exchange column was achieved with a 0 to 800 mM NaCl gradient. Pooled fractions of all antibody fragments were dialyzed against PBS. Protein purity was checked by non-reducing SDS-15% PAGE. Protein quantification was determined by the classic Bradford method.

2.2.6 In vitro binding studies of anti-IN antibody fragments

Relative binding affinities of purified anti-IN antibody fragments were determined via enzyme-linked immunosorbent assay (ELISA) after coating the wells with 500 ng of purified recombinant HIV-1 IN protein, HIV-1 protease (PR) or BSA, overnight at 4ºC. Wells were blocked for one hour at 37ºC with BSA 3% in PBS. Purified anti-IN scFvs were added to the wells for further 1 h incubation at 37ºC. After washing the wells with PBS, HRP-conjugated anti-HA mAb (Roche) was used for detection. The results were measured by optical density at 405 nm and performed in triplicate. Specificity recognition of IN by anti-IN scFvs was next demonstrated by western-blot analysis. HIV-1 IN and HIV-1 PR proteins at several amounts were separated by PAGE and transferred to nitrocellulose membranes. After blocking, proteins were probed with each purified anti-IN scFv clones and then with HRP-conjugated anti-HA mAb (Roche), as a secondary antibody.

Proteins were visualized using the ECL system (Amersham Pharmacia). BM10 is an anti-LANA scFv and was used as an irrelevant control antibody in both assays [221].

2.2.7 Mapping HIV-1 IN epitopes

To explore the IN epitope, purified anti-IN antibody fragments were tested for binding to a set of linear 20-mer peptides representing the entire HIV-1 IN sequence (AIDS Research and Reference Reagent Program). Briefly, covalink ELISA plates (Nunc) were coated overnight with 5 μg of each peptide. After blocking with 3% BSA in PBS, purified anti-IN ScFv were added to the wells and incubated 1 h. After washing the wells with low ionic buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.1% Triton X-100) or high ionic buffer (500 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100), HRP-conjugated anti-HA mAb (Roche) was used for detection. All steps were performed at room temperature. The results were measured by optical density at 405 nm and performed in triplicate. BM10 was used as an irrelevant control antibody.

2.2.8 DNA substrates for IN activity assays

The 3'-end processing substrate consisted of the terminal 21 bp from the U5 end of viral DNA was prepared by using the oligonucleotide RZ132 (5'-GTGTGGAAAATCTCTAGCAGT-3') and AE118 (5'-ACTGCTAGAGATTTTCCACA-3') [329]. RZ132 was treated with alkaline phosphatase and 5’ end labeled with [γ-^{32}P] ATP by T4 olynucleotidekinase.

The radiolabeled oligonucleotide was then annealed to AE118 and the unincorporated nucleotide was removed by centrifugation through a quick spin column (Millipore). The pre-cut DNA substrate for the strand transfer assay was prepared by *NdeI* digestion of pNde675 plasmid and resulted in a 675bp DNA linear fragment flanked by 31bp of HIV-1_{NL4-3} U5 LTR and U3 LTR sequences at each ends. The pre-cut DNA fragments were treated with alkaline phosphatase and then 5' end labeled with [γ-^{32}P] ATP by T4 polynucleotidekinase. The target DNA used in the strand transfer integration assay was the supercoiled pBR322 (Invitrogen).

2.2.9 IN activity assays

All IN activity assays were performed essentially as described previously with minor modifications [328,329]. For the 3'-end processing assay the reaction mixtures (25 µl final volume) were assembled by incubating 200 nM integrase on ice in 20 mM MOPS, pH 7.2, 0.1 mg/ml BSA, 7.5 mM $MnCl_2$, 10% glycerol and 10 mM 2-mercaptoeth. These components were preincubated on ice for 15 min with anti-IN ScFv at various IN/scFv ratios. Then, 20 nM oligonucleotide substrate was added. Samples were further incubated 1 h at 37ºC and reactions were quenched by adding 20 µl of sequencing gel loading dye (95% formamide, 10 mM EDTA, 0.03% bromophenol blue and 0.03% xylene cyanol). A 2.5 µl aliquot of each reaction mixture was subjected to electrophorese in a 20% denaturating polyacrylamide gel. Gels were dried, exposed to imaging plates and visualized and quantified with a Fuji BAS-2500 bio-imaging analyzer. For the strand-transfer assay reaction mixtures (25 µl final volume) were

assembled by incubating 80 nM integrase on ice in 20 mM HEPES, pH 7.5, 12% DMSO, 5 mM DTT, 10% PEG-6000, 10 mM $MgCl_2$, 20 mM $ZnCl_2$ and 100 mM NaCl. These components were preincubated on ice for 15 min with anti-IN ScFv at various IN/scFv ratios. After addition of 10 nM DNA pre-cut substrate, samples were further incubated on ice 30 min. Then 500 ng target plasmid DNA pBR322 was added. After a further 30 min preincubation on ice, the reaction was initiated by transfering to 37ºC and incubation was continued for 90 min. The reactions were stopped by addition of 0.1% SDS, 10 mM EDTA and 5 µg of proteinase K. A 2.5 µl aliquot of each reaction mixture was subjected to electrophoresis in a 0.8% agarose gel in 1×TBE buffer. Gels were dried, exposed to imaging plates and visualized and quantified with a Fuji BAS-2500 bio-imaging analyzer.

2.2.10 Expression of anti-IN antibody fragments in eukaryotic cells

After expression studies in *E. coli* TOP10F and analysis of binding activities, genes encoding anti-IN scFv's were transferred into pcDNA3.1/Zeo$^+$ (Invitrogen) by *NotI* and *XhoI* digestion. A methionine initiation codon was added into all ScFv by PCR as well a sequence encoding the HA-tag sequence (YPYDVPDYA) at the C-terminus. The primers used for cloning in pcDNA3.1/Zeo$^+$ were: Babe ScFv5 5'-GGCATGGGGGCCCAGGCGGCCCAGCTC-3' and Babe ScFv3 5'-GCCACCACCCTCCTAAGAAGC-3'. Anti-IN ScFv were also cloned into pcDNA3.1/Zeo$^+$ with a nuclear localization signal (NLS). The NLS sequence was added at the N-terminal end of each fragment by using the primers: Babe ScFv5NLS 5'-GGCATGGGGGCCCAGGCGGCCCAGCTC-3' and

Babe ScFv3 5'-GCCACCACCCTCCTAAGAAGC-3'. To analyze the intrabodies expression in eukaryotic cells, 293T cells (1-2×10^6) were transfected by a standard calcium phosphate method with 5 μg of plasmids encoding the scFv of interest. Forty-eight hours post-transfection, 293T cells were washed with 5 ml of cold PBS. Cells were lysed in 1 ml 50 mM Tris (pH 8.0), 100 mM NaCl, 1% Nonidet P-40 containing protease inhibitors (Roche) for 60 min on ice. The lysates were cleared by centrifugation for 30 min at 14,000×g, separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Western Blot was performed with HRP-conjugated anti-HA mAb.

2.2.11 Immunofluorescence staining

HeLa CD4 cells (0.5×10^6/well) transfected by calcium phosphate method with 4 μg of plasmids were fixed in PBS with 4% paraformaldehyde for 10 min at room temperature, permeabilized with PBS plus 0.1 % Triton X-100 for 5 min, and washed with PBS plus 2% fetal calf serum before staining. For IN analysis, fixed cells were incubated with HIV-1 anti-IN polyclonal antibody #7375 (1:40) for 90 min at 37°C, washed with PBS and then incubated with Alexa-conjugated goat anti-rabbit immunoglobulin antibody (1:200) for 30 min at 37°C. For immunostaining of scFv, direct immunofluorescence was performed with rhodamine-conjugated anti-HA mAb (5 μg/ml). For double immunofluorescence staining of intrabodies and IN protein, rhodamine-conjugated anti-HA mAb was used in combination with anti-IN polyclonal antibody #7375 and Alexa-conjugated goat anti-rabbit immunoglobulin antibody at similar concentrations. Slides were

mounted with DAPI (4,6 diamidino-2-phenylindole) Vectashield (Vector Labs, Burlingame, CA) to stain nuclei, and cells were visualized using an Olympus IX-50 inverted microscope, Ludl BioPoint filter wheels and a 12-bit V-scan cool CCD (Photonic Science). Integrated control of filter wheel and image acquisition is achieved by Image-Pro Plus 4.0 and Scope-Pro 3.1 (Media Cybernetics). Settings for image acquisition (camera exposure time, filters, time interval and storing modes) were determined by custom-made macros. Images were collected with Olympus 40x or 100x plan apo objectives (NAs= 0.95 and 1.4 respectively).

2.2.12 One step viral replication analysis

One step viral replication experiments were performed into HeLa CD4 LTR-β-Gal cells. Briefly, HeLa CD4 LTR-β-Gal cells (1×10^6/well) were transfected by calcium phosphate method with 4 μg of plasmids encoding anti-IN ScFv. After 24 h of transfection, HIV-1_{NL4-3} at a multiplicity of infection (MOI) of 1.0 was used to infect cells for 5 h. Cells were washed with pre-warmed serum free medium and cultured for 48 h. The ability of anti-IN ScFv to inhibit early steps of replication was measured by quantification of the β-galactosidase activity in cell lysates, using a colorimetric assay based on the cleavage of chlorophenolred-β-Dgalactopyranoside (CPRG) by β-galactosidase. HeLa CD4 LTR-β-Gal cells were washed with PBS and lysed with lysis buffer (50 mM Tris (pH 8.0), 100 mM NaCl, 1 % Nonidet P-40), supplemented with protease inhibitors (Roche). After incubation for 30 min on ice, CPRG reaction buffer (6 mM in lysis buffer) was added to the cell lysates and incubated for

2 h at 37ºC. The results were measured by optical density at 570 nm. BM10 was used as an irrelevant control antibody.

2.2.13 Single round infectivity assay and western-blot analysis of anti-IN scFv and IN packaging into viral particles

To further evaluate the inhibition of HIV-1 infectivity by anti-IN ScFv, 293T cells (1-2× 10^6/well) were cotransfected by calcium phosphate method with 8 μg of HIV-1_{NL4-3} and 4 μg of plasmids encoding anti-IN ScFv. Forty eight hours post-transfection, the viral supernatants were normalized for the same TCID50 and used to infect subconfluent HeLa CD4 LTR-β-Gal cells in 96-well plates. Forty eight hours after infection, the ability of anti-IN ScFv to inhibit HIV-1 infection was measured by quantification of the β-galactosidase activity in cell lysates as described above. To analyze virion protein contents, the HIV-1 particles used in the infectivity assay were analyzed by Western Blot. Briefly, viral supernatants produced in the cotransfected 293T cells were harvested and cleared by low speed centrifugation. Virion particles were concentrated by ultracentrifugation and then separated on 15% acrylamide gels. For detection of scFv, proteins were probed with HRP-conjugated anti-HA mAb. For detection, of IN and p24, proteins were probed with anti-IN polyclonal antibody #7375 and anti-p24 mAb, respectively. Subsequently, blots were probed with species-specific HRP-conjugated secondary antibodies.

2.2.14 Generation of stable anti-IN-scFv-Jurkat cell clones

pcDNA3.1-anti-IN-scFv plasmids were digested with *SfiI*, and the anti-IN scFv genes were cloned between the two *SfiI* sites of pMX-

KRAB8FPBS2puro, replacing the KRAB8FPBS2 zinc finger gene. pMX-anti-IN-scFv and pMD-G plasmids were co-transfected into the Gag-Pol-293 packaging cell line (Clontech) using the standard calcium phosphate method. After 48 h of incubation, culture supernatants were used for transduction of Jurkat cells. After 3-4 weeks of selection in a gel matrix containing puromycin, stable anti-IN-scFv-Jurkat clones were isolated and analyzed for expression of anti-IN antibody fragments by western blot analysis with HRP-conjugated anti-HA mAb.

2.2.15 HIV-1 challenge of stable anti-IN-scFv-Jurkat cell clones

Anti-IN-scFv-Jurkat clones were infected at a MOI of 0.1 to 0.5 for 6 h at 37°C, washed, and then cultured for up to 20 days. To monitor infection, aliquots were taken from the cultures at the indicated time points and p24 levels were determined using a HIV-1 p24 ELISA (Innovagen). Cellular proliferation and viability of infected cells were analyzed with tetrazolium salt WST-1 (Roche) according to the manufacturer's protocol. To further evaluate if HIV-1 viral replication could be inhibited during late stages, viral supernatants at day 12 were normalized by the same TCID50 and subjected to ELISA RT activity assay system (RetroSys™ RT Activity Kit) following by manufacturer’s instruction (Innovagen).

2.3 Results

2.3.1 Selection of specific anti-IN antibody fragments

Two pairs of rabbits from the New Zealand White strain were immunized and boosted four times with 200 μg of purified HIV-1 IN protein. ELISA analyses of the rabbit sera from both animals showed that the immunization along time resulted in a strong immune response against HIV-1 IN (Figure 2.2). Rabbits were euthanized and bone marrow and spleen were extracted for total RNA preparation and cDNA synthesis. To generate the chimeric scFv library, specific oligonucleotide primers covering all known variable rabbit antibody family sequences were used to amplify VH and VL gene segments [324]. The purified variable regions products were assembled into scFv format by overlapping PCR. The recombinant phagemid pComb3x was transformed into *E. coli* ER2538 cells to yield 8.3×10^7 individual clones. The phage library displaying the scFv fragments was then panned against immobilized HIV-1 IN protein using a solid-phase as described in Material and Methods section.

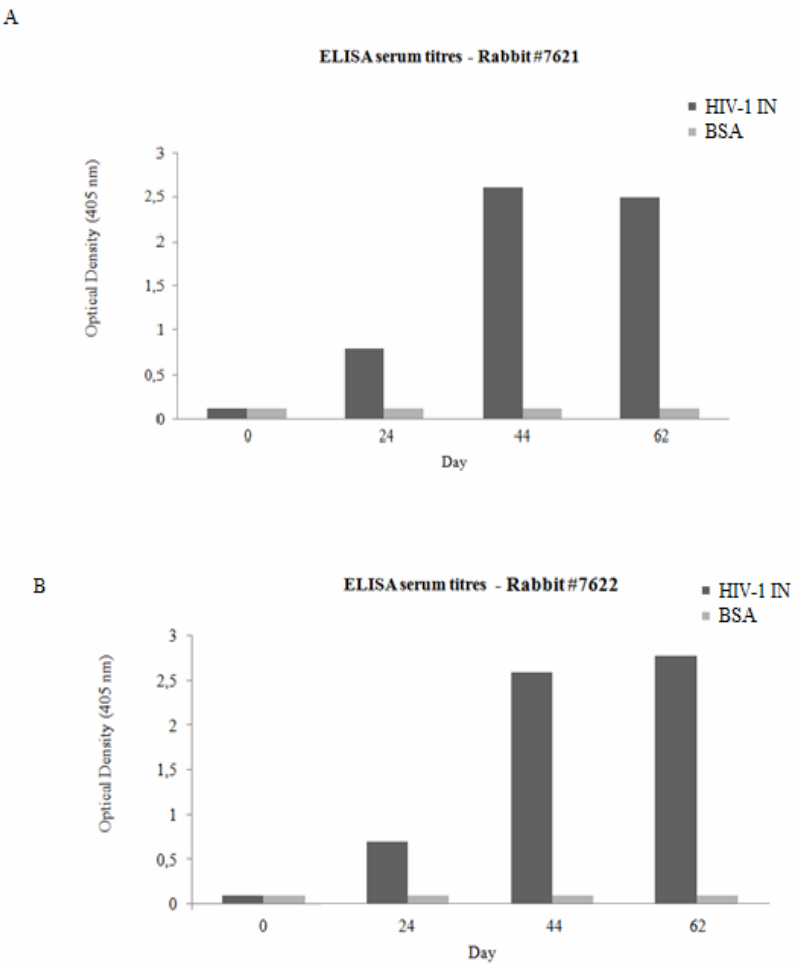


Figure 2.2 - Titration of serum antibodies A) - Rabbit # 7621 and B) - Rabbit # 7622. Antisera from the immunized animals was analyzed for binding to 200 ng of HIV-1 IN protein by ELISA using HRP-conjugated goat anti-rabbit Fc polyclonal antibody as secondary antibody (PIERCE). Serum titre is defined by the highest limiting dilution recognized by the antigen (1:10000).

From the final phage-display panning, 150 scFv phage-clones were randomly selected for ELISA to evaluate their binding activity against HIV-1 IN (Figure 2.3). A total of 22 clones with higher-than-background signal were isolated and sequenced. We observed that 14 clones shown sequence variations in the CDR regions and therefore were chosen for further expression and binding characterization (data not shown).

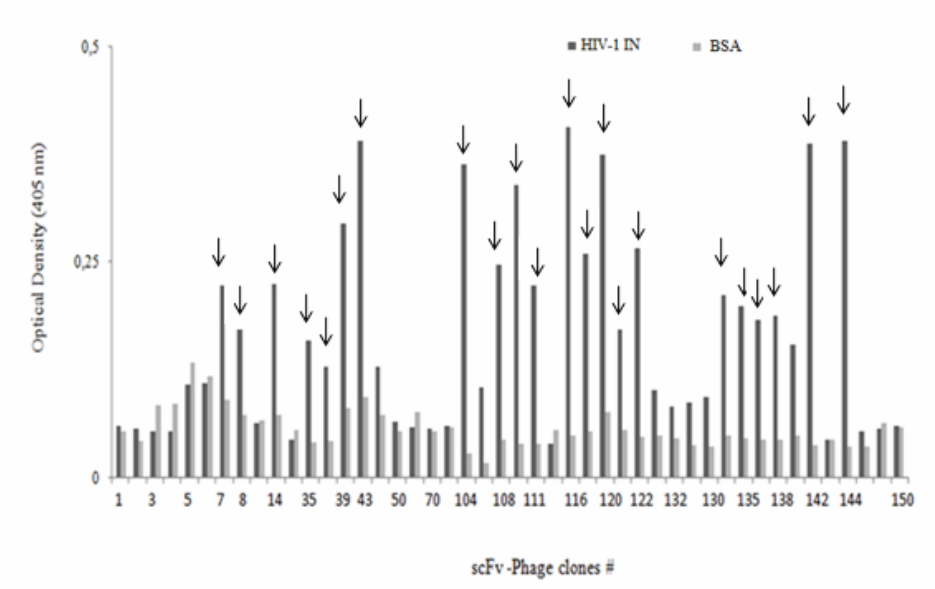
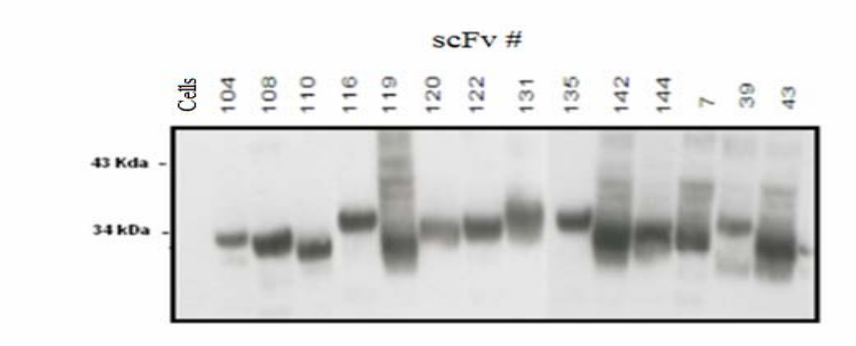


Figure 2.3 - Relative binding affinities of scFv phage-clones. A total of 150 scFv phage-clones were randomly selected for ELISA to evaluate their binding activity against HIV-1 IN. HRP-conjugated anti-M13 mAb was used as secondary antibody. Results were measured by optical density at 405 nm. A total of 22 clones with higher-than-background signal were isolated (shown with an arrow).

2.3.2 Relative binding affinity and specificity of anti-IN antibody fragments

To determine if the binding efficiency of recombinant scFvs to the HIV-1 IN protein were correlate with those of scFv phage-clones, we expressed the different 14 clones obtained in the periplasm of the non-suppressor *E. coli* strain TOP10F. After 18 hours of IPTG induction, cells were lysed and the soluble fraction was subjected to immobilized metal affinity chromatography (IMAC) and anion exchange chromatography as described in Material and Methods section. All scFvs were highly expressed in the form of soluble proteins (Figure 2.4A) and typical yields of pure scFv (> 95 %) were in the range of 1 ± 0.1 mg/L of bacterial culture. Relative binding affinity and specificity of anti-IN scFv fragments were assessed by ELISA and Western Blotting. As shown in Figure 2.4B, all anti-IN scFv clones specifically bound to HIV-1 IN protein. In contrast, no binding to IN protein was observed with an irrelevant scFv (clone BM10) that specifically recognizes the latency-associated nuclear antigen-1 (LANA1) from Kaposi sarcoma–associated herpes virus [221]. Moreover, experiments showed near background signals and lower non-specific binding of all anti-IN scFvs to HIV-1 protease protein (PR) and bovine serum albumin (BSA).

A



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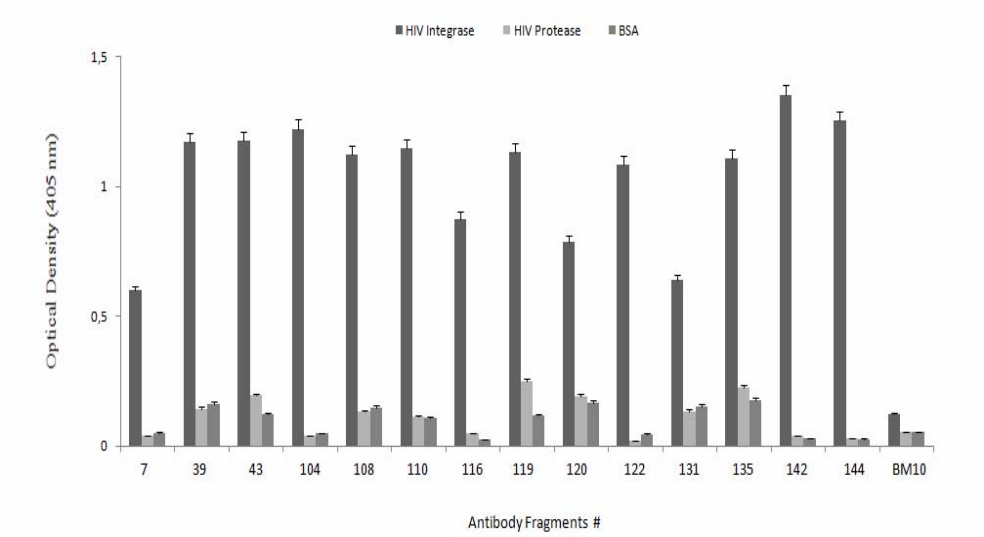


Figure 2.4 - Expression and relative binding affinities of anti-IN scFv fragments. (A) *E. coli* TOP 10F bacteria expressing anti-IN scFvs after induction with 0.5 mM IPTG at 37°C for 18 hours. Antibody fragments were extracted from the periplasmic space as described in Material and Methods. After separation on a 15% SDS-PAGE gel and blotting, antibody fragments were detected with HRP-conjugated anti-HA mAb (Roche). Molecular weight is indicated in kDa. (B) The anti-IN scFv fragments were used for evaluating relative binding affinities to 200 ng of HIV-1 IN, PR and BSA by ELISA. The anti-LANA1 scFv (BM10) was used as an irrelevant control antibody. Results were measured by optical density at 405 nm. Data represents results of three independent experiments.

To further test binding specificity of scFv to IN protein, four scFv clones with high binding activity to IN (clones 104, 135, 142 and 144) and one that gave a weaker signal (clone 7) were chosen to carry out the immunoblotting assays. We have separated HIV-1 IN and PR proteins by SDS-PAGE and transferred to nitrocellulose membranes. After blocking, proteins were probed with purified anti-IN scFvs and then with HRP-conjugated anti-HA mAbs, as a secondary antibody. As an irrelevant control antibody we have used the anti-LANA1 scFv (BM10). As demonstrated in Figure 2.5, all anti-IN scFvs specifically recognize the 32 kDa HIV-1 IN protein. As expected by our ELISA results, only the high binding clones could detect low concentrations of IN. Importantly, recombinant anti-IN scFv did not recognize HIV-1 PR and no bands were detected using the anti-LANA1 scFv (BM10). Our ELISA and immunoblotting results showed that scFv clones 7, 104, 135, 142 and 144 specifically bound to HIV-1 IN and therefore were chosen for further antibody characterization and functional studies.

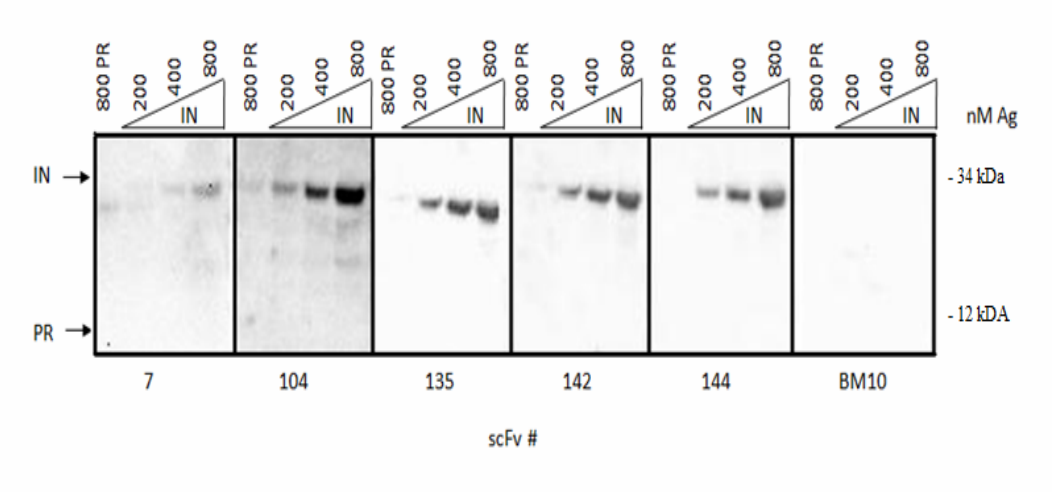


Figure 2.5 - Detection of antigens by western-blot. Purified HIV-1 IN protein at several amounts was separated by 15% SDS-PAGE and transferred to nitrocellulose membranes. For immunodetection, purified anti-IN scFv clones 7, 104, 135, 142 and 144 were used. HRP-conjugated anti-HA mAb was used as a secondary antibody. HIV-1 PR at 800 nM was used to test anti-IN specificity and anti-LANA1 scFv (BM10) was used as an irrelevant control antibody. Molecular weight is indicated in kDa.

2.3.3 Mapping HIV-1 IN epitopes

We observed that the 5 selected anti-IN scFvs apparently recognized a continuous epitope(s) of IN, since they reacted with denatured IN on immunoblots. In order, to identify the epitopes recognized by the different antibodies, a set of 30 overlapping synthetic peptides covering the entire IN molecule were used. Each peptide contained 20 residues, with the first 10 residues overlapping those found in the peptide immediately preceding it in the primary sequence. Interactions between the scFvs and the synthetic peptides were analyzed by ELISA as described in the Material and Methods section. We verified that the background signal was routinely quite low, that

no peptide binding was observed with anti-LANA1 scFv (clone BM10) and an unexpected pattern of peptide reactivity for the five anti-IN scFvs. As shown on Table 2.2, all anti-IN scFv clones bound to peptide 4338 (residues 126 to 145), and peptide 4343 (residues 176 to 195) and also to two upstream peptides, 4346 and 4347 (residues 206 to 235). Since all clones bound simultaneously to three separate regions in the linear sequence of IN, we were concerned that unspecific peptide binding was occurring with anti-IN scFv. Therefore, ELISA epitope mapping was repeated with a high ionic washing buffer (Material and Methods section). With these conditions, a small decrease in the relative antigen-binding affinity was observed (data not shown); although the same epitope binding patterns were maintained for all anti-IN scFv clones.

Table 2.2 – Epitope mapping of anti-IN scFv. To perform epitope mapping, purified anti-IN scFv clones 7, 104, 135, 142 and 144 were tested for binding to a set of 30 overlapping synthetic peptides covering the entire HIV-1 IN protein sequence. Covalink ELISA plates were coated with 5 ug of each peptide and incubate overnight at 4ºC. The plates were then blocked with BSA and incubate with purified antibody fragments. After washing the wells with low ionic buffer or high ionic buffer (Material and Methods), HRP-conjugated anti-HA mAb was used for detection. The same binding pattern was observed in both washing conditions. The anti-LANA1 scFv (BM10) was used as an irrelevant control antibody. The black box highlights the scFv specific binding to the different IN peptide regions. The + symbol means binding and the – symbol means no binding.

Peptide #	Amino acid Position	Amino acid Sequence	scFv # 7	104	135	142	144	BM10
4324	1-5	EQVDKLVSAGIRKVLFLDGI	-	-	-	-	-	-
4325	6- 15	IRKVLFLDGIDKAQDEHEKY	-	-	-	-	-	-
4326	6-25	DKAQDEHEKYHSNWRAMASD	-	-	-	-	-	-
4327	16-35	HSNWRAMASDFNLPPVVAKE	-	-	-	-	-	-
4328	26-45	FNLPPVVAKEIVASCDKCQL	-	-	-	-	-	-
4329	36-55	IVASCDKCQLKGEAMHGQVD	-	-	-	-	-	-
4330	46-65	KGEAMHGQVDCSPGIWQLDC	-	-	-	-	-	-
4331	56-75	CSPGIWQLDCTHLEGKVILV	-	-	-	-	-	-
4332	66-85	THLEGKVILVAVHVASGYIE	-	-	-	-	-	-
4333	76-95	AVHVASGYIEAEVIPAETGQ	-	-	-	-	-	-
4334	86-105	AEVIPAETGQETAYFLLKLA	-	-	-	-	-	-
4335	96-115	ETAYFLLKLAGRWPVKTIHT	-	-	-	-	-	-
4336	106-125	GRWPVKTIHTDNGSNFTGAT	-	-	-	-	-	-
4337	116-135	DNGSNFTGATVRAACWWAGI	-	-	-	-	-	-
4338	126-145	VRAACWWAGIKQEFGIPYNP	+	+	+	+	+	-
4339	136-155	KQEFGIPYNPQSQGVVESMN	-	-	-	-	-	-
4340	146-165	QSQGVVESMNKELKKIIGQV	-	-	-	-	-	-
4341	156-175	KELKKIIGQVRDQAEHLKTA	-	-	-	-	-	-
4342	166-185	RDQAEHLKTAVQMAVFIHNF	-	-	-	-	-	-
4343	176-195	VQMAVFIHNFKRKGGIGGYS	+	+	+	+	+	-
4344	186-205	KRKGGIGGYSAGERIVDIIA	-	-	-	-	-	-
4345	196-215	AGERIVDIIATDIQTKELQK	-	-	-	-	-	-
4346	206-225	TDIQTKELQKQITKIQNFRV	+	+	+	+	+	-
4347	216-235	QITKIQNFRVYYRDSRNPLW	+	+	+	+	+	-
4348	226-245	YYRDSRNPLWKGPAKLLWKG	-	-	-	-	-	-
4349	236-255	KGPAKLLWKGEGAVVIQDNS	-	-	-	-	-	-
4350	246-265	EGAVVIQDNSDIKVVPRRKA	-	-	-	-	-	-
4351	256-275	DIKVVPRRKAKIIRDYGKQM	-	-	-	-	-	-
4352	266-285	KIIRDYGKQMAGDDCVASRQ	-	-	-	-	-	-
4353	276-288	AGDDCVASRQDED	-	-	-	-	-	-

Therefore, we went to analyze if the three IN regions (residues 126-145, residues 176-195 and residues 206-235 – Figure 2.6A) were closely located in the folded IN structure and could therefore form a shared epitope. Although, the structure of full-length HIV-1 IN protein has not been determined, a crystal structure of the catalytic core and C-terminal is already available [251]. Observation of this structure prompt us to the conclusion that the three epitope regions are close together in the folded structure and indeed form a cavity (Figure 2.6B). To our knowledge we believe that our scFv fragments constitute the first set of anti-IN antibodies that recognize and bind simultaneously to epitope regions in catalytic and C-terminus domains of IN.

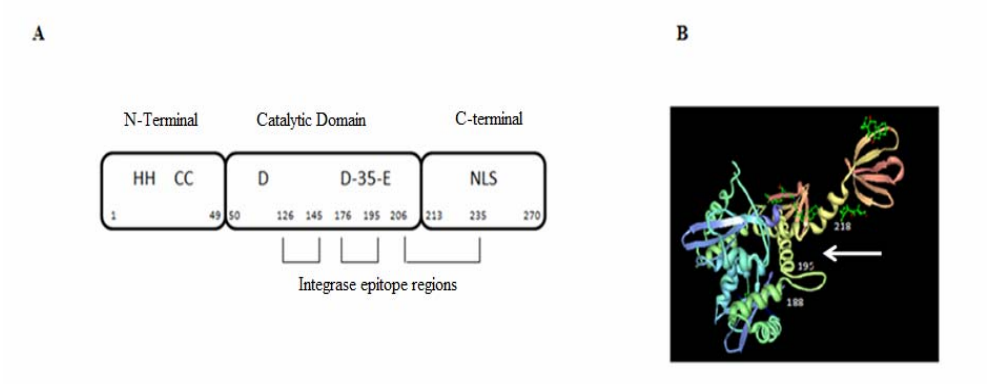


Figure 2.6 – Schematic representation of HIV-1 IN and its interactions with anti-IN scFvs. (A) Linear model of HIV-1 IN protein and its domains recognized by anti-IN scFv clones 7, 104, 135, 142 and 144. The IN enzyme comprises the N-terminal domain (NTD, aa 1–49), catalytic core domain (CCD, aa 50–212) and C-terminal domain (CTD, aa 213–288). The H and C residues within the NTD are additionally conserved among retrotransposon integrase proteins. The D and E residues in the CCD form the DDE motif. The nuclear localization signal (NLS) is localized in the C-Terminal domain. All anti-IN scFv tested recognize and bind simultaneously to residues 126 to 145 (peptide 4338), and residues 176 to 195 (peptide 4343) and also to the upstream residues 206 to 235 (peptides, 4346 and 4347). (B) Model of the crystal structure of HIV-1 catalytic and C-terminal domains (PDB code 1EX4)[251] showing the epitopes regions recognized by the anti-IN scFvs. The probable binding site of scFvs to HIV-1 IN is indicated by a white arrow.

2.3.4 Anti-IN scFv strongly inhibit the strand transfer reaction

Integration of retroviral DNA into the host chromosomal DNA involves two chemical steps. The newly synthesized bluntended viral DNA first undergoes 3´- end processing. In this reaction, two nucleotides are removed from each 3´- end. Next, the exposed hydroxyl groups attack a pair of phosphodiester bonds on opposite strands of the target DNA to complete the

strand transfer step. Both the 3´- processing and DNA strand transfer activities of HIV-1 IN can be recapitulated *in vitro* with DNA substrates that mimic the viral DNA ends. Therefore, to study the effects of our scFvs on the *in vitro* activities of IN, 3'-end processing and strand transfer assays were performed in the presence of our anti-IN scFv. As shown in Figure 2.7A, none of the anti-IN scFvs tested interferes in the 3'-end processing reaction since we did not observed any inhibition in the -2 cleavage product from the 21-base pair oligonucleotide substrate that represents the viral U5 end of viral DNA. In fact, not even a minimal inhibitory effect was observed when anti-IN scFv were added to IN at molar ratios of 1:4 (800nM). To evaluate the effect of each anti-IN scFv in the strand transfer reaction we have used a preprocessed DNA substrate. As shown in Figure 2.7B scFv 104, 135, 142 and 144 clearly inhibited the strand transfer reaction as demonstrated by the decrease of the half-site and full-site (concerted) integration of the ^{32}P-labeled preprocessed DNA substrate into the target DNA (pBR322). Indeed, these four antibody fragments inhibited more than 50% of the IN activity when present in the reaction in a ~twofold molar excess over IN (200 nM).

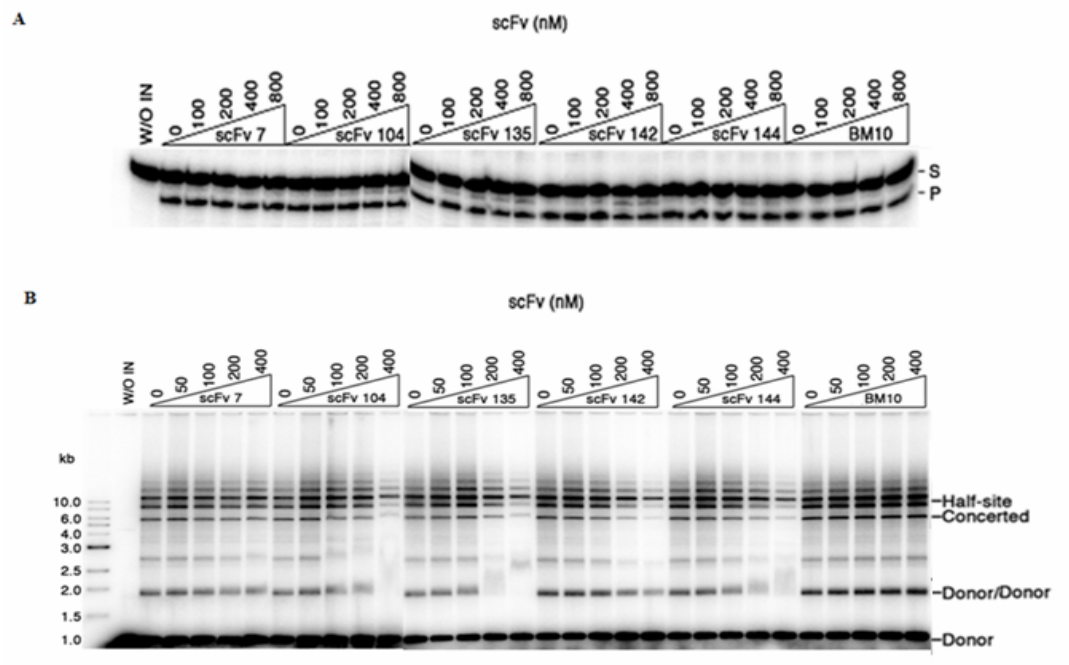


Figure 2.7 – Effect of anti-IN scFv binding on *in vitro* activities of HIV-1 IN. (A) Anti-IN scFvs do no inhibit the 3´-end processing reaction of HIV-1 IN. IN at 200 nM was preincubated on ice with each scFv at various scFv/IN molar ratios. Radioactively labeled oligonucleotide substrate was added, and the reactions were allowed to proceed at 37ºC. Each reaction mixture was subjected to electrophorese in a 20% denaturating polyacrylamide gel. The position of the 21-base pair oligonucleotide substrate (S) and the -2 cleavage product (P) are indicated. As shown no inhibition was observed in the 3´-end processing. (B) – Anti-IN scFvs strongly inhibit the strand transfer process of HIV-1 IN. Each purified scFv was preincubated with 80 nM IN at various scFv/IN molar ratios on ice. Radioactively labeled preprocessed DNA substrate and supercoiled target DNA (pBR322) were added and incubated as described in the Material and Methods section. Each reaction mixture was subjected to electrophoresis in a 0.8% agarose gel. Half-site integration involves the insertion of one LTR end per target, and full-site integration involves the concerted insertion of two LTR ends per target. The positions of the concerted integration product, the half-site product, and products resulting from integration of viral DNA

substrate into itself (*Donor/Donor*) are indicated. In both assays, anti-LANA1 scFv (BM10) was used as an irrelevant control antibody and gels were dried, exposed to imaging plates and visualized and quantified with a Fuji BAS-2500 bio-imaging analyzer.

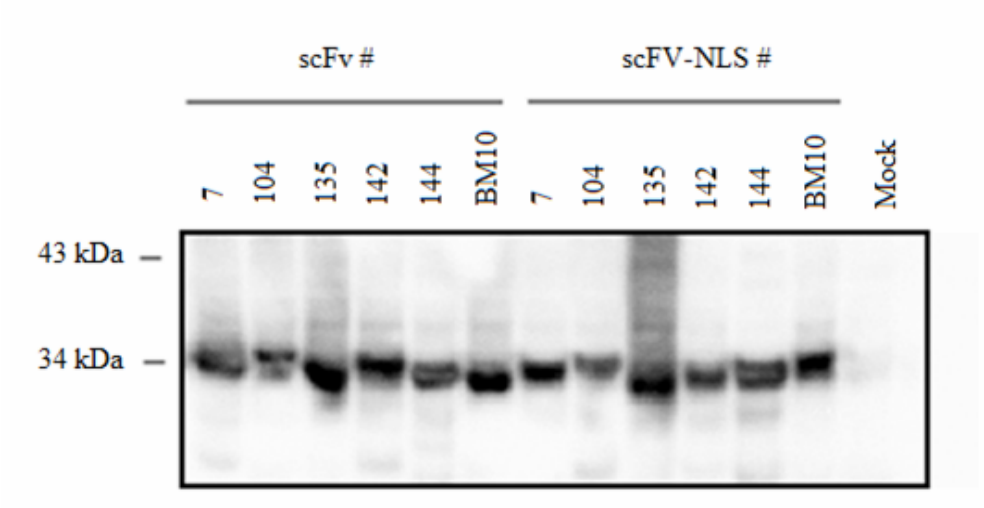
At higher concentrations (400 nM) scFv-104 and scFv-142 inhibited the reactions almost completely. On the other hand, scFv-7 had a weak inhibitory effect, and scFv anti-LANA1 had no detectable effect on the strand transfer activity of IN as expected. Therefore, our scFv 104, 135, 142 and 144 shown to have an important inhibitory effect on the strand transfer reaction meaning that they might be efficiently used as intrabodies to block the integration process during the HIV-1 replication.

2.3.5 Anti-IN scFv are efficiently expressed in eukaryotic cells and co-localize with IN

After analysis of inhibition of HIV-1 IN activity *in vitro*, genes encoding anti-IN scFv fragments with and without a nuclear localization signal (NLS) were transferred into pcDNA3.1/Zeo^{+}. To evaluate the expression of our anti-IN scFv in eukaryotic cells, 293T cells (1-2x 10^{6}) were transfected by a standard calcium phosphate method with each scFv plasmid. The data in Figure 2.8A demonstrates that all anti-IN scFv clones are highly expressed. To understand better where they are localized we have performed an immunofluorescence assay in HeLa cells using the scFv-142 and scFv-142-NLS. As shown in Figure 8B, scFv-142 is efficiently expressed in the cytoplasm and the scFv-142-NLS is expressed in the nucleus of eukaryotic cells. Therefore, these results demonstrate that our intrabodies can efficiently

be delivered to the destined compartment. We then evaluate the potential co-localization of both antibody formats expressed together with the HIV-1 IN protein. As shown in Figure 8C, when the IN protein is expressed with clone scFv-142 or scFv-142-NLS, we can observe co-localization of IN and scFv in both cases in the nucleus. Since, HIV-1 IN protein has been reported to localize predominantly in cell nucleus, and to play a role in nuclear import of the HIV-1 pre-integration complex, we can speculate that IN NLS domain can efficiently cause re-location within the nucleus of anti-IN intrabodies.

A



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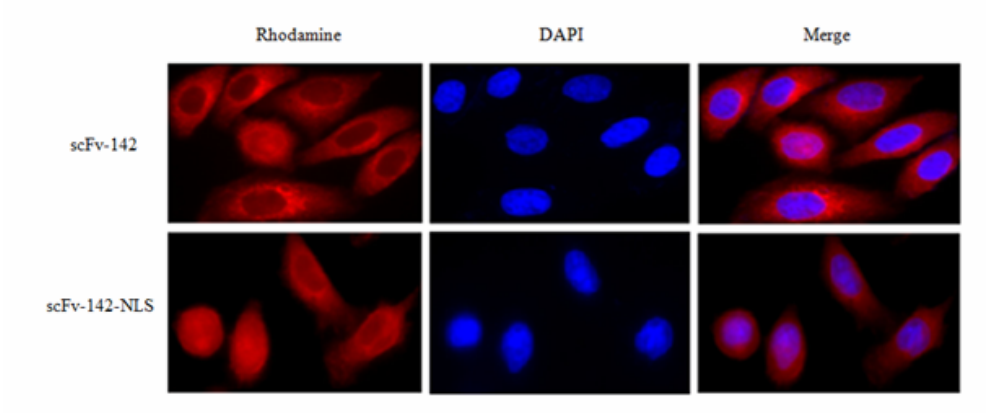

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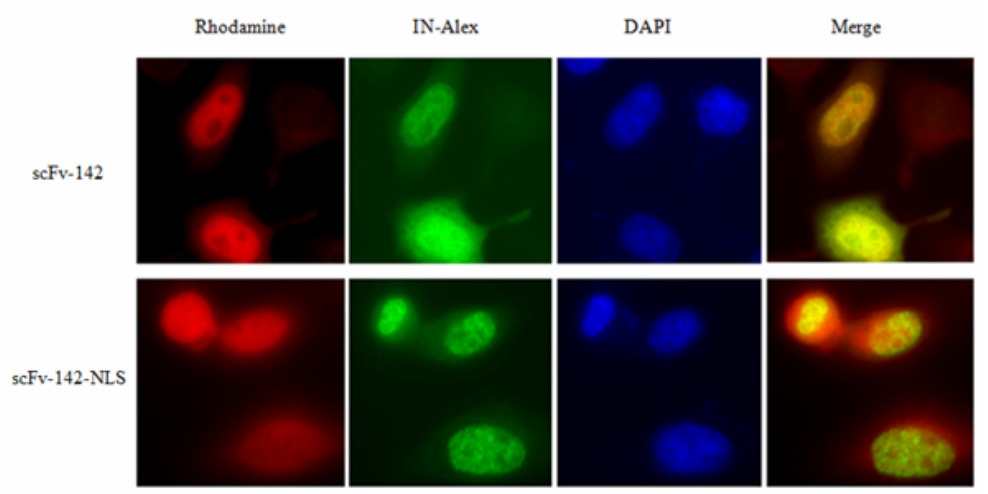


Figure 2.8 - Expression and co-localization of anti-IN intrabodies in eukaryotic cells. (A) Anti-IN intrabody expression vectors were transfected into 293T cells and after 48 hours cells were lysed and cleared by centrifugation. Proteins were separated by 15% SDS-PAGE and visualized by Western blot probed with HRP-conjugated anti-HA mAb. Mock lysates of 293T cells were used as controls in Western Blot analysis. Molecular weight is indicated in

kDa. (C) Transfected HeLa cells expressing scFv-142 and scFv-142-NLS were stained with rhodamine-conjugated anti-HA mAb. ScFv is shown in red (rhodamine) and IN in green (Alexa). (B) Transfected HeLa cells expressing scFv-142 or scFv-142-NLS and HIV-1 IN protein were stained with rhodamine-conjugated anti-HA mAb and anti-IN polyclonal serum plus Alexa-conjugated goat anti-rabbit immunoglobulin antibody as described in Materials and Methods. ScFv is shown in red (rhodamine) and IN in green (Alexa). Overlay of scFv and IN panels results in a yellow signal at sites of co-localization. Immunofluorescence microscopy was performed with the imaging setup described in Material and Methods using the appropriate excitation and emission filters.

2.3.6 HIV-1 replication can be inhibited by both cytoplasm and nucleus localized anti-IN scFv intrabodies

To evaluate whether the expression of anti-IN scFv fragments in either cytoplasm or nucleus could prevent HIV-1 replication, a one step viral replication assay in HeLa CD4 LTR-β-Gal cells was performed. The results shown in Figure 2.9 represent the percentages of viral replication relatively to the value obtained for HIV-1_{NL4-3} infection of control HeLa CD4 LTR-β-Gal cells (positive control, C+). As shown, both cytoplasmic and nuclear expressions of anti-IN scFv clones 104, 135, 142 and 144 inhibit the HIV-1 viral replication to 70-80%. The, scFv-7 in turn had a less prominent on the reduction of viral replication (only ~20% of the wild-type). Expression of anti-LANA1 scFv (BM10) with or without NLS had no inhibitory effect on HIV-1 replication. The biological activities of anti-IN scFv are correlated directly with our data described above, indicating that those clones that showed the stronger relative binding affinities and strand transfer inhibition are those that present the most efficient anti-HIV-1 activities. All together, our data indicates that the expression of anti-IN scFvs in eukaryotic cells

specifically neutralize IN activity prior to integration and, thus, has an effect on the integration process itself. Therefore, our experiments clearly demonstrate that HIV-1 replication can be blocked at early stages of viral replication in cells due to expression of anti-IN scFv intrabodies.

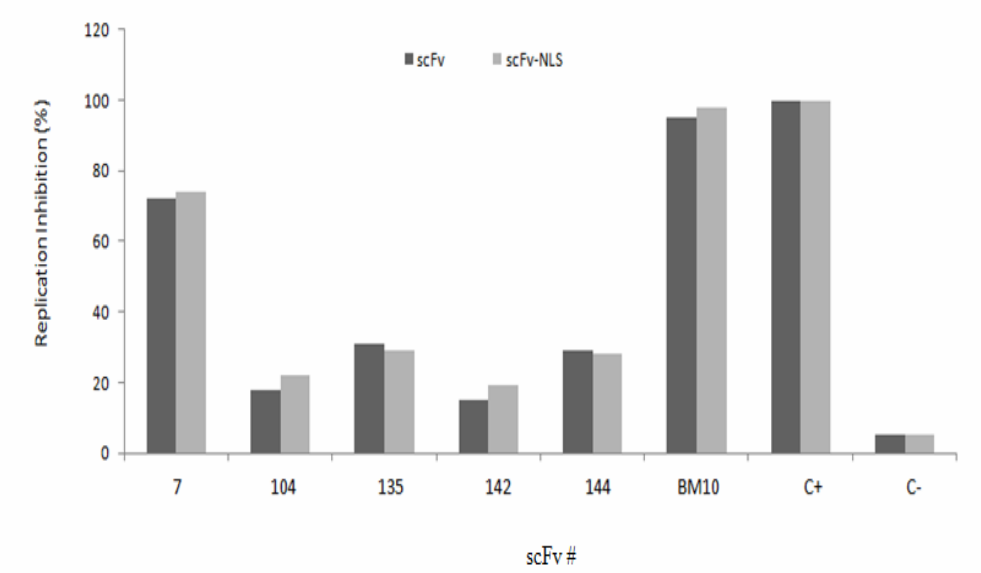


Figure 2.9 - Neutralization of IN function in one-cycle replication assay. Values represent the percentages of one cycle-replication in HeLa CD4 LTR-β-Gal cells relative to the value obtained for the wild-type. Cells were transfected with plasmids encoding anti-IN antibody fragments as described in Material and Methods. The ability of anti-IN antibody fragments to inhibit a single round of replication was measured by quantification of the β-galactosidase activity in cell lysates, using a colorimetric assay based on the cleavage of chlorophenolred-β-Dgalactopyranoside (CPRG) by β-galactosidase as described in the Material and Methods. Anti-LANA1 scFv (BM10) was used as an irrelevant control antibody; C^+, HIV-1_{NL4-3} infection of control HeLa CD4 LTR-β-Gal cells; C^-, uninfected HeLa CD4 LTR-β-Gal cells. The results were measured by optical density at 570 nm and data are representative of three independent experiments.

2.3.7 Anti-IN scFv intrabodies inhibit HIV-1 infectivity and are incorporated in viral particles

The three viral enzymes (reverse transcriptase, integrase and protease) are encoded within the HIV *pol* gene and translated as a polyprotein. The IN protein is then released from the polyprotein by the HIV protease during maturation of the virion. Therefore, if our anti-IN scFv intrabodies bind to the IN in the context of Gag-Pol polyprotein, problems in virion assembly or maturation might occur. Within this context, to evaluate if our anti-IN intrabodies could also inhibit the viral replication at late stages, 293T cells were cotransfected with pHIV-1_{NL4-3} and anti-IN scFv plasmids. The viral production was then analyzed by measurement of p24 amount in the culture supernatant by an HIV-1 p24 ELISA. We observed that the expression of any of the intrabodies into the cytoplasmic compartment did not affect significantly the virion production (data not shown). Resultant viruses were then normalized for the same TCID50 and used to infect HeLa CD4 LTR-β-Gal cells, where the level of infectivity was measured by quantification of the β-galactosidase activity (CPRG assay). The results shown in Figure 2.10 represent the percentages of viral infectivity relative to the value obtained for HIV-1_{NL4-3} infection of control HeLa CD4 LTR-β-Gal cells (positive control, C+). We observed that anti-IN scFv clone 104, 135, 142 and 144 have a pronounced reduction of 85% to 60% in viral infectivity compared to wild-type virus. The scFv-7 had only a reduced effect in HIV-1 infectivity (only 10% less than the wild-type level). The irrelevant anti-LANA1 scFv (BM10) did not have any effect in HIV-1 infectivity demonstrating the

specificity of our anti-IN scFvs. Taken together, these results suggest that anti-IN intrabodies can also block late stages of HIV-1 replication.

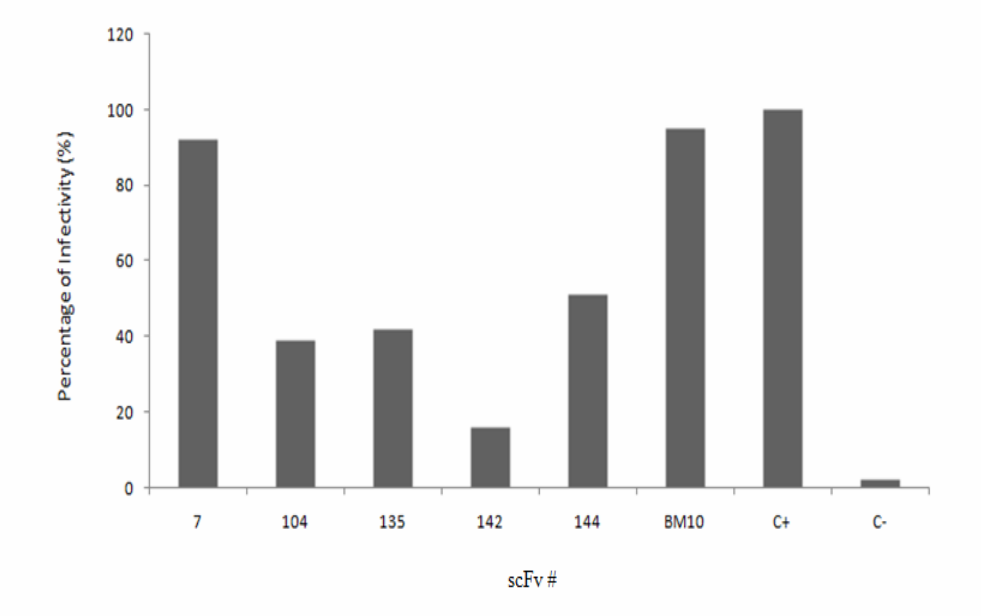


Figure 2.10 – Inhibition of HIV-1 infectivity in HeLa cells. A single cycle infectivity assay was performed with HIV-1 $_{NL4-3}$ virions produced in the presence of anti-IN antibody fragments. Values represent the percentages of infectivity in HeLa CD4 LTR-β-Gal cells relative to the value obtained for the wild-type. The infectivity of the viruses normalized for p24 was measured in HeLa CD4 LTR-β-Gal cells by quantification of the β-galactosidase activity in cell lysates, using a colorimetric assay based on the cleavage of chlorophenolred-β-Dgalactopyranoside (CPRG). BM10 was used as an irrelevant control antibody; C^+, HIV-1_{NL4-3} infection of control HeLa CD4 LTR-β-Gal cells; C^-, uninfected HeLa CD4 LTR-β-Gal cells. The results were measured by optical density at 570 nm and data are representative of three independent experiments.

To determine whether these defects in viral infectivity could be attributed to assembly or maturation problems, immunoblots protein analysis was performed for each virion produced in the presence of anti-IN scFvs. As demonstrated in Figure 2.11, all samples contained at the same intensity a 32 kDa IN band, showing that expression of anti-IN scFv intrabodies did not affect the incorporation of Gag-Pol polyprotein into the virion. In addition, we can also see that scFv 104, 135, 142 and 144 could be detected in virions. Indeed, clone 142 seems to be strongly incorporated. In contrast, scFv-7 and anti-LANA1 scFv (BM10) were not detectable in virions. Therefore, this result indicates that scFv-7 is not efficiently incorporated into virions, which might explain our results at levels of Figure 2.10. The data in Figure 2.11 also demonstrates that all scFvs were equally expressed in co-transfected 293T cells. Taken together all our results, we can speculate that when viral particles are produced in the presence of high yields of cytoplasmic anti-IN scFv intrabodies, they might be able to interact with the IN protein in the context of the Gag-Pol polyprotein and therefore be incorporated into the viral particles. As a consequence, infectivity of these virions will be presumably reduced since the incorporated anti-IN scFv fragment will neutralize the function of intravirion IN.

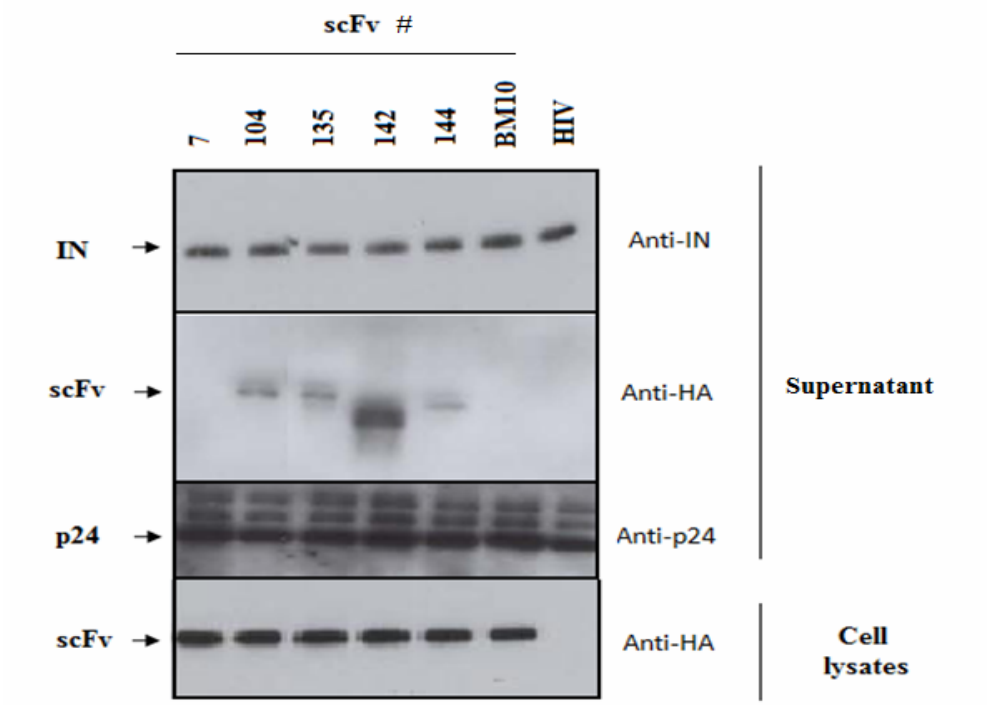


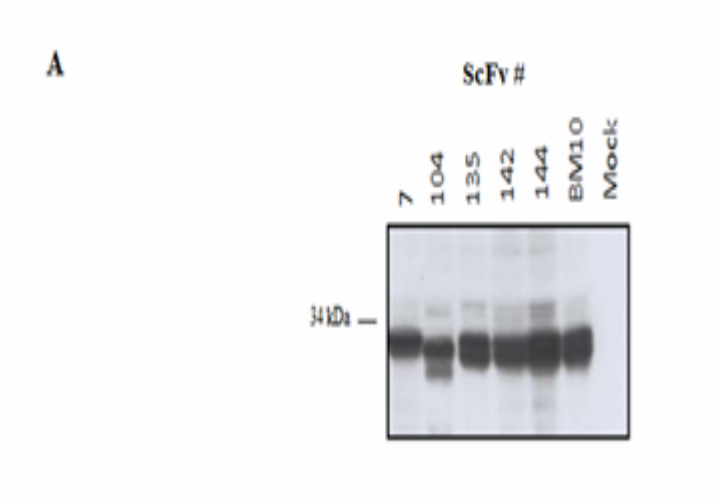
Figure 2.11 - Immunoblot analysis of anti-IN scFv and IN packaging within virions. Each viral supernatant of 293T cells co-transfected with HIV-1_{NL4-3} and antibody plasmids were collected, concentrated by ultracentrifugation and then separated on 15% SDS-PAGE gel. For detection of scFvs, proteins were probed with HRP-conjugated anti-HA mAb. For detection, of IN and p24, proteins were probed with anti-IN polyclonal antibody serum and anti-p24 mAb, respectively. Subsequently, blots were probed with species-specific HRP-conjugated secondary antibodies. The scFv expression was also analyzed in the 293T co-transfected cells (cell lysates).

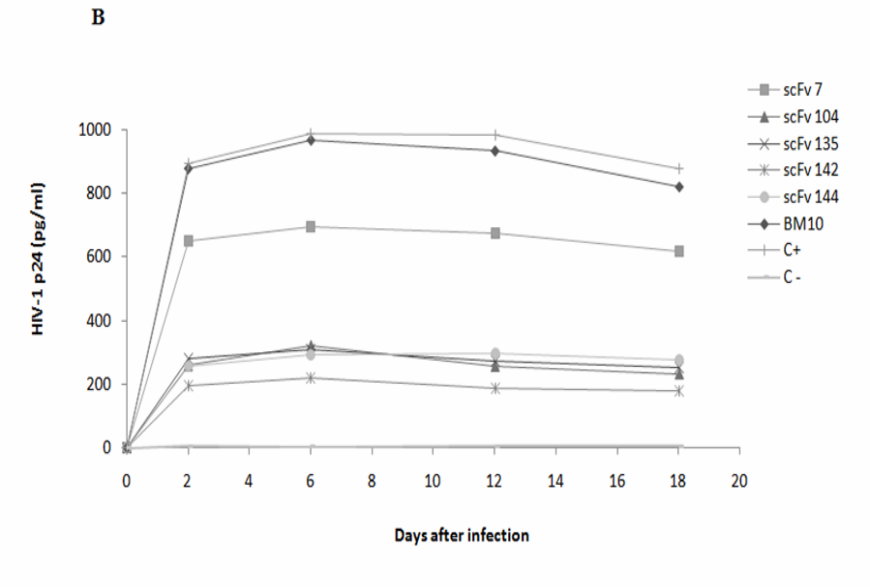
2.3.8 Jurkat stably cells expressing anti-IN intrabodies are protected from HIV-1 infection

The experiments described above examined the biological activity of anti-IN scFvs in a transient assay under conditions in which most virus transmission occurs by cell-to-cell spread. To study whether anti-IN scFv intrabodies might be able to inhibit IN function during several rounds of HIV-1 replication, Jurkat cell lines expressing independently scFv intrabodies were developed. For this generation, retroviral vectors encoding anti-IN intrabodies were used to transduce Jurkat cells. As a control, a retroviral vector encoding the irrelevant anti-LANA1 (BM10) was also used. As shown in Figure 2.12A, resultant Jurkat-scFv cell lines showed homogeneous and stable expression of intrabodies. To determine if the intracellular expression of anti-IN scFvs was able to prevent HIV-1 replication in susceptible human T cells, Jurkat intrabody-expressing cell lines were challenged with the HIV-$1_{NL4-3.}$ Infectivity assays were performed with an MOI of 0.1-0.5 to mimic natural infections [330]. The infected cell cultures were maintained for up to 20 days and to monitor infection, aliquots were taken at the indicated time points and HIV-1 p24 antigen levels were determined by ELISA. Parental non-transduced Jurkat cell lines supported vigorous replication of HIV-1 (positive control, C+), as shown by the initial increases in HIV-1 p24 antigen, which peaked at approximately day 12 (Figure 2.12B). Importantly, in the case of Jurkat scFv-104, scFv-135, scFv-142 and scFv-144, low levels of HIV-1 p24 antigen were observed in the supernatants. On days 6 to 18 post-infection, these cells showed approximately 60-80% inhibition of HIV-1 p24 antigen production

compared with the parental Jurkat cells. The anti-IN scFv-7 in turn demonstrated a more modest inhibitory effect on HIV-1 replication. In addition, expression of control anti-LANA1 scFv (BM10) in Jurkat cell lines had no significant inhibitory effect on HIV-1 replication, indicating that our anti-IN scFv are specific for IN inhibition. Previous studies have shown that intracellular antibody expression has no obvious negative effect on cell viability or proliferation. Nevertheless, we quantified cell proliferation and cell viability of infected scFv-Jurkat cells and compared them to non-infected cells. The assay consists of a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche). We observed that the kinetics of WST-1 metabolism in scFv-Jurkat cells infected with HIV had similar levels of proliferation in comparison with non-infected cell lines (data not shown). The inhibition results obtained here indicate that HIV-1 replication was dramatically reduced in Jurkat cells at early stages by expression of our anti-IN intrabodies. To further evaluate if HIV-1 viral replication could also be inhibited during late stages by our anti-IN scFv, the viruses resulting from infection were normalized by the same TCID50 and used to measure the RT activity in the virions as described in Material and Methods at day 12. As demonstrated in Figure 2.13, the virions released from Jurkat cells expressing scFv-anti-LANA (BM10) showed RT activities similar as the wild-type virions. In contrast, virions released from Jurkat cells expressing scFv-104, scFv-135, scFv-142 and scFv-144, showed a pronounced reduction in the RT activities compared to wild-type virus. A more reduced effect in RT activity was obtained with anti-IN scFv-7 (only 10% less than the wild-type level), as already expected. We can also see that scFv-142 has

the stronger activity in the inhibition of the HIV-1 RT activity. In conclusion, our data demonstrates that the scFv 104, 135, 142 and 144 generated from immunized rabbits with HIV-1 IN can simultaneously block early and late stages of HIV-1 replication.





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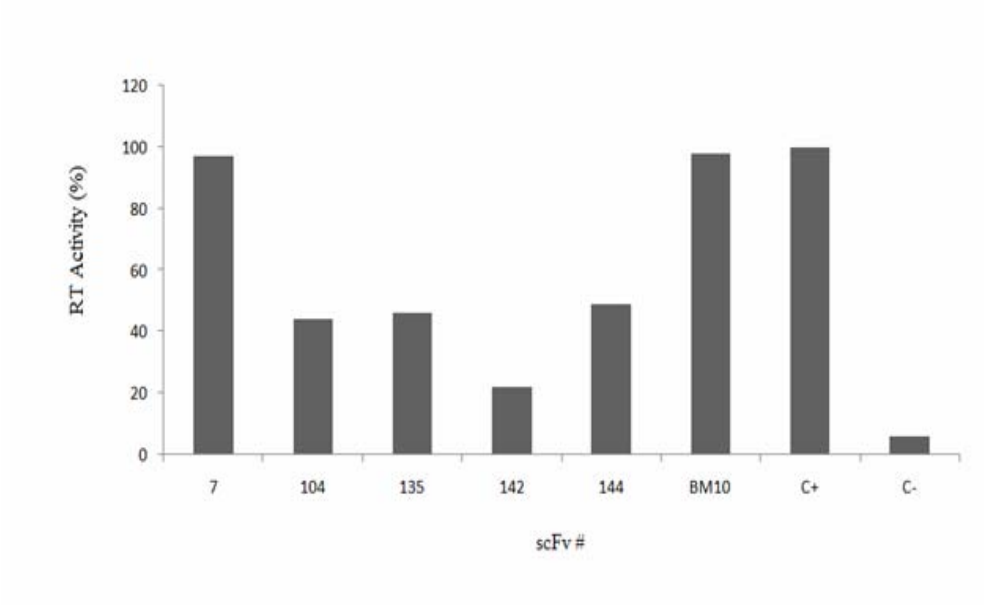

Figure 2.13 – HIV-1 challenge of stable anti-IN-scFv-Jurkat cell clones. (A). Western-blot of cell lysates of stable Jurkat cell lines expressing anti-IN ScFv and anti-LANA1 scFv (clone BM10). Lysates of Jurkat cells not expressing scFv were also used in Western blot as negative controls (mock). (B) Stable Jurkat cell clones expressing anti-IN scFv intrabodies were infected with HIV-1_{NL4-3} at a MOI of 0.1 to 0.5. The cultures were maintained for up to 20 days, and to monitor infection aliquots were taken at the indicated time points to determine p24 levels by ELISA. BM10 was used as an irrelevant antibody; C^+, HIV_{NL4-3} infected cells; C^-, uninfected cells. The data is representative of two independent experiments. (C) RT activity levels were detected in viral supernatants at day 12. Viral supernatants were normalized by the same TCID50 and subjected to ELISA RT activity assay system as described in Material and Methods. Values represent the percentages of virions RT activity relative to the value obtained for the wild-type. BM10 was used as an irrelevant control antibody; C^+, HIV_{NL4-3} virion RT activity; C^-, uninfected supernatant RT activity.

2.4 Discussion

Controlling HIV infection continues to be a major challenge in both underdeveloped and developed nations. Although the drug cocktails used in HAART have markedly changed the profile of progression to AIDS in HIV-infected individuals, drug failures continue to occur as a consequence of viral resistance and other complications arising from a lifelong regimen of chemotherapy. Therefore, the development of new modes of inhibition is of paramount importance and has been the focus of recent research efforts. Within this context, gene therapy has captured the interest of a number of investigators as an attractive addition to conventional pharmacologic therapies since; alteration of the host cell could potentially confer permanent suppression of viral replication, after infection, or could provide protection against viral infection. We have explored this approach by developing intrabodies to the HIV-1 IN protein. IN catalyzes the integration of viral cDNA into the host chromosome. Moreover, there are no cellular homologs to IN and the reactions catalyzed by IN are unique. Thus, scFv-based strategies directed against IN may be an effective approach to inhibit this crucial step of the viral replication cycle.

In the present study, we have for the first time immunized rabbits with HIV-1 IN and developed a combinatorial scFv phage-library against IN. While the generation of rabbit monoclonal antibodies by hybridoma technology has also been reported [331], the phage display approach with its inherent linkage of phenotype and genotype provides ready access to antibody sequences and facilitates further *in vitro* optimizations such as humanization

or affinity maturation. Furthermore, with combinatorial scFv libraries we have the potential to display the entire immunological record of an individual, allowing the detection and recovery of any antibody ever made, irrespective of whether it is currently being produced.

The results presented here shown that we were able to identify 5 new scFv intrabodies that specifically recognize and bind HIV-1 IN protein. These scFvs bound simultaneously to two epitope regions in the catalytic domain (amino acids 126-145 and 176-195) and to one upstream region in the C-terminal domain (amino acids 206-235) of IN protein. To our knowledge the scFv fragments described in this work constitute the first set of anti-IN antibodies that recognize and bind simultaneously to three epitope regions in IN. As mentioned above, these epitope regions are close together in the folded IN structure and form a cavity. Therefore, it seems that with our combinatorial scFv library we were able to identify a panel of new anti-IN scFv fragments that can reach targets not easily accessible by conventional antibodies molecules, such as small pockets or canyons in the IN protein. Moreover, since our anti-IN intrabodies bind simultaneously to three epitope regions in the IN protein, the development of HIV-1 escape mutants will be minimized. Our results have also shown that strongly binding of anti-IN scFvs inhibit the strand transfer reaction when these epitopes are target, whereas no effect was observed in the 3'end processing. We can assume that these epitope regions of IN are essential to the strand transfer activity but not to the 3'end processing. Thus, the data obtained here can be used to better understand the intracellular function of IN and determine

which IN residues and protein domains can be effectively target to inhibit HIV-1 IN function.

The expression of anti-IN scFvs in eukaryotic cells showed that our intrabodies could be correctly folded as soluble proteins and were efficiently delivered into the cytoplasm and nucleus compartments. We also showed that both anti-IN scFv formats co-localize with the IN in the nucleus, indicating that the IN NLS domain localized in the C-terminal region was not blocked by the intrabodies. Moreover, these results suggest that the IN must contain a strong NLS domain, as we were not able to trap the IN in cytoplasm when anti-IN scFv and IN are co-expressed. By using a single-round viral assay, we demonstrated that HIV infectivity was strongly inhibited in cells transiently expressing anti-IN scFv-104, scFv-135, scFv-142 and scFv-144 clones in either the cytoplasmic or nuclear compartments. These results demonstrated that the stronger anti-IN scFv binders specifically neutralize the IN activity prior to integration and, thus, have an effect on the integration process itself. Therefore, our scFv-strategy can be used to block early stages of viral replication, due to their capacity to interfere with the establishment of a provirus. Moreover, we also found that these intrabodies were able to interact with the IN protein in the context of the Gag-Pol polyprotein and could be incorporated into the viral particles. As a result, these virions were shown to be less infectious than the wild-type virus, meaning that our intrabodies can also interfere in late stages of viral replication.

To further analyze that our intrabodies were also able to block the IN function during multiple rounds of infection, we developed stable Jurkat cell lines expressing our anti-IN scFv. Results of these experiments extend previous findings that scFv intrabodies can be stably expressed and do not affect cell viability or proliferation. Second, we demonstrate that Jurkat stable cells expressing intrabodies were highly protected from HIV-1 infectivty, indicating that anti-IN scFvs were capable to block the IN activity during multiple rounds of infection. Thirdly, we observed that those virions that escape and were produce in the presence of our intrabodies shown a pronounced reduction in the RT activities compared to wild-type virus. It has been shown that IN also plays an important role during the reverse-transcription steps of HIV-1 infection, possibly through physical interactions with RT [332,333]. Indeed, in a recent study Zhu *et al* have shown that the C-terminal domain of wild-type HIV-1 IN strongly interacts with RT [334]. Although the mechanism(s) of inhibition during late stages of viral replication are not totally uncovered in these studies, it is in our contention that the reduction in RT activity and consequently in the viral infectivity can be the result of an intravirion activity of our anti-IN scFvs.

In summary, the present study strongly suggests that combinatorial rabbit scFv phage-libraries can be efficiently used to identify scFv with new epitope binding characteristics. Moreover, our data provide proof-in-principle that rabbit anti-IN intrabodies can be designed to block early and late stages of HIV-1 replication. Thus, anti-IN intrabodies can be potential agents for HIV-1 gene therapy approaches and also constitute new tools to better understand the structure and function of HIV-1 IN.

CHAPTER 3

Characterization of a Rabbit VL Single-Domain Intrabody Against the HIV-1Vif Protein

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Manuscript in preparation

Abstract

Intracellular antibodies have particular promise in the area of functional genomics and in the long term, may even find an enormous broad therapeutic application in gene therapy setting. The scFv is usually viewed as the suitable format for intracellular expression in eukaryotic cells. However, in the reducing environment of the cytoplasm the intrachain disulfide bridges cannot form and only very soluble and stably expressed antibody fragments will be able to fold correctly in sufficient amounts to be active as intrabodies. Within this context, there is a strong interest in the ability to express functional antibodies in this environment. Recent approaches have shown that VH, VHH and camelized VH single-domain antibodies alone may be useful as intrabodies, with improved intracellular expression. In the present study, we now show that a rabbit VL domain can also be very potentially as intrabody. Our VL single-domain was derived from an anti-Vif scFv that was recently developed from immunized rabbits to HIV-1 Vif protein. The results demonstrate that our VL single-domain could be expressed soluble at high yields, preserve the antigen-binding activity and specificity in the absence of the parent VH domain and show favourable biophysical properties. The expression in eukaryotic cells also showed that rabbit VL single-domain could correctly be folded as soluble protein in the reducing environment and could strongly neutralize HIV-1 infectivity. Therefore, the present study strongly suggests that rabbit VL domains have enormous value as intracellular antigen recognition units.

3.1 Introduction

Over the last two decades, monoclonal antibodies (mAbs) have become the most powerful tools for basic research, diagnostics and therapy [103,105-107]. Advances in recombinant DNA technology and protein engineering has made possible to manipulate the genes encoding antibodies and to express antibody binding fragments in eukaryotic cells [109]. These intracellularly expressed antibodies, termed intrabodies, are typically formulated as single chain Fv (scFv) fragments which comprise immunoglobulin variable (V) domains of heavy (H) and light (L) chains held together by a short peptide linker [109-111]. Intrabodies can be genetically fused with known intracellular protein trafficking signals and thereby be directed to different subcellular comportments [30]. The binding of an intrabody to an intracellular target protein has the potential to block, suppress, alter or even enhance the process mediated by that molecule [31-33]. This new approach have particular promise in the area of functional genomics and in the long term, may even find an enormous broad therapeutic application in gene therapy setting. The strong inhibiting potential of intracellular antibodies has been demonstrated in the field of cancer, HIV, transplantation, autoimmune and neurodegenerative diseases [121,131,133-140,144-147].

The recombinant scFv molecule is usually viewed as the suitable format for intracellular expression in eukaryotic cells. However, in the reducing environment of the cytoplasm the intrachain disulfide bridges cannot form and only very soluble and stably expressed antibody fragments will be able to fold correctly in sufficient amounts to be active as intrabodies [29].

Moreover, the interaction of VH and VL single-domains by the interface is weak and may aggregate in the cytoplasm being targeted to proteosome degradation. Within this context, there is a strong interest in the ability to express functional antibodies in this environment and only recently several different approaches started to emerge with such requirements. These approaches include *in vivo* selections for intrabody-antigen interaction based on two hybrid screenings, and construction of antibody libraries using randomized CDRs on scFv frameworks that have been selected for high solubility and stability in an intracellular environment[152-157,190]. Recently, single-domain antibodies (dAbs) are becoming a major strategy to generate effective intrabodies.

Single-domain antibodies are the smallest functional antigen-binding fragments of an antibody, consisting of only the variable heavy (VH) or variable light domain (VL) [126]. Each single-domain contains only three of the six complementarity determining regions (CDRs) from an antibody. However, this can be sufficient for antigen binding specificity and high affinity, as seen in camelids [127]. Members of this family have a class of antibodies which contain no light chains and have functional antigen binding regions consisting of a single unpaired heavy chain variable domain, referred to as VHH [127,128]. Recent studies have shown that single-domains antibodies can be easily produced as recombinant proteins in microbial cell culture and appear to be more soluble and stable [126,128]. In addition, these smaller antibody molecules might, reach targets not easily accessible by conventional antibodies molecules, such as enzyme active sites and canyons in viral and infectious disease biomarkers [126,129].

Thus an antibody molecule can be easily reduced in size to a single functional variable domain and be used as an intrabody.

The first study of a single-domain tested as intrabody was reported in 2003 by Tanaka *et al* [130]. In this work the authors, demonstrated that VH single-domains could be highly expressed in mammalian cells and exhibited specific antigen recognition *in vivo*. Moreover, VH selected for binding to the RAS protein could inhibit RAS-dependent oncogenic transformation of NIH3T3 cells [130]. We have also recently developed a minimal rabbit VH fragment with intrabody properties derived from an anti-Vif scFv that was engineered to mimic camelid antibody domains [121,131]. These camelized VH single-domain intrabodies were highly expressed in reducing environments and exhibited a strong neutralization of HIV infectivity [121]. Natural VHH antibodies have also been validated as intrabodies. In this case, Bax-specific VHH intrabodies were efficiently developed to prevent mitochondrial membrane potential collapse and apoptosis after oxidative stress in the host cells [132]. Although the first attention was directed to VH and VHH, VL single-domains are also becoming of considerable interest as intrabodies. In a recent study using a yeast surface display to isolate a scFv against amino terminus huntingtin (htt) protein, the binding site of the highest affinity variant was mapped to just the VL domain [135]. This was the first reported case where a scFv paratope mapped entirely to the VL domain. In addition, this human VL single-domain was markedly better expressed in the cytoplasm relative to its scFv counterpart and consequently effectively blocked aggregation off htt in a cellular model of Huntington's disease.

In the present work, we show that rabbit VL single-domains can be efficiently used as intrabodies. The VL scaffold was derived from an anti-Vif scFv that was recently developed from immunized rabbits to HIV-1 Vif protein and inhibited viral replication. Our results demonstrate that the anti-Vif VL single-domain preserve the antigen-binding activity and specificity in the absence of the parent VH domain. In addition, the VL single-domain was highly expressed in microbial cell culture and show favourable biophysical properties. The expression in eukaryotic cells also showed that the rabbit VL single-domain was correctly folded as soluble protein in the reducing environment and could strongly neutralize HIV-1 infectivity. Therefore, the present study suggests that rabbit VL single-domains may have an enormous value as intracellular antigen recognition units.

3.2 Material and Methods

3.2.1 Cloning of VL anti-Vif single-domain

A fragment encoding the anti-Vif VL domain was generated by PCR from the pComb3X phagemid vector containing the anti-Vif scFv gene (pComb3X-4BL). The following primers were used: SDVL-F 5'-CCT GGC CGG CCT GGC C GCCTGTGACGGTCAGCTGGGTCCC-3'and SDVL-R 5'-CCT GGC CGG CCT GGC C GCCTGTGACGGTCAGCTGGGTCCC-3'.The resulting PCR fragment was gel-purified, digested with the restriction endonuclease *Sfi*I, and cloned into the phagemid vector pComb3X. The pComb3X plasmid contains a suppressor stop codon and sequences encoding peptide tags for purification (6-His) and detection (HA). After expression studies in *E. coli* TOP10F and analysis of binding activities, the VL single-domain gene was transferred into pcDNA3.1/Zeo^{+} (Invitrogen). The primers used for cloning in pCDNA3.1/Zeo^{+} were: Babe ScFv5 5'-GGCATGGGGGCCCAGGCGGCCCAGCTC-3' and Babe ScFv3 5'-GCCACCACCCTCCTAAGAAGC-3'. We introduced a sequence encoding the HA-tag sequence (YPYDVPDYA) at the C-terminus, followed by a stop codon. The PCR products were cloned by NotI and XhoI into pCDNA3.1/Zeo^{+}. The Anti-Vif VL single-domain gene was also cloned into pHIVnPLAP-IRES-N+ in place of PLAP-IRES-nef. The primers used for cloning were VL-VH-NOT 5'–

ATAAGAATGCGGCCGCTAAACTATATGGGGGCCCAGGCGGCCGA GCTC-3' and 4ScFv-HIV-Xho 5'-CCGCTCGAGCGGGCCACCACCCTCCTAAGAAGC -3'.

3.2.2 Expression and purification of antibody fragments

To express and purify anti-Vif antibody fragments from the bacteria periplasmic space, each phagemid DNA was transformed into non-suppressor *E. coli* strain TOP10F. A fresh colony of each clone was grown at 30ºC overnight in SB medium containing 100 μg/ml of ampicillin. A 10 ml sample of cells was used to inoculate 1 liter of SB medium containing 100 μg/ml of ampicillin. Cells were grown at 30ºC until A_{600nm}= 0.9, induced by the addition of 0.5 mM IPTG and growth was continued for 18 hours. After induction, bacteria were harvested by centrifugation (4,000 × *g*, 4°C, and 15 min), resuspended in 50 ml equilibration buffer (20 mM NaH_2PO_4, 0.5 M NaCl, 30 mM imidazole (pH 7.4)), supplemented with protease inhibitors (Roche), and lysed by sonication. Cell debris were removed by centrifugation (40,000 × *g*, 4° C, 30 min), and the supernatant was filtered through a 0.2-μm syringe filter. Each antibody fragment was purified by nickel chelate affinity chromatography making use of the C-terminal 6His of pComb3X. Bound proteins were eluted with a linear imidazole gradient (0-300 mM imidazole in 20 mM NaH_2PO_4, 0.5 M NaCl (pH 7.4)). The appropriate fractions from IMAC were pooled, dialyzed against PBS and concentrated by Centricon columns (Millipore). Protein purity was checked by non-reducing SDS-15% PAGE. Protein

quantification was determined by measuring the optical density at 280 nm by the classic Bradford method.

3.2.3 ELISA measurements

To analyze relative antigen binding affinities of anti-Vif VL, VH, VHD and scFv 4BL antibody fragments, ELISA plates (Nunc) were coated with 100 ng of purified recombinant HIV-1 Vif protein, thyroglobulin or BSA, overnight at 4º C. Wells were blocked for one hour at 37ºC with BSA 3% in PBS. Purified anti-Vif VL, VH, VHD and scFv 4BL were added to the wells for further incubation. After washing the wells with PBS, HRP-conjugated anti-HA mAb (Roche) was used for detection. The results were measured by optical density at 405 nm and performed in triplicate.

3.2.3 Protein expression in reducing environment and alkylation with AMS

To assess the effect of the reducing environment on anti-Vif VL, VHD and scFv 4BL folding in the periplasm of *E. coli* Top10F we used a similar method as described by Missiakas *et al* [335] and Martineu *et al* [336]. Briefly, a fresh colony was grown at 30ºC overnight in SB medium containing 100 μg/ml of ampicillin. A sample of 1 ml of cells was used to inoculate 10 ml of SB medium containing 100μg/ml of ampicillin. Cells were grown at 30ºC until A_{550nm} = 0.9 and 5 mM DTT was added at the time of induction with 0.5 mM IPTG. After 18 hours induction, density of bacteria culture was normalized. Cultures were centrifuged (4000g, 30 min, 4º C) and cell pellets were resuspended in PBS containing protease inhibitors (Roche). Soluble

periplasmic proteins were extracted with lysozyme and collected by 15 min centrifugation at 14,000g. To confirm the redox state of the proteins in the periplasmic extracts we analyzed the alkylation of samples using the 4-acetamido-4'- maleimidylstilbene-2,2'- disulfonic acid (AMS) as described by Kobayashi *et al* [337] and Jurado *et al* [338]. Briefly, a final concentration of DTT 100 mM was added and then each sample was first incubated for 10 minutes on ice and then 10 minutes at 100º C. Samples were precipitated with TCA for one hour on ice followed by centrifugation at 14,000 g for 15 min at 4ºC. Pellets were washed with ethanol 70%, air dried and resuspended in 40 µl of an alkylating cocktail (150 mM Tris-HCl (pH 7.5), 2% (w/v) SDS, 15mM AMS). After one hour of incubation at 22º C, 40 µl of SDS-PAGE sample buffer (2x) lacking 2- mercaptoethanol were added to all samples. Periplasm extracts were analyzed by non-reducing 15% SDS-PAGE and Western Blot with HRP conjugated anti-HA mAb (Roche). As a control, alkylation of samples with no addition of DTT was performed.

3.2.4 Monitoring intrabody solubility by the antibody-CAT-fusion system

To further evaluate the solubility of each antibody fragments in a reducing environment we adapted the protein-CAT fusion system developed by Maxwell *et al* to our intrabodies [339]. First, each anti-Vif VH, VL, VHD and scFv 4BL genes were cloned into the *BglII* and *XbaI* cloning sites of the pCFN1. The pCFN1 vector was kindly provided by Dr Davidson. This plasmid includes a strong IPTG promoter (*P*trc), a strong translation start with an *NcoI* site at the initiator ATG codon, phage f1 and pBR322 origins

of replication, the ampicillin resistance gene, and the *lacI* q gene. To evaluate protein solubility, chloramphenicol resistance analysis were performed by transforming JM101 cells with each antibody CAT-fusion clone. Briefly, each transformation mixture was inoculated into 5 ml SOC and incubated at 37ºC for 1 hour. Next 10 ml of SB medium with 3 µl of 100 mg/ml ampicillin was added to each transformation. A total 15 ml of each culture was shaken for 1 hour at 37ºC. Subsequently 4.5 µl of 100 mg/ml ampicillin was added and cultures shaken for one hour at 37ºC. Then, 35 ml of SB medium with 35 µl of 100 mg/ml ampicillin was added and cultures grown overnight at 37ºC. The following day, 600 µl of each culture was used to inoculate 20 ml of SB medium containing 100 µg/ml ampicillin. Expression of CAT-fusion antibody fragments was induced by addition of 0.5 mM IPTG when the optical density of cultures reached 0.9. After 2 hours of induction, several dilutions of each clone were plated on agar plates containing 100 µg/ml ampicillin, 200 ug/ml IPTG and various concentrations of chloramphenicol. Plates were incubated at 37ºC for 16-20 hours. The level of resistance was quantified as the highest level of chloramphenicol at which colonies appeared after the 37 ºC incubation period.

3.2.5 Cell lines and Transfections

293T cells were maintained in DMEM Medium and H9, $H9_{38}$ LTR-CAT and Jurkat cells were maintained in RPMI 1640 Medium. Media was supplemented with 10% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) and 2 mM glutamine. All cell

cultures were maintained at 37°C in 5% CO_2. Tissue culture media and reagents were from BioWhitaker. To produce large amounts of HIV-1 particles, 4-5 x 10^6 293T cells were transfected by Fugene (Roche) according to the manufacturer's protocol with 2 μg of wild type HIV-1_{NL4-3}, pHIV-1_{NL4-3}Δ*vif* or pHIVnPLAP-IRES-N expressing anti-Vif antibody fragments.

3.2.6 Pulse - chase analysis

For pulse-chase experiments, 293T cells (1-2x 10^6) were transfected by Fugene (Roche) with 2 μg of VL, VH, VHD and scFv pcDNA3.1 plasmids. Thirty six hours post-transfection, cells were incubated with 1 ml of methionine/cysteine-free medium for 2 hours at 37ºC and metabolically labelled with similar medium containing 100 μCi [^{35}S] methionine/cysteine for 2 hours at 37ºC. After labelling, cells were washed 3 times with 1 ml DMEM supplemented with 40x excess methionine (1.2 mg /ml) and 20x excess cysteine (0.84 mg/ml) and incubated with the same medium for various time points. At each time point, cells were washed twice with cold PBS and lysed on ice for 1 hour with 400 μl lysis buffer containing protease inhibitors (Roche). Lysates were cleared by centrifugation and supernatant incubated overnight at 4ºC with anti-HA affinity matrix (Roche) and then immunoprecipitated proteins were separated by 15% SDS-PAGE. The gel was dried and subjected to autoradiography.

3.2.7 Replication complementation assay

A transient complementation assay was performed as previously described to provide a quantitative measure of the ability of wild-type Vif protein to complement a single-round of HIV-1 replication in *trans.*[51] Briefly, $H9_{38}$ LTR-CAT cells (10^6) and Jurkat (10^6) cells were cotransfected by Fugene (Roche), with 2 μg of pSVCATΔ*env*ΔVif, 2 μg of pVSVG, 2 μg of pSVLVif and either plasmids encoding anti-Vif scFv 4BL, VH, VHD or VL antibody fragments. The ability of antibody fragments to inhibit a single round of infection was measured by assaying for chloramphenicol acetyltransferase (CAT) activity in the transfected culture 9 days after transfection. CAT assay was performed by the *Quan*-T-CAT system (Amersham Biociences).

3.2.8 Cell-specific inhibition of HIV-1 replication

HIV-1 recombinant virus stocks encoding VL, VH, VHD and scFv antibody fragments were prepared by transfection of 293T cells. Forty eight hours post-transfection, the viral supernatants were normalized for the same TCID50 and used to infect permissive cells (Jurkat) and nonpermissive cells (H9). Cell cultures were maintained for up to 20 days and to monitor infection, aliquots were taken at the indicated time points to determine p24 levels by HIV-1 ELISA (Innotest). Permissive and nonpermissive cells were infected with HIV-1_{NL4-3} and HIV-1_{NL4-3} ΔVif as positive and negative controls, respectively. Cellular proliferation and viability of infected H9 and

Jurkat cells were analyzed with tetrazolium salt WST-1 (Roche) according to the manufacturer's protocol.

3.2.9 Gel-Filtration

Gel-filtration was performed using a Pharmacia FPLC system on a Superdex 200 HR 10/30 column. Samples containing variable concentration of VL single-domain were loaded in 100 µl of 50 mM sodium phosphate buffer (pH 7.4). The column was calibrated using molecular weight markers including bovine serum albumin (66 kDa), ovalbumin (45 kDa) and cytrochrome *c* (12.4 kDa).

3.2.10 GdnHCl-induced Unfolding/Refolding Equilibrium

The fluorescence spectrum of the VL single-domain was recorded at 25ºC with a Varian Cary/Eclipse fluorescence spectrophotometer. Unfolding transition curves were determined by reversible chemical denaturation of 10µM solutions of the VL single-domain mixed together in PBS (pH 7.4) with variable concentration of guanidinium chloride (GdnHCl), after overnight incubation at 4ºC. Under these conditions, the unfolding transition is completely reversible, and unfolding and refolding curves are identical within experimental error. The fluorescence emission spectrum was recorded from 295 to 420 nm with an excitation wavelength of 280 nm.

3.3 Results

3.3.1 Anti-Vif VL single-domain strongly inhibit HIV replication

The human immunodeficiency virus type-1 (HIV-1) *vif* gene encodes a 23 kDa protein that is essential for viral replication and spread in peripheral blood lymphocytes and primary macrophages, as well as in some established T-cell lines [20,21,300-304]. We recently demonstrated that anti-Vif scFv intrabodies are an effective approach to inhibit this crucial step of the viral replication cycle [131]. A specific anti-Vif scFv (clone 4BL) from immunized rabbits was shown to bind Vif intracellularly and inhibit viral replication. From this scFv scaffold, we then developed a minimal VH single-domain with intrabody properties that was engineered to mimic camelid antibody domains[121]. Non-specific binding of VH by its interface for the VL domain was prevented through amino acid mutations in framework 2 and 4 (V37F, G44E, L45R, W47G and W103R). These camelized VH single-domain intrabodies were highly expressed in reducing environments and exhibited a strong neutralization of HIV replication[121]. In the present study, we now evaluate if the anti-Vif VL single-domain can also be effective as an intrabody. To test this hypothesis two sets of HIV inhibition experiments were performed. The first experiment was performed by a transient *trans*-complementation assay in nonpermissive cells that require Vif function for HIV-1 replication. *B*riefly, *n*onpermissive $H9_{38}$ LTR-CAT cells were cotransfected with pSVCATΔ*env*ΔVif, pVSVG, pSVL-Vif and pcDNA3.1-anti-Vif-VL as described in Material and Methods section. To compare the VL inhibition with the VH, scFv 4BL and

the camelized VH (VHD) intrabody, anti-Vif VH, scFv-4BL and VHD antibody fragments were also transfected in $H9_{38}$ LTR-CAT cells. The HIV-1 virus particles produced in this assay result in only a single round of infection, as the packaged viral genome is defective for Env production. The efficiency of a single round of virus replication is quantitated by measuring the level of CAT enzyme activity in the infected cultures after 9 days, the minimum time for a detectable signal above background. As shown in Figure 3.1, in the absence of Vif, replication of *vif*-negative virus was ~ 90-fold lower than that of *vif*-positive virus. When anti-Vif scFv and VHD intrabodies are coexpressed in $H9_{38}$ cells the *trans*-complementation is reduced to 25% and 15% of the wild-type level, respectively. When the anti-Vif VL single-domain was coexpressed a strong reduction was also observed in the *trans*-complementation (~85% of the wild-type level). In contrast, the anti-Vif VH single-domain caused a much less significant reduction in viral replication. As a control, permissive cells (Jurkat) that do not require Vif function to HIV replication were cotransfected with pSVCATΔ*env*ΔVif, pVSVG, pSVL-Vif and plasmids encoding anti-Vif fragments. In this case, no inhibitory effect was observed on HIV-1 replication.

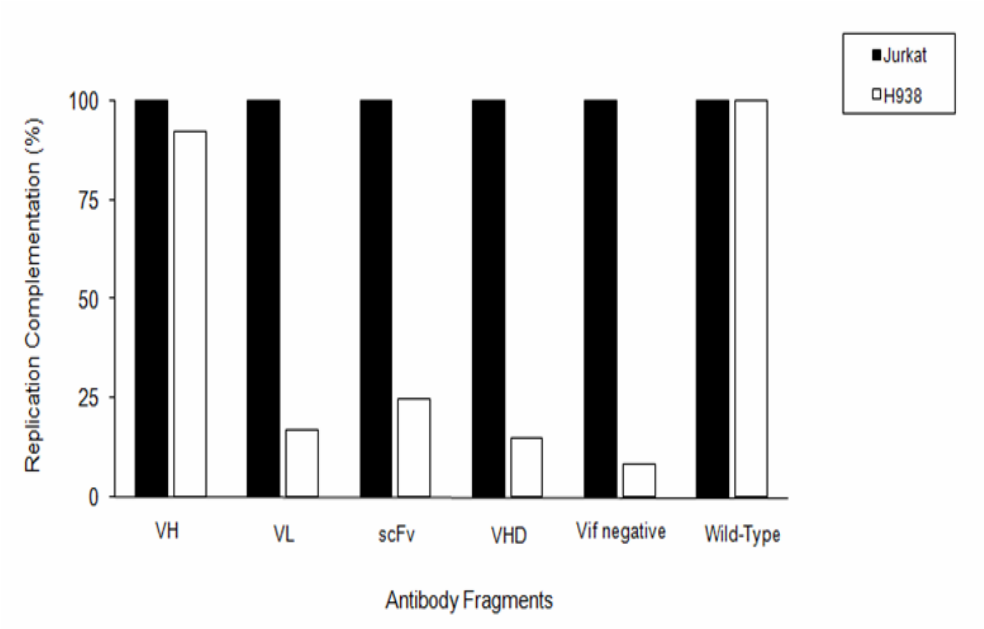
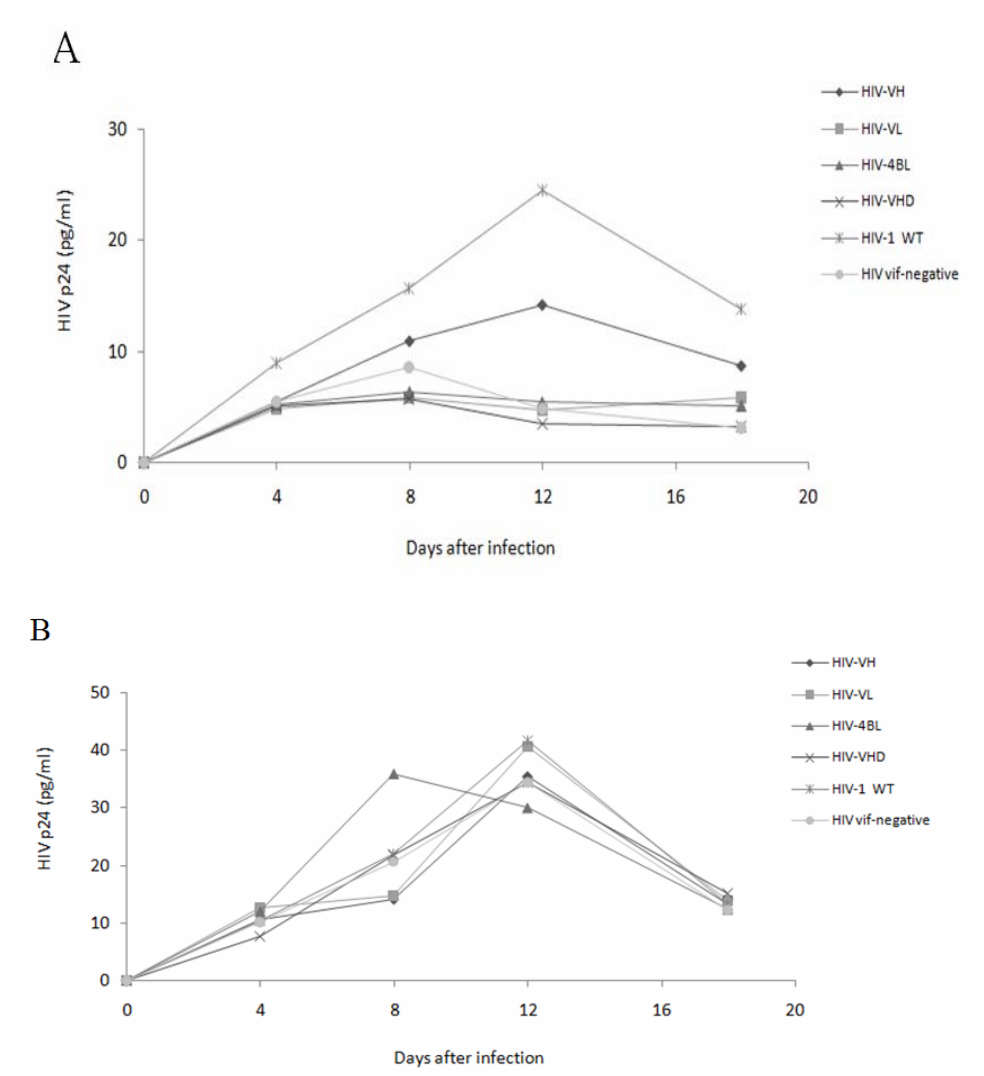


Figure 3.1 - Neutralization of Vif function in a *trans*-complementation assay. Values shown represent the percentages of replication-complementation in nonpermissive $H9_{38}$ cells (White bars) and permissive Jurkat cells (Dark bars) relative to the value obtained for the wild-type. Cells were cotransfected with pSVCATΔ*env*ΔVif, pVSVG, psvlVif, and either anti-Vif VL, VH, VHD or scFv 4BL plasmids as described in Material and Methods. The ability of anti-Vif antibody fragments to inhibit a single round of infection was measured by assaying for chloramphenicol acetyltransferase (CAT) activity in the cell cultures 9 days after transfection.

The *trans*-complementation assay examined the biological activity of anti-Vif antibody fragments in a transient assay under conditions in which most virus transmission occurs by cell-to-cell spread. To evaluate the specificity of viral inhibition in several rounds of replication in nonpermissive and permissive cell lines a second experiment was performed with recombinant HIV-1 viruses expressing anti-Vif fragments in *cis*. These recombinant HIV-1 viruses were generated from pHIVnPLAP-IRES-N+, by replacing the human placental alkaline phosphatase (PLAP) gene by VL, VH, VHD

and scFv-4BL, generating pHIV-VL, pHIV-VH, pHIV-D and pHIV-4BL. Each plasmid was then transfected in 293T cells, and high titre supernatants of each virus was obtained. HIV-1 supernatants normalized for the same TCID50 were used to infect permissive cells (Jurkat) and nonpermissive cells (H9) at a multiplicity of infection of 0.1 and their ability to replicate in these cells was assessed by performing standard virus growth curves. The cell cultures were maintained for up to 20 days and to monitor infection, aliquots were taken at the indicated time points and HIV-1 p24 antigen levels were determined by ELISA. As shown in Figure 3.2, nonpermissive cells did not support the spread of HIV-D, HIV-4BL and HIVΔ*vif*. When H9 nonpermissive cells were infected with HIV-VL virus the same pattern of virus replication inhibition was observed. In contrast, the permissive cell line Jurkat supported replication of all viruses used in this experiment. We have previous shown that intracellular antibody expression has no obvious negative effects on cell viability or proliferation. Nevertheless, we quantified cell proliferation and cell viability of infected permissive and nonpermissive cells compared with non-infected cells. The assay consists of a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells (Roche). The kinetics of WST-1 metabolism showed that H9 and Jurkat cells infected with HIV expressing anti-Vif antibody fragments have similar levels of proliferation compared with non-infected cell lines (Figure 3.2 C and D). The two inhibition assays described above clearly demonstrate that the anti-Vif VL single-domain intrabody strongly neutralize HIV replication. The VL single-domain tested in this study derived directly from the anti-Vif scFv 4BL scaffold. Therefore, to further understand why this VL single-domain was

so effective as an intrabody in the absence of the parent VH domain and without any camelization process, several experiments were performed to characterize the VL domain in more detail.



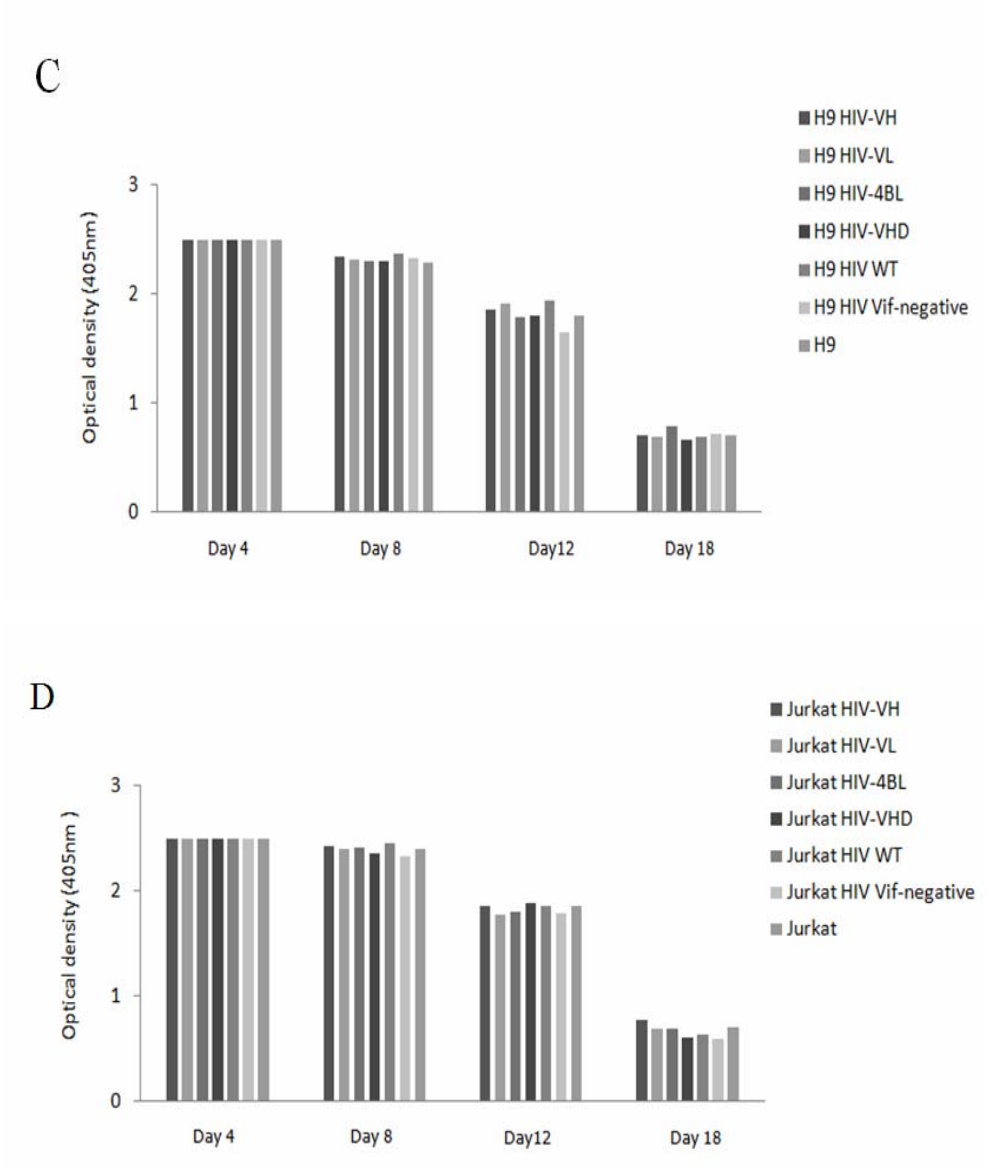


Figure 3.2 - Cell-specific inhibition of HIV-1 replication in the presence of anti-Vif antibody fragments. Replication of HIV-1 encoding anti-Vif VL, VH, VHD and scFv 4BL antibody fragments was assessed in permissive and nonpermissive cells. Cell-specific inhibition was started with HIV-1_{NL4-3} encoding anti-Vif antibody fragments produced after transfection of 293T cell lines with full-length proviral DNA as described in Material and

Methods. (A) - Nonpermissive H9 cells were infected with HIV-VL, HIV-VH, HIV-VHD and HIV-4BL. (B) - Permissive Jurkat cells were infected with HIV-VL, HIV-VH, HIV-VHD and HIV-4BL. Infection of H9 and Jurkat cells with wild-type HIV-1_{NL4-3} and with HIV-1_{NL4-3} ΔVif were used as positive and negative controls, respectively. The cultures were maintained for up to 20 days, and to monitor infection aliquots were taken at the indicated time points to determine p24 levels by ELISA. The data are representative of two independent experiments. (C) - H9 cells proliferation kinetics with WST-1 reagent. (D) - Jurkat cells proliferation kinetics with WST-1 reagent. The results of cellular proliferation and viability were measured according to the manufacturer's protocol (Roche) by optical density at 405 nm.

3.3.2 Expression and relative binding affinity of the anti -Vif VL single-domain

The aim of the first experiment was to compare the expression yields and the relative binding affinities of the anti-Vif VL with VH, VHD and scFv 4BL antibody fragments. Briefly, to express each antibody in the bacteria periplasmic space, phagemid DNA´s were transformed into non-suppressor *E. coli* strain TOP10F. After IPTG induction, samples of bacteria were taken just prior to induction of protein expression (non-induced) and at 2**,** 4**,** 6, 12 and 18 hours**.** Cells were normalized for the same OD_{600}, lysed and then each soluble protein fraction was separated by 15% SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western Blot with HRP-conjugated anti-HA mAb. As shown in Figure 3.3A, all antibody fragments were expressed in the periplasm as soluble proteins. However, VHD and VL single-domains were clearly the two antibody fragments that showed the higher expression and purification yields. Purification yields of soluble protein from 1 liter bacterial culture were 0.6 ± 0.1 mg in the VH

construct, 2 ± 0.1 mg in scFv 4BL and 8 ± 0.1 mg in VL and VHD. The VHD was previously demonstrated to be highly expressed as a soluble protein due to the VH camelization process that was applied to mimic camelid antibody domains. In the case of the VL, no camelization process or protein engineering was applied. Therefore, it seems likely that the VL single-domain can be naturally expressed as soluble protein at high levels in the absence of the parent VH domain.

To analyze and compare the binding activity of the isolated VL domain with VH, VHD and scFv 4BL antibody fragments, an ELISA was performed as described in the Material and Methods section. As shown in Figure 3.3B, the VL single-domain showed similar binding patterns to that of the VH and VHD. Moreover and as previous observed with VH[121], the VL also displayed a ~ 50- fold lower relative affinity for Vif as compared to the parental anti-Vif scFv. Control experiments showed near background signals and lower non-specific binding of all single-domains to bovine serum albumin (BSA) and thyroglobulin, similar to that of anti-Vif scFv. This suggests that the binding of VL and VH domains antibodies is mediated through the CDRs loops and is not mediated *via* some other part of the molecule, for example the putative hydrophobic surface which is normally covered by the corresponding paired VH or VL domain in conventional antibodies. Therefore, the decrease in the relative binding affinity of the VL single-domain is not dramatic and still allows constant specific binding to HIV-1 Vif protein as observed previously for the VH and VHD intrabodies [121].

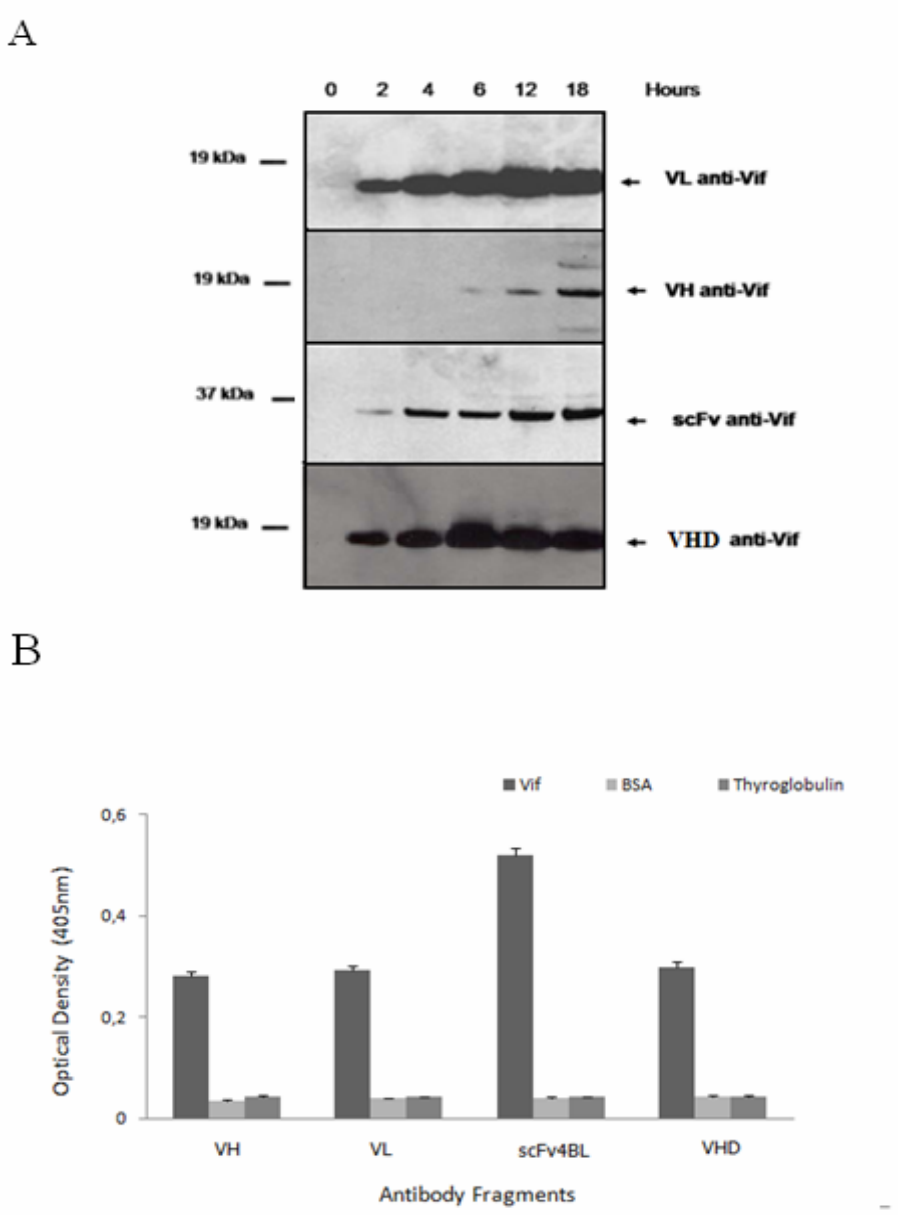


Figure 3.4 - Expression and relative binding affinities of anti-Vif VL, VH VHD and scFv 4BL antibody fragments. (A) *E. coli* TOP 10F bacteria expressing anti-Vif VL, VH, scFv 4BL and VHD at 2, 4, 6,12 and 18 hours after induction with 0.5 mM IPTG at 30°C. Antibody fragments were separated by 15% SDS-PAGE and visualized by Western blot

probed with HRP-conjugated anti-HA monoclonal antibody. Molecular weight is indicated in kDa. (B) The anti-Vif VL, VH, scFv 4BL and VHD antibody fragments were used for evaluating relative binding affinities to 100 ng of Vif protein, thyroglobulin and BSA by ELISA. Results were measured by optical density at 405 nm. Data represents results of three independent experiments; anti-Vif scFv 4BL was used as positive control. As shown, all single-domains have similar binding patterns to Vif antigen, but less than scFv 4BL. Background levels were detected using the control antigens BSA and thyroglobulin.

3.3.3 Expression and redox state of anti-Vif antibody fragments in reducing environment

Antibody domains carry a highly conserved internal disulfide bond, connecting both β-sheets of the β-sandwich structure [105]. Functional antibody fragments that fold under reducing conditions or which no longer require cysteine residues (Cys) for folding, give a number of significant advantages as intrabodies. The aminoacid sequence of the light and heavy chain variable chain of the anti-Vif scFv 4BL is shown in Figure 3.4. The VL sequence of the scFv 4BL belongs to the $V\kappa_1$ family. Most rabbit light chains have an extra disulfide bridge that links the variable and constant domains in addition to the two conserved intrachain disulfide bridges. In the case of the anti-Vif VL single-domain we can observe that this additional Cys is present [218,219]. Moreover, we can also detect two additional Cys present in the CDR3. The presences of Cys in CDRs are usually observed in camelid antibody fragments [127,128]. However, in our laboratory we also observe that this characteristic is quite common with rabbit antibodies (data not shown). The VH sequence of the scFv 4BL belongs to the VH1 family and only the two constant intrachain disulfides bridges are present.

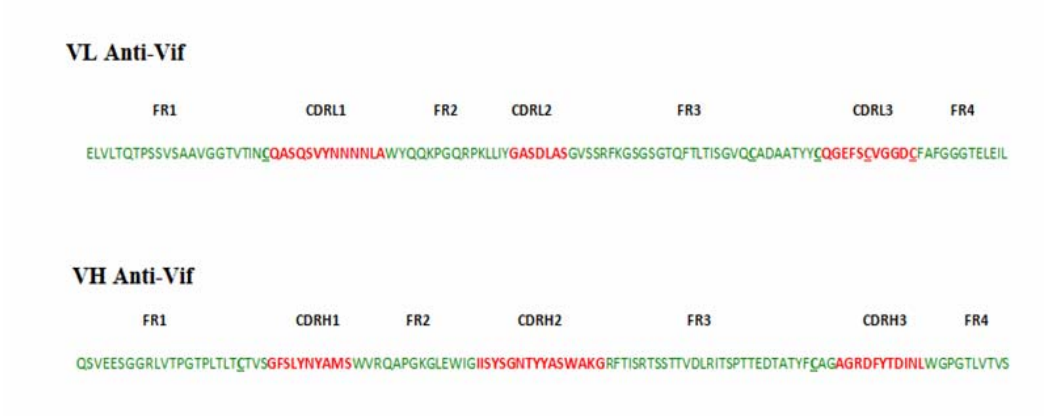


Figure 3.4 – Amino acid sequence of rabbit anti-Vif VL domain. The frameworks, CDRs and the amino acid numbering are as defined by Kabat *et al*. The Cys are underlined.

In order to determine if our anti-Vif antibody fragments do not require the cysteine residues to be correctly folded in a reducing environment, production of VL, VH, VHD and scFv 4BL were induced in the periplasm and 5mM of reducing agent DTT was added to the growth medium at the time of induction [335,336]. This concentration of DTT has been described to be sufficient for mimic the reducing environment of cytoplasm and not impair the growth rate of bacteria cells. To analyze the oxidation state of the Cys residues in anti-Vif antibody fragments, soluble periplasmic extracts were prepared as described in Material and Methods section and incubated with the 4-acetamido-4'-maleimidylstilbene-2, 2'- disulfonic acid (AMS) [337,338]. AMS covalently binds to free thiol groups in proteins, increasing their molecular mass and preventing any further oxidation [338]. Due to the low expression yields and high tendency for aggregation of VH domain, it was only possible to examine and determine the redox state for the VL, VHD

and scFv 4BL antibody fragments. As the data in Figure 3.5 demonstrates, in the reducing DTT environment of periplasm the VL, VHD and scFv fragments could correctly be fold as soluble proteins and were alkylated by AMS (reduced form). In contrast, antibody fragments expressed in non-reducing environment were not alkylated by AMS indicating their oxidized form. Therefore, these data indicate that, in the reducing environment the VL, VHD and scFv antibody fragments can tolerate the loss of intra-domain disulfide bonds and be correctly folded as soluble protein in high yields.

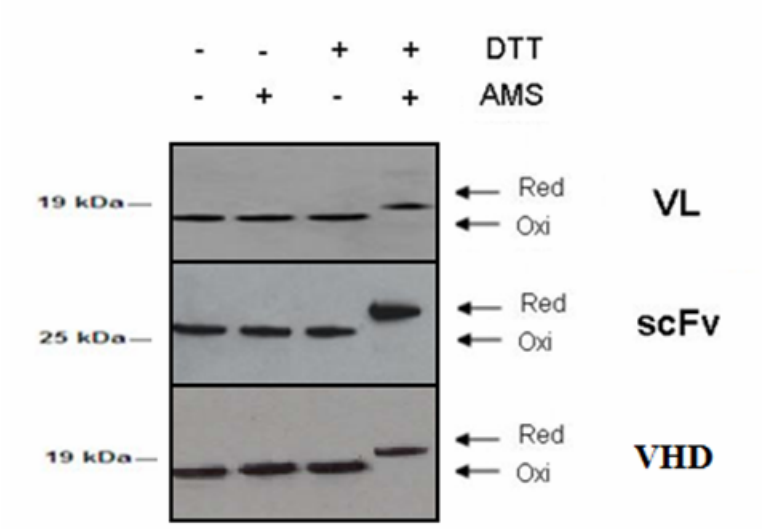


Figure 3.5 - Analysis of soluble fractions of *E. coli* expressing anti-Vif VL, VHD and scFv 4BL antibody fragments in reducing environment and alkylation with AMS. To mimic the reducing environment of the cytoplasm in the *E. coli* TOP 10 F bacteria periplasm, 5mM DTT was added to medium at the time of induction. The redox state of each antibody fragment was analyzed by AMS-alkylation. The alkylated (reduced, red) and nonalkylated (oxidized, oxd) forms of antibody fragments from periplasmic space were prepared as described in Material and Methods, separated by their different mobility in nonreducing 15% SDS-PAGE gel and visualized by Western blot probed with HRP-

conjugated anti-HA mAb. The arrow labelled DTT indicates the presence (+) or the absence (-) of 5mM DTT in the growth medium. As shown, reduced and alkylated proteins are expressed at higher molecular weight compared to oxidized forms when DTT is present. All antibody fragments expressed with DTT are reduced in their cysteine residues. Molecular weight is indicated in kDa.

3.3.4 Monitoring intrabody solubility by the antibody-CAT-fusion system

In a recent study Maxwell *et al* developed a simple *in vivo* system for assessing protein solubility that involves expressing a fusion of a protein or protein domain of interest with chloramphenicol acetyltransferase (CAT), the enzyme responsible for conferring bacterial resistance to chloramphenicol [339]. CAT is a highly soluble homotrimeric protein of 25 kDa molecular weight that has been shown to maintain activity when fused to various other proteins. With this system the authors demonstrate that *E. coli* cells expressing fusions of an insoluble protein to CAT exhibit decreased resistance to chloramphenicol compared to fusions with soluble proteins [339]. To test if this system could also be able to differentiate which anti-Vif intrabody presents the higher solubility in a reducing environment, the VH, VL, VHD and scFv 4BL genes were cloned into the pCFN1-CAT fusion vector (kindly provided by Dr. Maxwell). Each antibody-CAT-fusion plasmid was transformed into JM101 cells and protein expression was induced as described in Material and Methods section. At the end of the induction period cells were diluted and plated on agar plates at increasing chloramphenicol concentrations. The level of resistance was quantified as the highest level of chloramphenicol where colonies appear after an

overnight incubation at 37ºC. Cells without pCFN1-CAT plasmid (negative control) did not grow on chloramphenicol concentration above 10 µg/ml, whereas cells with pCFN1-CAT plasmid (positive control) grew without detectable differences at chloramphenicol concentration as high as 600 µg/ml (Table 3.1). Cells transformed with the pCFN1-VH-CAT showed decreased growth even at 10 ug/ml and did not grow above 20 µg/ml chloramphenicol concentration. In contrast, cells transformed with the pCFN1-scFv4BL-CAT grew at 400 µg/ml chloramphenicol concentration and cells with either the pCFN1-VL-CAT or pCFN1-VHD-CAT constructs grew at 600 µg/ml chloramphenicol concentration.

Table 3.1 - Chloramphenicol resistence conferred by intrabodies CAT-fusion constructs

Protein fused	Solubility	Maximal CAT resistence µg/mL
None	----	600
VH	Insoluble domain	20
VL	Soluble domain	600
scFv	Soluble domain	400
VH-D	Soluble domain	600

These results clearly demonstrate that the chloramphenicol resistances displayed by cells transformed by the pCFN1 derivates were affected by the solubility of each antibody fragment fused to CAT. As expected, the VH domain was shown to be highly insoluble and with high tendency for aggregation. On the other hand, the scFv, VHD and VL antibody fragments revealed to be highly soluble when expressed in a reducing environment.

Moreover, we can observe that among these three antibody fragments, the VL and VHD were those who revealed to be more soluble and correctly folded. Within this context, the VL single-domain alone and without any camelization process appears to be as soluble and stable as the VHD. The results presented here clearly demonstrated that the CAT-fusion system can differentiate soluble from insoluble intrabodies when expressed under reducing conditions. As a result, this system might also be a useful approach for direct *in vivo* screening of soluble and stable intrabody fragments.

3.3.5 Steady-state expression and turnover measurements

In a previous study we demonstrated that the biological activities of the anti-Vif VH antibody fragments were directly correlated with their intracellular solubility and stability [121]. In addition, the steady-state level and long protein half-lifes of camelized VH were shown to be higher than the wild-type VH and thus were far more effective in neutralization of the HIV-1 replication. To further understand why the anti-Vif VL single-domain was so effective as an anti-HIV-1 intrabody, steady-state expression level and turnover rates were measured by pulse-chase experiments in 293T cells Figure 3.6 shows the pulse-chase analysis for the VH, VHD, VL and scFv 4BL antibody fragments.

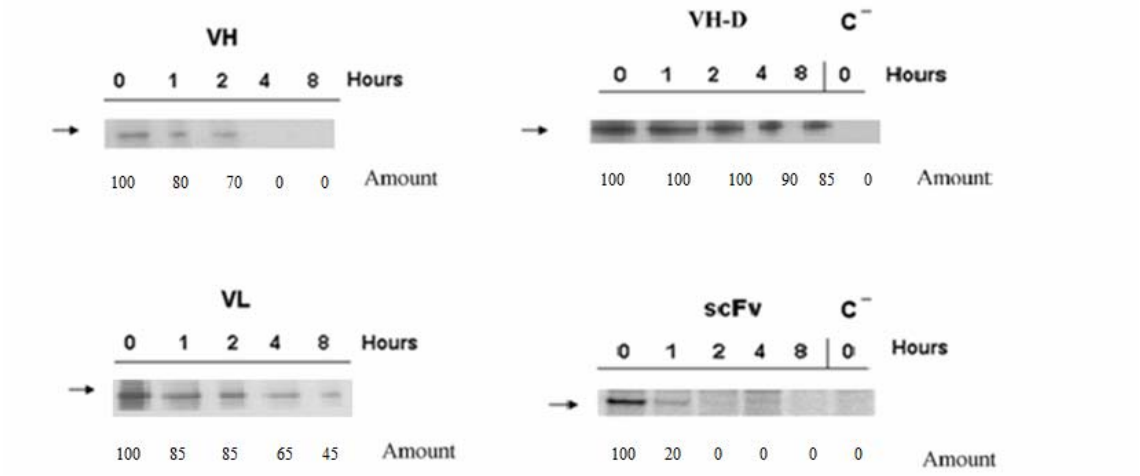


Figure 3.6 - Pulse-chase analysis of anti-Vif VH, VL, VHD and scFv 4BL in transfected 293T cells. At 36 hours following transfection, 293T cells were pulse-labeled in [^{35}S]-methionine/cysteine for 2 hours at 37ºC and chased for various times with DMEM medium supplemented with 40x excess methionine (1.2 mg /ml) and 20x cysteine (0.84 mg/ml). At each time point, cells were lysed in buffer with 50 mM Tris (pH 8.0), 100 mM NaCl, 1% Nonidet P-40 and immunoprecipitated with anti-HA affinity matrix (Roche). The proteins were separated by 15% SDS-PAGE and visualized by autoradiography. Lysates of 293T cells not expressing antibody fragments at time 0 were used in Western blot as a negative control (C^-). Chase times (in hours) are indicated on top. The amount of protein expressed at all time points was determined by optical density relative to the protein expressed at time 0.

As previously demonstrated, the wild-type VH domain shows a lower steady-state level and a relatively short protein half-life (< 2 hours). With respect to the scFv, a high protein steady-state level was observed in the first time point. However, it was not possible to detect any scFv protein quantity after two hours of labelling. The scFv was, therefore, shown to be rapidly degraded and hence its protein half-life was demonstrated to be relatively short (< 1 hour). In contrast, the anti-Vif VHD and VL intrabodies

were found to be the most stable, showing the higher steady-state level and long protein half-life. Moreover, it should be noted that although the VHD single-domain showed a relatively higher steady-state accumulation at 8 hours, its higher stability was only possible to achieve due to the camelization process that was applied. In contrast, the high VL intracellular stability was naturally obtained without any process of protein engineering. Given these results, it can be speculated that the underlying reason that makes the VL single-domain so effective as the VHD intrabody is its capacity to be highly soluble in a reducing environment.

3.3.6 Equilibrium transition experiments of VL single-domain

To confirm that *in vivo* protein results were consistent with *in vitro* protein stability data, the thermodynamic stability of the anti-Vif VL single-domain was examined by GdnHCl equilibrium denaturation experiments. The thermodynamic parameters were calculated using the six-parameter fit on the plot of concentration of GdnHCl versus fluorescence intensity, giving curves consistent with two-state behavior [340]. As shown in Figure 3.7A, the VL domain started to unfold at 1.75 M GdnHCl, where VL is monomeric in solution as indicated by gel-filtration analysis (Figure 3.7B).

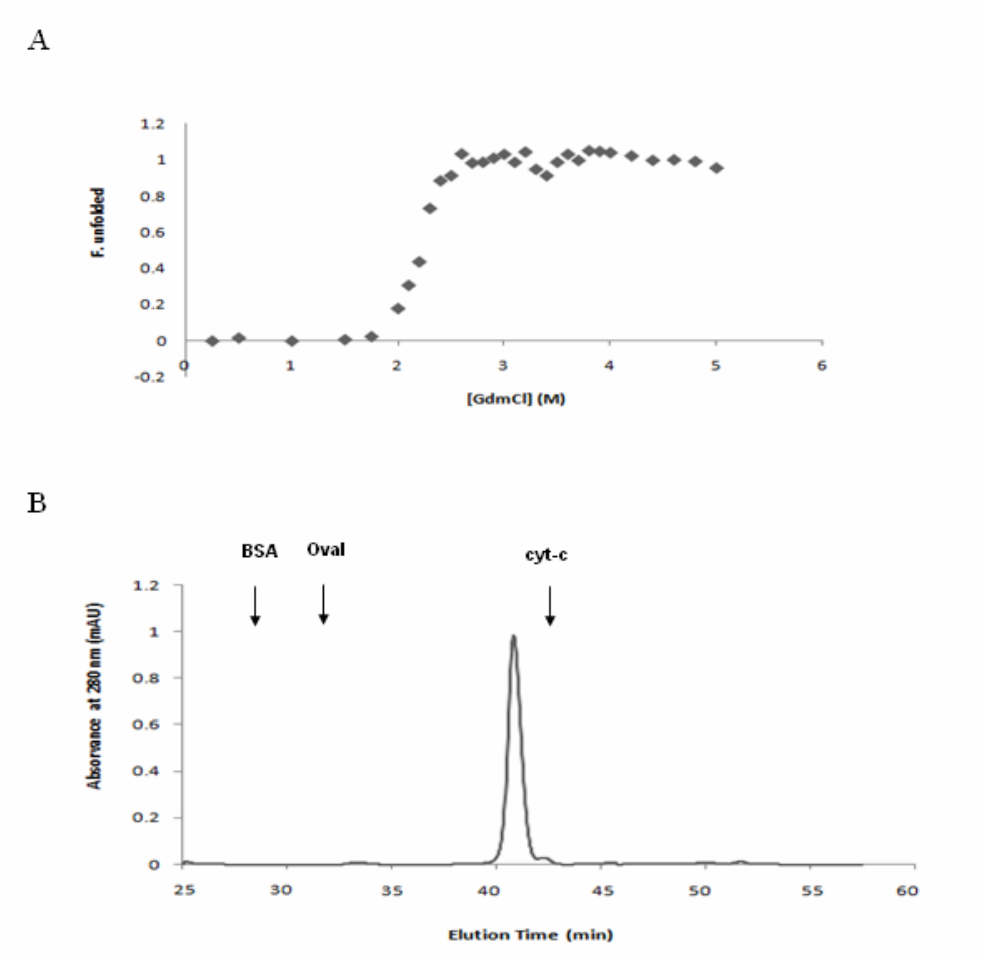


Figure 3.7 – (A) – GdnHCl denaturation of anti-Vif VL single-domain antibody. Unfolding transition curves were determined by reversible chemical denaturation of 10µM solutions of the VL single-domain mixed together in PBS (pH 7.4) with variable concentration of guanidinium chloride (GdnCl), after overnight incubation at 4ºC. The fluorescence emission spectrum was recorded from 295 to 420 nm with an excitation wavelength of 280 nm. **(B) - Analytical gel filtration of anti-Vif VL single-domain in** 50 mM sodium phosphate buffer (pH 7.4) on a Superdex 200 HR 10/30 column at a concentration of 10µM. Arrows indicate elution time of molecular mass standards: bovine serum albumin (66 kDa), ovalbumin (45 kDa) and cytrochrome c (12.4 kDa).

Therefore, the transition is influenced only by the stability of the monomeric VL domain and is not affected by multimerization equilibria. The VL single-domain displayed an unfolding cooperativity (m value) of 18.8 kJ mol^{-1} M^{-1} and a free energy upon unfolding (ΔG_{N-U}) of 42.4 kJ mol^{-1}. The range of the m value can be compared to that expected for proteins of this size (14–15 kDa), and indicate that the VL have the cooperativity expected for a two-state transition [340]. Recently, Ewert *et al* published a study where the biophysical properties of human VL single-domains families were intensely characterized [341]. In this study the authors shown that Vk3 were the most stable VL domain with a ΔG_{N-U} of 34.5 kJ mol^{-1}, followed by Vk1 with 29.0 kJ mol^{-1} and Vk2 and Vλ1 with 24.8 kJ mol^{-1} and 23.7 kJ mol^{-1}, respectively. The least stable VL domains were Vλ2 and Vλ3 with a ΔG_{N-U} of 16.0 kJ mol^{-1} and 15.1 kJ mol^{-1}. When we compare these results with the anti-Vif VL single-domain ΔG_{N-U} of 42.4 kJ mol^{-1} we can clearly note that our rabbit VL scaffold was more stable than the human VL domains. Therefore, the *in vitro* thermodynamic data measured by GdnHCl equilibrium denaturation experiments also indicates the anti-Vif VL single-domain is extremely stable. However, it was already shown by others that the disulphide bond significantly contributes to the stability of antibody domains. Since our goal is to evaluate why the VL single-domain is so effective as an intrabody, further studies are in progress to measure the VL ΔG_{N-U} under reducing conditions. In addition, VH, scFv and VHD thermodynamic stabilities are also being determined so we can compare all the anti-Vif intrabodies stability.

3.4 Discussion

The recombinant single-chain antibody fragment (scFv) is usually viewed as the suitable format for intracellular expression in eukaryotic cells. The purpose of these antibody fragments is to bind a specific protein and thereby inhibit a biological response. However, in the reducing environment of the cytoplasm the intrachain disulfide bridges cannot form and only very soluble and stably expressed antibody fragments will be able to fold correctly in sufficient amounts to be active as intrabodies.[21] Moreover, the interaction of single-domains VH and VL by the interface is weak and may aggregate in the cytoplasm being targeted to proteosome degradation.

Recently, single-domain antibodies (dAbs) have been considered a promising scaffold structure for construction of intrabodies. Single-domain antibodies are the smallest functional antigen-binding fragments of an antibody, consisting of only the variable heavy (VH) or variable light domain (VL). The VH domain has been shown to significantly contribute to the formation of the antigen-binding site. However, the efficient expression of VH fragments is usually confronted with folding problems, low solubility and high tendency for aggregation caused by the exposure of the hydrophobic VH/VL interface upon removal of the VL domain. Nevertheless, these problems seem to have been overcome, or at least greatly reduced for some mouse and rabbit VH domains by methods of genetic engineering that confer the structural properties of the VHH domain of camelids. The strong inhibiting potential of several VH and VHH intrabodies has been demonstrated in the field of cancer, HIV autoimmune

and neurodegenerative diseases [121,130-132]. In contrast, the VL-domain has not been extensively studied as an independent source for intrabodies construction. The study of Colby *et al* is to our knowledge, the only study that reports the construction of a human VL single-domain as an intrabody [135].

In a recent study we have developed a minimal scaffold VH fragment with intrabody properties derived from an anti-Vif single-chain antibody that was engineered to mimic camelid antibody domains [121]. We found that camelization of a rabbit anti-Vif VH domain renders it more soluble and enables higher levels of intracellular expression. As a result, these camelized VH intrabodies revealed higher steady-state levels and long protein half-lifes and strongly neutralize the HIV-1 infectivity. In the present work, we now show that rabbit VL single-domains can also be efficiently used as intrabodies. Our results demonstrate that the anti-Vif VL single-domain was highly soluble and resistant to aggregation when expressed in *E. coli*. When, expressed under reduction conditions the VL single-domain was shown to tolerate the loss of intra-domain disulfide bonds and be correctly folded as soluble protein in high yields. Moreover, although there was a small decrease in the antigen-binding, VL scaffold was shown to preserve its specificity and activity. Therefore, the anti-Vif VL intrabody demonstrated to be so effective in the neutralization of HIV replication as the VHD. These results, together with observations by our group and others suggest once again that the protein turnover rate and the steady state accumulation are the most critical factors for the effectiveness of an intrabody [121, 221, 138, 140]. At the end the concentration of intrabody

inside the cell and the affinity for the antigen will govern the extent of its binding and consequent neutralization of function.

In conclusion, in the present study we now demonstrate that a rabbit VL single-domain antibody can be highly expressed as soluble protein in the absence of the parent VH domain and without any process of protein engineering. Since several other rabbit VL single-domains have already been characterized in our laboratory and shown to be extremely robust, we can consider that rabbit VL single-domains have also an enormous value as intracellular antigen recognition units.

CHAPTER 4

Cell type-specific targeting with Sindbis pseudotyped lentiviral vectors displaying anti-CCR5 single-chain antibodies

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4.0 Abstract

Lentiviral vectors are among the most efficient tools for gene delivery into mammalian cells. A major goal of lentiviral gene delivery systems is to develop vectors that can efficiently target specific cell types. In the present work, we attempt to generate viral particles for targeting gene delivery. We have used CCR5-positive cells as the target for our strategy. Therefore, we developed a novel Sindbis pseudotyped lentiviral vector where the Sindbis receptor binding envelope protein was modified to directly encode a single-chain antibody fragment (scFv) against the CCR5 chemokine receptor. We have generated two chimeric scFv-Sindbis envelopes varying the length of peptide linker that connect the heavy chain and light chain of anti-CCR5 scFv. The two chimeric scFv-Sindbis envelopes were successfully incorporated into lentiviral-derived vectors and the resulting pseudotyped viral particles showed specific targeting to CCR5-expressing cells. However, our data demonstrate that the length of the peptide linker significantly affect the efficiency of infection. Pseudotyped viral particles, which display single-chain antibody fragments with longer peptide linkers, allowed higher titers of infection. The present study can be a model strategy for specific gene delivery mediated by lentiviral vectors pseudotyped with Sindbis envelope displaying scFv that recognize specific cellular surface proteins. Furthermore, this strategy has the potential to become a powerful approach for targeting gene delivery in anti-HIV gene therapy due to the important role of CCR5 expression in disease progression.

4.1 Introduction

Recent approaches of gene therapy have been proposed and developed for overcoming inborn errors of metabolism as well as in the treatment of cancer and HIV infection [149,342]. In a large number of trials, the tools of gene delivery consist of lentiviral vectors [206]. One of the main challenges faced by lentiviral gene delivery systems is the development of vectors that can efficiently target specific cell types [200, 206]. The most common approach developed to date, changes the tropism of envelope lentiviral receptor-binding domain with a cell specific ligand, or a single-chain antibody fragment that recognizes and binds to specific cell surfaces molecules [199-208]. Some of theses approaches have allowed some degree of cell-type-specific viral entry, however, the envelope alterations in most strategies also affect viron assembly and lead to low fusion activity and viral titers [205,209,343,344]. Within this context, there is a strong interest for the ability to develop new and improved strategies allowing a lentiviral cell specific targeting.

Recently, Morizono *et al* reported an approach to improve lentiviral targeting by exploring some properties of the Sindbis virus, a member of the Alphavirus genus [208]. This virus can be produced at very high titers, shows high levels of expression and, unlike retroviruses, the fusogenic protein can fuse to cells independently of the receptor binding protein [202,208,345,346]. The Sindbis virus encodes two transmembrane envelope proteins, E1 and E2 [208]. Although E1 and E2 form a heterodimer that function as a unit, the E1

domain is responsible for pH-dependent fusion and E2 for binding to receptor cells [208,346]. Morizono *et al* demonstrated that lentiviral vectors can be pseudotyped with Sindbis virus envelope modified with a Fc interaction domain of protein A (ZZ domain), to bind a monoclonal antibody against a specific cell surface antigen. This strategy provides enrichment in terms of level of infection and specificity [208]. Thus, these new vectors have received considerable attention for use as cell-specific targeting. In the present study, we aim to extend the utility and advantages of this new approach. For this purpose, we have generated a chimeric scFv-Sindbis pseudotyped lentiviral vector to be tested for specific gene delivery.

The entry of human immunodeficiency virus (HIV) requires a specific interaction of the viral envelope glycoprotein and a cell receptor complex formed by CD4 and a chemokine receptor, CXCR4 or CCR5 [48,49,347]. HIV variants that use CXCR4 are typically detected at the later stages, and are associated with a rapid decline in CD4+ T cells and progression to AIDS [348-350]. In contrast, CCR5-tropic variants dominate the early stages of HIV infection and frequently persist during the entire course of the disease [349,351,352]. Therefore, CCR5-positive cells are the critical first targets in HIV infection and the CCR5 expression is key in disease progression [148,220]. Hence, the CCR5 can be a useful molecule for gene therapy targeting. A single-chain antibody fragment (scFv) specific to human CCR5 has recently been developed and characterized in detail [148,220]. We therefore

sought to apply this scFv for targeting specific cell types that express CCR5. With this objective, we modified the Sindbis envelope by inserting the ST6 scFv into the Fc-binding position (ZZ domain) previously reported by Morizono *et al*. Our results confirm that the modified scFv-Sindbis virus envelope can pseudotype lentiviral-derived vectors and, most importantly, we show that it is possible to target lentiviral vectors specifically to cells that express CCR5 chemokine receptor. Furthermore, this strategy has the potential to become a powerful approach for targeting gene delivery in anti-HIV gene therapy due to the important role of CCR5 expression in disease progression.

4.2 Material and Methods

4.2.1 Cloning and plasmids

A recombinant anti-CCR5 specific single-chain antibody fragment (ST6) was kindly provided by Carlos Barbas III. This scFv was isolated from an immunized rabbit by using the phage display approach and has been characterized in detail [148,220]. A DNA fragment encoding the ST6 scFv was generated by PCR amplification, using as template the phagemid pComb3X-ST6, which contains a gene encoding the anti-CCR5 scFv. The scFv was modified at 5' and 3' end with the following primers introducing the *BstEII* sites: ST6-BstEII-F 5'-AGGAGGAGCAGGTAACCGGAGGCGGGGCCCAGGCGGCCGAGCTC-3' and ST6-BstEII-HA-B 5'-GGTGGTTTGGTGGTTACCCCACCTCCCAGAAGCGTAGTCCGGAACGTCGTACGGGGTACTGGCCGGCCTGGCCACTAGTG-3'. The resulting PCR fragment was gel-purified, digested with the restriction endonuclease BstEII, and cloned into the BstEII sites of the Sindbis virus envelope protein expression vector, pIntron Sindbis ZZ [208], generating the pIntron Sindbis-ST6SL. The pIntron Sindbis ZZ, kindly provided by Irvin S. Y. Chen, contains BstEII cloning site, inserted between the amino acids 71 and 74 of E2 [208]. With this cloning step, we modified the Sindbis ZZ envelope by inserting the ST6 scFv into the Fc-binding domain (ZZ) of

pIntron Sindbis ZZ. Original VL and VH fragments of ST6 scFv are covalently linked with a peptide linker of seven aminoacids (GGSSRSS). We constructed also the ST6 scFv with a long peptide linker of 18 aminoacids (SSGGGGSGGGGGGSSRSS). The following primers were used: RSC-F 5'-GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC-3' and ST6linkerB 5'-CCCACCACCGCCCGAGCCACCGCCACCAGAGGAGGAAGATCTAGAGGAACCACC ; RSC-B 5'-GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG-3' and ST6linkerF 5'-TCCTCTGGTGGCGGTGGCTCGGGCGGTGGTGGGGGTGGTTCCTCTAGATCTTCC. The purified PCR products were assembled by another PCR using the following primers: RSC-F 5'-GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC-3' and RSC-B 5'-GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG-3'. The resulting overlap-PCR product encodes the ST6 scFv in which the N-terminal VL region is linked with the VH region through the 18-aa peptide linker named ST6LL scFv. The DNA fragment was gel-purified, digested with the restriction endonuclease SfiI, and cloned into the appropriately cutted vector pComb3X. This scFv cDNA was then modified at its 5' and 3' with the primers ST6-BstEII-F and ST6-BstEII-B to introduce the *BstEII* sites. The resulting digested PCR fragments were cloned into the BstEII

sites of the plasmid pIntron Sindbis ZZ, generating the pIntron Sindbis-ST6LL.

4.2.2 Expression and purification of anti-CCR5 single-chain antibody fragments

To express and purify anti-CCR5 single-chain antibody fragments (ST6SL and ST6LL) from periplasmic space, phagemid DNA was transformed into the non-suppressor *E. coli* strain Top10F. A fresh colony of each scFv was grown at 37ºC overnight in SOB medium containing 100 μg/ml of ampicillin. A 5 ml sample of cells was used to inoculate 500 ml of SOB medium containing 100 μg/ml of ampicillin. Cells were grown at 37ºC until $O.D_{600nm}$ 0.9, induced by the addition of 0.5 mM IPTG and growth continued for 16 hours. After induction, cells were centrifuged for 30 min at 4000×g and for cell disruption pellets were resuspended in 30 ml of 20 mM Tris-HCl buffer (pH 8.0), 0.7 M sucrose and supplemented with protease inhibitors (Roche). After one hour on ice, 5 ml of a 2 mg/ml lysozyme solution in 0.1 M EDTA (pH 8.0) was added. Cells were incubated for 30 min on ice and the soluble periplasmic extract was collected by 15 min centrifugation at 14000×g. The periplasmic expressed scFv antibody fragments was purified by nickel chelate affinity chromatography using the C-terminal His_6-tag. The eluted fraction was concentrated by Centricon columns (Millipore). Purified scFv were analyzed by SDS-PAGE followed

by Coomassie Blue staining and Western Blot with HRP-conjugated anti-HA monoclonal antibody (Roche). Protein quantification was determined by measuring the optical density at 280 nm by the classic Bradford method.

4.2.3 Cell lines

293T, U87-CD4 and U87-CD4.CCR5 cells were maintained in Dulbecco´s Modified Eagles Medium. U87-CD4 and U87-CD4.CCR5 cells were obtained from the AIDS Research and Reference Reagent Program. Media was supplemented with 10% fetal calf serum (FCS), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and 2 mM glutamine. Human PBMC from healthy HIV-seronegative donors were treated for 48 hours with phytohemagglutinin (PHA) and then cultured in RPMI 1640 medium supplemented with 20% FCS and 50 U of interleukin-2 per ml (Roche). All cell cultures were maintained in a humidified incubator at 37°C in 5% CO_2. Tissue culture media and reagents were from BioWhitaker.

4.2.4 Pseudotyped Viral Particles

For generation of Sindbis pseudotyped lentiviral vectors expressing the luciferase reporter gene, a lentiviral plasmid HIV-LucΔ*env* and pIntron Sindbis-ST6SL or pIntron Sindbis-ST6LL were used. The HIV-LucΔ*env* vector was kindly provided by Irvin S. Y. Chen. The resulting pseudotyped viruses were named HIV-LucΔ*env/*Sindbis-ST6SL and HIV-

LucΔ*env*/Sindbis-ST6LL. For generation of Sindbis pseudotyped lentiviral-derived vectors expressing the enhanced green fluorescent protein (EGFP) reporter gene, pRRL.SIN-EGFP, plasmid expressing viral structural and enzymatic proteins, pCMVR8.9, and pIntron Sindbis-ST6SL or pIntron Sindbis-ST6LL, were used. The packaging plasmid pRRL.SIN-EGFP, and the vector plasmid pCMVR8.9 were kindly provided by Didier Trono. The resulting pseudotyped viruses were named HIVEGFP/Sindbis-ST6SL and HIVEGFP/Sindbis-ST6LL. Vesicular stomatitis virus (VSV) and Sindbis ZZ envelope were also used as positive and negative controls, respectively. All viral particles were produced by cotransfection of 293T cells ($5x10^6$) with Fugene reagent according to the manufacturer's protocol (Roche). The virus-containing supernatant was collected 48 hours post-transfection, normalized by p24 antigen levels and used immediately or frozen at –70ºC for later use.

4.2.5 Infection assays

For infection assays, cells were seeded in 6-well plates at density of $1x10^6$ cells/well. After 24 hours, cells were infected overnight with 1 ml of pseudotyped lentiviral vectors normalized for the same p24 antigen levels (300 pg/ml), expressing the luciferase reporter gene (HIV-LucΔ*env*) or expressing the enhanced green fluorescent protein (EGFP) reporter gene (pRRL.SIN-EGFP). The medium was replaced and cell targeting was evaluated by luciferase assay in U87-CD4 and U87-CD4.CCR5 cells, and

by flow cytometry in U87-CD4, U87-CD4.CCR5, 293T and PBMC cells. Luciferase assay was performed 72 hours after infection, according to the manufacturer's protocol (Promega). In flow cytometry, cells were detached from wells at 96 hours after infection, washed with PBS and incubated for 1 hour at 4ºC with BSA. Following two washing steps, cells were incubated 1 hour at 4ºC with rabbit anti-human CCR5 monoclonal antibody. After two additional washing steps, cells were incubated 1 hour at 4ºC with rhodamine-conjugated anti-rabbit antibody and then analyzed by flow cytometry.

4.2.6 Western Blot Analysis

Supernatants from transfected 293T cells containing pseudotyped lentiviral-derived vectors expressing EGFP (RRL.SIN-EGFP) reporter gene were harvested and concentrated by ultracentrifugation. The amount of virus was normalized by p24 antigen levels (70ng/sample). Pellets were resuspended in SDS-PAGE sample buffer, subjected to 10% SDS-PAGE and electroblotted onto nitrocellulose membranes. Western Blot was performed with a rabbit polyclonal antibody against the structural protein of Sindbis virus and HRP-conjugated anti-rabbit IgG antibody. The polyclonal anti-Sindbis antibody was kindly provided by Pavel Osten and Sondra Schlesinger [355]. Western Blot analysis was also performed with HRP-conjugated mouse monoclonal antibody directed against the p24 antigen. Proteins were visualized by using the ECL system (Amersham Pharmacia

Biotech) according to the manufacture's instructions.

4.2.7 Binding assays

To analyze if soluble anti-CCR5 ST6SL and ST6LL scFv recognize and bind specifically to CCR5 chemokine receptor, U87-CD4.CCR5 and U87-CD4 cells were detached by non-enzymatic treatment with cell dissociation buffer (GIBCO) and distributed to polypropylene tubes ($1x10^6$ cells per sample). After incubation with BSA for 30 min at 4ºC, cells were washed with PBS and preincubated for 1 hour with purified ST6LL or ST6SL antibody fragments. Following another washing step with PBS, cells were incubated with anti-HA fluorescein isothiocyanate-conjugate antibody and then analyzed by flow cytometry (FACS). As positive control, cells were stained with rabbit anti-human CCR5 antibody followed by fluoresceine-conjugated anti-rabbit IgG.

4.2.8 Competition assay

To test the specificity of viral infection with scFv-Sindbis envelope, competition assays were performed. Briefly, U87-CD4.CCR5 cells ($1x10^6$) were preincubated for 1 hour with increasing amounts of purified ST6SL and ST6LL antibody fragments before infection. Following two washing steps with DMEM medium, cells preincubated with ST6SL scFv were

infected overnight with 1 ml of HIV-LucΔ*env*/Sindbis-ST6SL and cells preincubated with ST6LL scFv were infected overnight with 1 ml HIV-LucΔ*env*/Sindbis-ST6LL. Three days postinfection, cells were lysed and the supernatant analyzed for luciferase activity according to the manufacturer's protocol (Promega). To control the assay, U87-CD4.CCR5 cells were preincubated with increasing amounts of ST6LL scFv antibodies before infection with HIV-LucΔ*env*/VSV and HIV-LucΔ*env*/Sindbis ZZ pseudotyped virus.

4.3 Results

4.3.1 Construction of Sindbis pseudotyped lentiviral vectors displaying anti-CCR5 single-chain antibody fragments

It was previously shown by Morizono *et al* that the envelope of Sindbis virus can pseudotype lentivirus-derived vectors [208]. When Sindbis envelope was modified to contain the Fc-binding region of protein A (ZZ domain) between aminoacids 71 and 74 of the E2 protein, the pseudotyped vector significantly enhanced its specificity when used in combination with monoclonal antibodies that recognize and bind to specific cell surfaces antigens [208].

In the present work, to develop lentiviral-derived vector particles with specificity of gene delivery for CCR5-expressing cells, we attempted to design a novel Sindbis pseudotyped vector where the Sindbis E2 receptor binding envelope protein was modified to directly encode a single-chain antibody fragment (scFv) against the CCR5 chemokine receptor (Figure 4.1A). An anti-CCR5 recombinant antibody fragment (ST6) was developed and characterized in detail by Carlos Barbas III and co-workers [148,220]. ST6 specifically bound to the N-terminal extracellular domain of CCR5 (amino acids 16-18) and was originally derived from a Fab phage display library [148,220]. To be applied as intrabody, the ST6 Fab was previously converted to

a single-chain fragment in which the VL and VH fragments were covalently linked with a peptide linker of seven amino acids (GGSSRSS) [148]. Expression of ST6 with this short linker was reported to result in dimeric scFv domains [148].

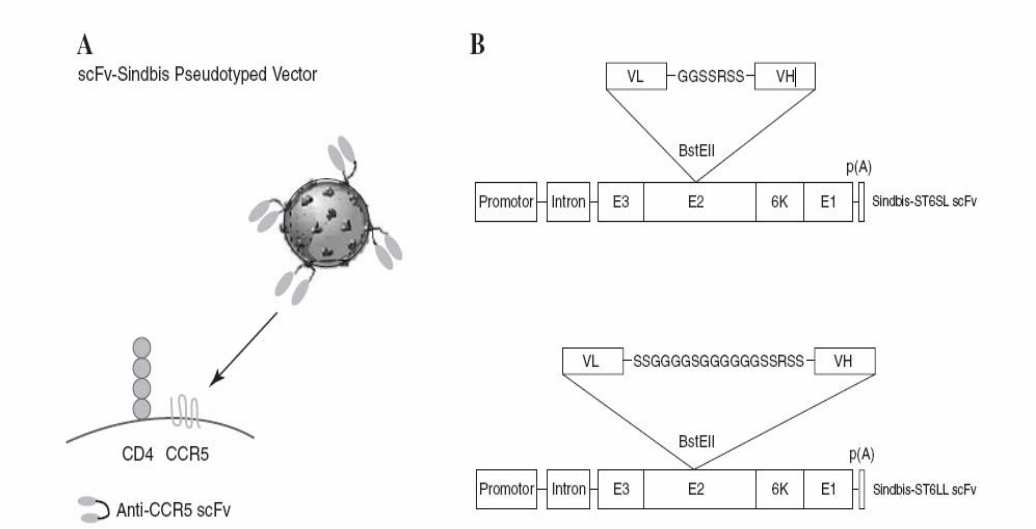

Figure 4.1 (A) - Schematic illustration for cell type-specific targeting with Sindbis pseudotyped lentiviral vectors displaying anti-CCR5 single-chain antibodies. (B) - Schematic representation of chimeric scFv-Sindbis envelope constructions developed in this study. The pIntron Sindbis ZZ was kindly provided by Irvin S.Y. Chen [208]. The Sindbis envelope encodes two transmembrane envelope proteins, E1 and E2. E3 is a leader sequence for E2 protein and 6K is the leader for E1. The Sindbis envelope is initially synthesized as a polypeptide and then cleaved by cellular proteases to produce the E3, E2, 6K and E1 proteins [208]. The pIntron Sindbis ZZ plasmid contains the cytomegalovirus immediate early promoter, the intron II of the rabbit β-globin, the polyadenylation signal and a BstEII cloning site between aminoacids 71 and 74 of the E2 glycoprotein. A scFv (ST6) derived from a Fab phage display library obtained from immunized rabbits, which

recognizes the N-terminal extracellular domain of CCR5 chemokine receptor x, was cloned between the BstEII sites of the expression vector of Sindbis virus envelope. The VL and VH fragments of ST6SL are covalently linked with a peptide of seven aminoacids (GGSSRSS). The VL and VH fragments of ST6LL are covalently linked with a long linker of 18 aminoacids (SSGGGGSGGGGGGSSRSS).

The length of linker was crucial for the association state of the scFv molecule, as longer linkers favor monomeric molecules and short linkers favor dimers [324]. ScFv with long and short linkers usually have different binding patterns and stabilities [131,353]. Consequently, the display and conformation of ST6 anti-CCR5 scFv with short and long linkers on the Sindbis envelope can be different and influence the specificity of binding to the chemokine receptor. Therefore, to avoid conformation constraints we also constructed the ST6 scFv with a long peptide linker of 18 aminoacids (SSGGGGSGGGGGGSSRSS). To allow cell specific targeting, we modified the Sindbis envelope by inserting the anti-CCR5 ST6SL and ST6LL scFv into the Fc-binding domain (ZZ) between aminoacids 71 and 74 of the E2 glycoprotein (Fig 4.1.B). The rationale for these two constructs was to determine whether the length of the linker influences the display of chimeric single-chain Sindbis envelope and/or infectivity of respective viral particles.

4.3.2 Production and incorporation of chimeric scFv-Sindbis envelope proteins into lentiviral particles

To demonstrate that chimeric Sindbis-ST6SL and Sindbis-ST6LL envelope glycoproteins was successfully incorporated into the lentiviral-derived vector, immunoblot analysis was performed for each construct with a polyclonal antibody against the structural proteins of Sindbis virus [355]. Briefly, 293T cells were transfected with pRRL.SIN-EGFP, pCMVR8.9 and pIntron Sindbis-ST6SL, pIntron Sindbis-ST6LL, pIntron Sindbis ZZ or pVSV. At 48 hours post-transfection, pseudotyped virions were harvested and concentrated by ultracentrifugation. The viral amount was normalized by p24 antigen levels (70 ng/sample) and pellets were analyzed by Western-blot. As shown in Figure 4.2, the chimeric scFv-envelope glycoproteins showed a band near 97 kDa, confirming the expected size of E2 fused to anti-CCR5 scFv domain. The HIVEGFP/Sindbis ZZ virions showed a lower molecular weight band near 66 kDa which is the estimated molecular mass of the Sindbis ZZ chimeric glycoprotein. These results demonstrate that the chimeric scFv-Sindbis envelope proteins can be expressed, processed and incorporated into lentiviral-derived vectors.

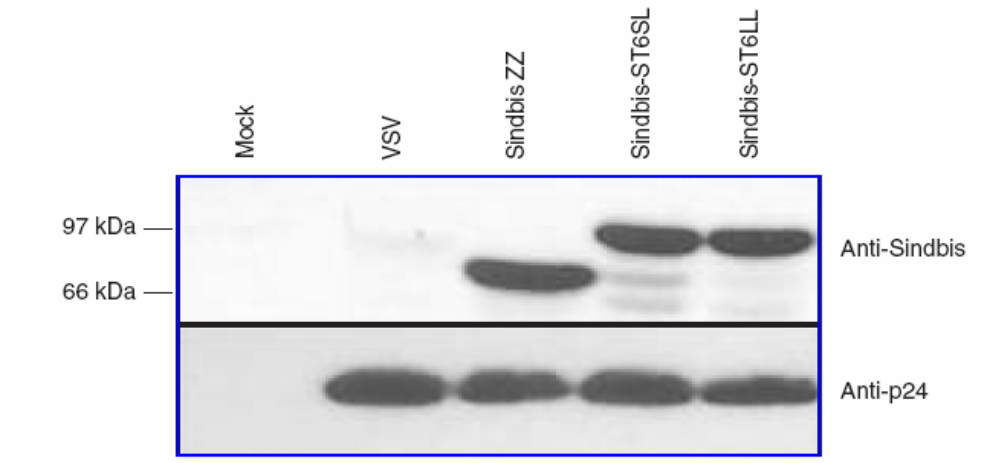

Figure 4.2 - Detection of lentiviral particles pseudotyped with chimeric scFv-Sindbis envelope proteins. Supernatants from transiently transfected 293T cells were collected, ultracentrifuged and normalized for p24 antigen (70 ng/sample). Pellets were resuspended in SDS-PAGE sample buffer, subjected to 10% SDS-PAGE and electroblotted onto nitrocellulose membranes as described in Material and Methods. Western Blot was performed with rabbit polyclonal anti-Sindbis antibody and HRP-conjugated anti-rabbit IgG antibody. Western Blot analysis was also done with HRP-conjugated mouse monoclonal antibody directed against the p24 antigen. The molecular weight is shown in kDa.

4.3.4 Targeting chimeric scFv-Sindbis pseudotyped lentiviral vectors to CCR5-expressing cells

Three sets of experiments were performed to test the specificity of infection. First, the efficiency of cell targeting was analyzed with U87-CD4 and U87-CD4.CCR5 cells by luciferase expression. U87-CD4.CCR5 is a U87-CD4

cell line expressing the CCR5 chemokine receptor (AIDS Research and Reference Reagent Program). Pseudotyped viral particles HIV-LucΔ*env* containing each of the chimeric envelope proteins (VSV, Sindbis ZZ, Sindbis-ST6SL or Sindbis-ST6LL) were incubated with target cells, and the efficiency of targeting of each construct were analyzed by luciferase activity. The results shown in Figure 4.3 represented the percentages of infection relative to the value obtained for the pseudotyped HIV-LucΔ*env*/VSV vector. The HIV-Luc Δ*env*/Sindbis-ST6SL and HIV-LucΔ*env*/Sindbis-ST6LL viral particles specifically infected U87-CD4 cells expressing the CCR5 chemokine receptor (Figure 4.3), but did not infect non-CCR5 expressing U87-CD4 cells. In contrast, the pseudotyped HIV-LucΔ*env* vector displaying the Sindbis ZZ chimeric envelope was unable to infect CCR5-expressing cells at detectable levels. These results demonstrated that pseudotyped lentiviral-derived particles with Sindbis envelope containing anti-CCR5 scFv specifically infect CCR5-expressing cells. However, as the data in Figure 4.3 shows, the targeting of HIV-LucΔ*env*/Sindbis-ST6SL to U87-CD4.CCR5 cells (14%) was lower than the HIV-LucΔ*env*/Sindbis-ST6LL (52%). Pseudotyped vectors were also used to infect other CCR5-expressing cell lines like P4R5 cells (AIDS Research and Reference Reagent Program). The infection efficiency of HIV-LucΔ*env*/Sindbis-ST6SL and HIV-LucΔ*env*/Sindbis-ST6LL to P4 cell line expressing the CCR5 chemokine were similar to those obtained with U87-CD4.CCR5 cells (data not shown). Therefore, these preliminary results

indicated that the linker length between the VH and VL domains may have a significant influence on the efficiency but not specificity of infection.

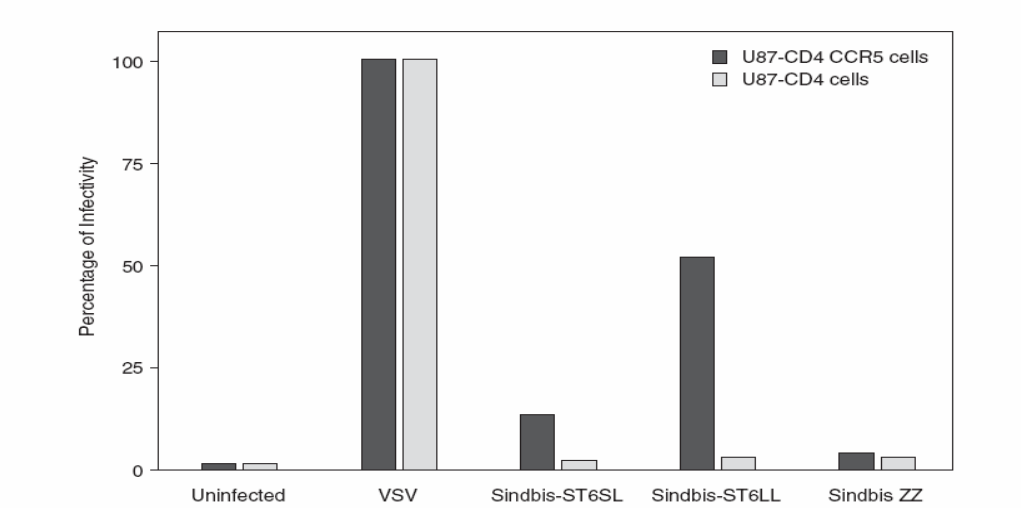

Figure 4. 3 - Targeting of scFv-Sindbis pseudotyped lentiviral particles to U87-CD4, U87-CD4.CCR5. Supernatants of HIV-LucΔ*env* containing each of the chimeric envelope proteins (VSV, Sindbis ZZ, Sindbis-ST6SL or Sindbis-ST6LL) from producer cells were normalized for p24 antigen (300 pg/ml) and incubated overnight with the target cell line ($1x10^6$ cells), as described in Material and Methods. Efficiency of targeting for each pseudotyped vector was analyzed for luciferase activity 72 hours after infection according to the manufacturer's protocol (Promega). Values shown represent the percentages of infection relative to the value obtained for the pseudotyped VSV lentiviral vector. U87-CD4.CCR5 is a U87-CD4 cell line expressing the CCR5 chemokine receptor (AIDS Research and Reference Reagent Program). As shown luciferase activity is only detected in CCR5-expressing cells infected with ST6SL and ST6LL chimeric Sindbis envelope virus. Data represents the results of two independent experiments.

To quantify the efficiency of lentiviral targeting, U87-CD4.CCR5 and U87-CD4 cells were transduced with the lentiviral-derived vector, pRRL.SIN-EGFP. This vector has the enhanced green fluorescent protein (EGFP) as the reporter gene that allows detection and enumeration of transduced cells by flow cytometry [191,198,354]. Therefore, specificity of gene delivery was quantified by FACS analysis for EGFP expression 96 hours after infection with pseudotyped lentiviral-derived vectors. As shown in Figure 4.4, HIVEGFP/Sindbis-ST6SL and HIVEGFP/Sindbis-ST6LL specifically infected CCR5-expressing cells. In contrast, no transduction was observed when U87-CD4.CCR5 was infected with pseudotyped HIVEGFP/Sindbis ZZ viral particles. Background levels of EGFP expression were detected in U87-CD4 cells transduced with these viruses. Virus titers in U87-CD4.CCR5 were 3.4×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6SL and 7.2×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6LL. Consistent with our previous experiments, these results showed that targeting of CCR5-expressing cells was dependent of anti-CCR5 single-chain antibody display on Sindbis envelope. However, as indicated in Figure 4.4, the transduction levels obtained with Sindbis envelope displaying the ST6 with a longer linker (72%) was higher than that with Sindbis displaying the ST6 with the short linker (34%).

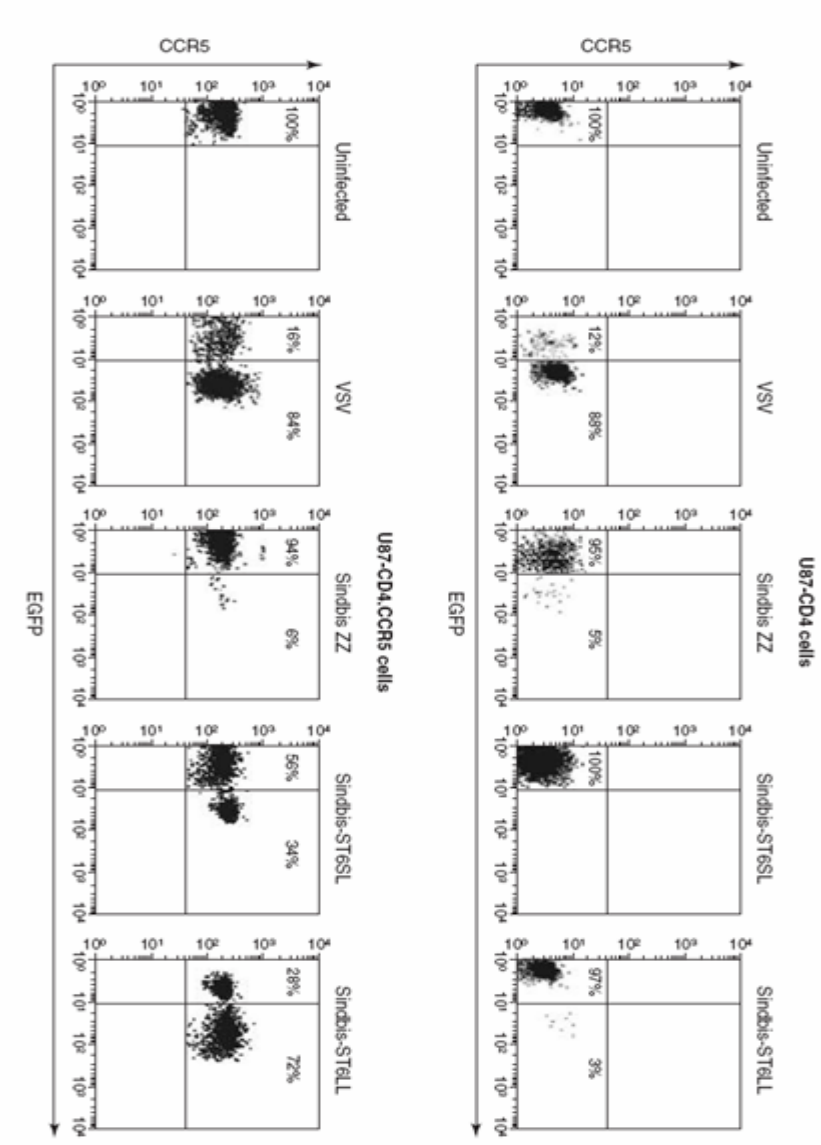

Figure 4.4 (Legend next page)

Figure 4.4 - High titers of scFv-Sindbis pseudotyped lentiviral particles are able to transduce CCR5-expressing cells. Lentiviral-derived vectors (1 ml) expressing EGFP and pseudotyped with VSV, Sindbis ZZ, Sindbis-ST6SL and Sindbis-ST6LL envelopes were normalized for p24 antigen (300 pg/ml) and incubated overnight with U87-CD4 cells and U87-CD4.CCR5 cells ($1x10^6$), as described in Materials and Methods. Four days after infection, cells were stained with rabbit anti-human CCR5 antibody plus rhodamine-conjugated anti-rabbit IgG. Cells were analyzed for CCR5 staining and EGFP expression by flow cytometry. The viral titer was calculated according to the formula: transducing units (TU)/ml = number of cells counted before infection × percentage of transduced cells reported from flow cytometry analysis/ volume (ml) of viral supernatants. The percentage of transduced cells was calculated as the number of events in the upper right panel divided by the total number of events in the right and left upper panels. The titers obtained were 3.4×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6SL and 7.2×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6LL. Data are representation of two independent experiments.

Other groups and ours have reported that scFv with longer and short linkers usually lead to different antigen-binding properties and protein stabilities [131,353]. Therefore, to examine if ST6SL and ST6LL antibody fragments recognize and bind to CCR5 chemokine expressed at cell surface, a binding assay with purified soluble anti-CCR5 antibody fragments was performed. Briefly, U87-CD4 and U87-CD4.CCR5 cells were detached from flasks, blocked and incubated with purified ST6SL and ST6LL antibody fragments. Following a washing step, cells were incubated with anti-HA fluorescein isothiocyanate-conjugated antibody and analyzed by flow cytometry. The results shown in Figure 4.5, demonstrated that both anti-CCR5 scFv were

able to recognize CCR5 chemokine receptor at similar levels. The fluorescence intensities observed with each single-chain antibody fragment was comparable to those seen when an IgG anti-CCR5 was used as a CCR5-specific probe (Figure 4.5). In contrast, no binding was detected when anti-CCR5 scFv were used with U87-CD4 cells. These results suggested that soluble ST6SL and ST6LL have similar specificity for binding to CCR5-expressing cells and do not shown influence of increased avidity of ST6SL. Although these experiments cannot rigorously establish a precise mechanism why Sindbis-ST6LL pseudotyped lentiviral derived vectors have higher titers, the most likely explanation was that the ST6LL scFv fused into the E2 protein of Sindbis envelope is able to undergo an optimal conformational for VH-VL paratopes interaction allowing superior binding to the native CCR5 chemokine receptor.

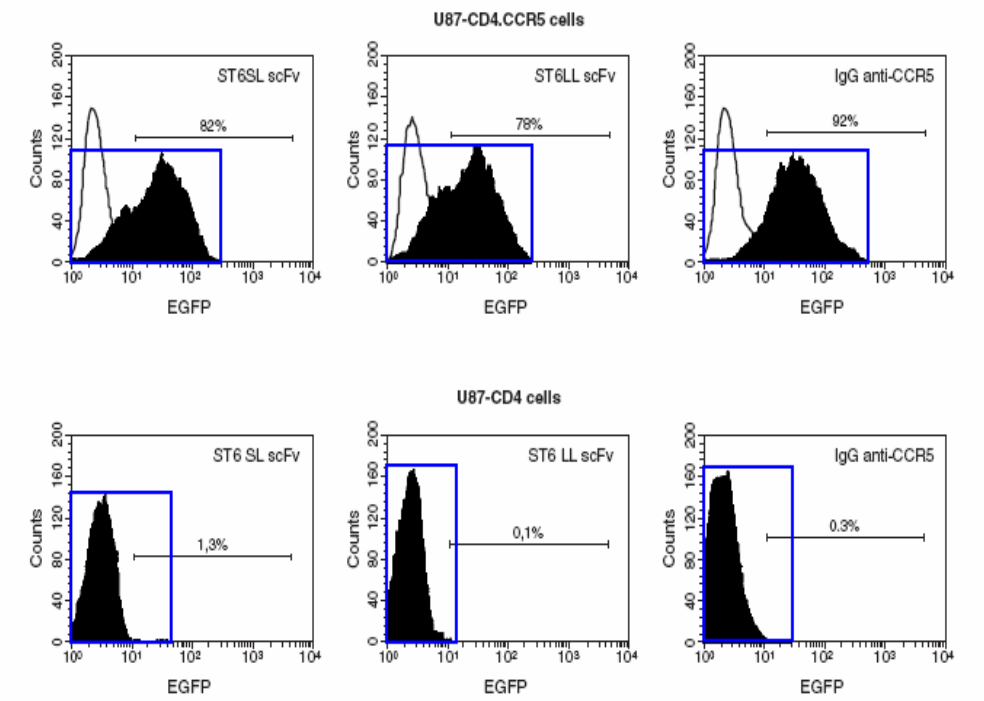

Figure 4.5 - Purified Anti-CCR5 scFv binds specifically to CCR5-expressing cells. U87-CD4.CCR5 and U87-CD4 cells ($1x10^6$ cells per sample) were incubate with soluble ST6LL or ST6SL purified antibody fragments. Following a washing step, cells were stained with anti-HA fluorescein isothiocyanate-conjugate antibody and analyzed by flow cytometry (FACS), as described in Material and Methods. As positive control for CCR5 expression, cells were stained with rabbit anti-human CCR5 antibody followed by fluoresceine-conjugated anti-rabbit IgG. Data represents the results of two independent experiments.

As alphavirus vectors have been shown to infect cells from different species *in vitro*, the extent of infection of human cells for which Sindbis viruses have tropism requires further investigation with chimeric scFv-Sindbis lentivirus vectors [356,357]. To assess the ability of Sindbis ZZ, Sindbis-ST6SL and Sindbis-ST6LL to mediate viral transduction to 293T cells, pseudotyped lentiviral vectors expressing EGFP were used and infection levels were evaluated. As shown in Figure 4.6, pseudotyped HIVEGFP viral particles containing each of the chimeric envelope proteins (Sindbis ZZ, Sindbis-ST6SL or Sindbis-ST6LL) were unable to infect 293T cells at detectable levels. In contrast, when 293T cells were infected with VSV-pseudotyped lentiviral vector, 72% of cells were trasnduced. These results were consistent with those of Morizono *et al*., where a reduced infectivity was observed with Sindbis lentiviral vector [208]. Therefore, this data suggest that lentiviral vectors pseudotyped with chimeric-Sindbis envelope are unable to target cells for which Sindbis virus have tropism.

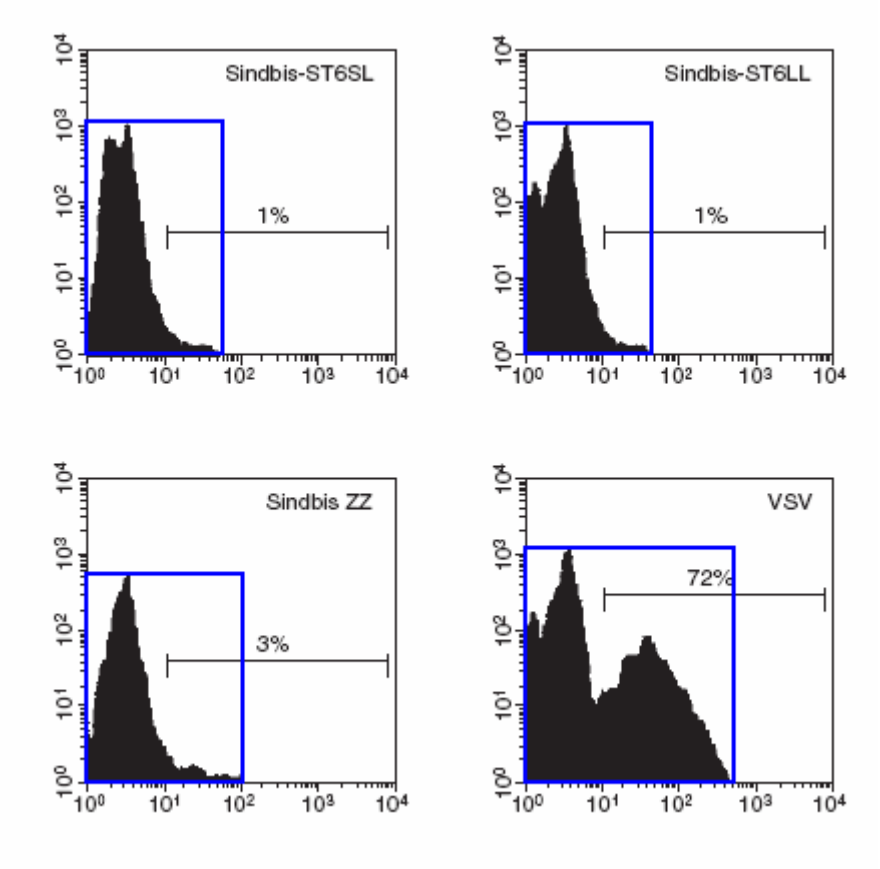

Figure 4.6 - Targeting of pseudotyped lentiviral particles to 293T. Lentiviral-derived vectors (1 ml) expressing EGFP and pseudotyped with VSV, Sindbis ZZ, Sindbis-ST6SL or Sindbis-ST6LL envelopes were normalized for p24 levels (300 pg/ml) and incubated overnight with 293T cells ($1x10^6$), as described in Materials and Methods. Four days after infection, viral infectivity was evaluated by flow cytometry (FACS), as described in Materials and Methods. As shown, lentiviral infection in 293T cells was only observed with VSV pseudotyped vectors.

4.4.5 Specificity viral infection with chimeric scFv-Sindbis envelope

To validate if the specificity of infection with scFv-Sindbis pseudotyped lentiviral vectors was mediated by the display of anti-CCR5 single-chain antibody fragments, competition experiments were performed with purified ST6SL and ST6LL scFv. We hypothesized that the addition of increasing amounts of anti-CCR5 scFv to the target cells would block the CCR5 chemokine receptor, preventing viral infection. Briefly, U87-CD4.CCR5 cells were preincubated before infection with increasing amounts of purified ST6SL or ST6LL antibody fragments. Following a washing step, cells preincubated with ST6SL scFv were infected with HIV-LucΔ*env*/Sindbis-ST6SL and cells preincubated with ST6LL scFv were infected with HIV-LucΔ*env*/Sindbis-ST6LL. As a control, cells preincubated with ST6LL antibody fragments were also infected with HIV-LucΔ*env*/VSV and HIV-LucΔ*env*/Sindbis ZZ vectors. Three days postinfection, cells were lysed and luciferase activity was determined. The results shown in Figure 4.6 demonstrate that increasing amounts of anti-CCR5 scFv added to the CCR5-expressing cells before infection blocked infectivity of HIV-LucΔ*env*/Sindbis-ST6SL and HIV-LucΔ*env*/Sindbis-ST6LL, respectively (Figure 4.7). No inhibition of infection was observed when those cells were preincubated with similar amounts of an irrelevant scFv (anti-Vif scFv) (data not shown). In contrast, no infection was observed when U87-CD4 cells were preincubated with similar amounts of ST6SL or ST6LL scFv,

reflecting the absence of target molecule for pseudotyped lentivirus (data not shown). As shown in Figure 4.7 the addition of ST6LL to U87-CD4.CCR5 cells in any concentration did not inhibit infectivity of HIV-LucΔ*env*/VSV. The data showed that blocking of viral infectivity was mediated by antibody-specific inhibitory effects. Therefore, the specific targeting of CCR5-expressing cells by chimeric scFv-Sindbis pseudotyped lentiviral vectors were mediated by the anti-CCR5 scFv display.

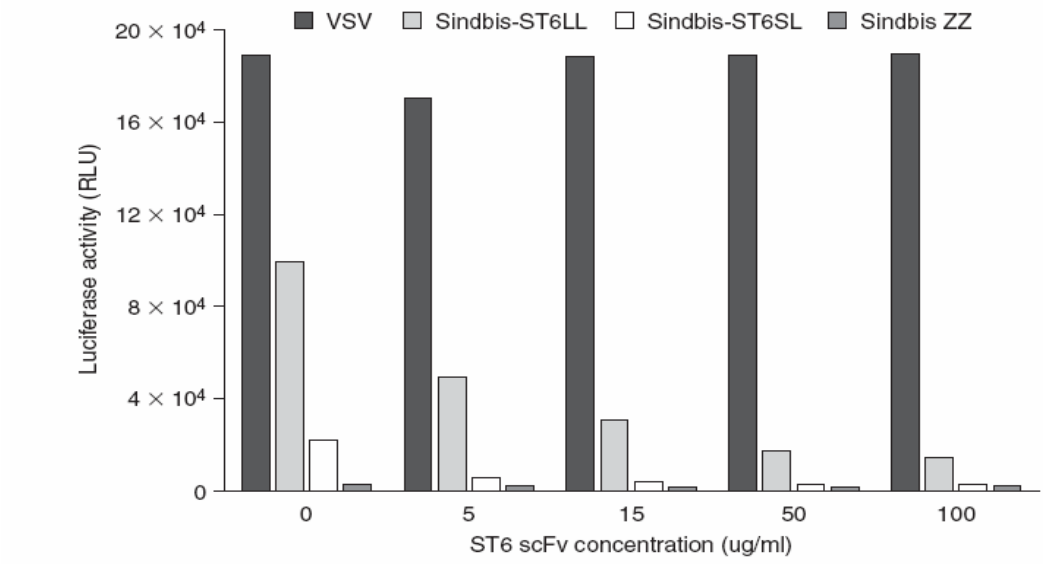

Figure 4.7 - Competition assay shows the specificity of infection with Sindbis pseudotyped lentiviral particles displaying anti-CCR5 single-chain antibody fragments. U87-CD4.CCR5 cells ($1x10^6$) were preincubated with increasing amounts (μg/ml) of purified ST6SL and ST6LL antibody fragments before infection. Cells preincubated with ST6SL scFv were infected with HIV-LucΔ*env*/Sindbis-ST6SL and cells preincubated with ST6LL were infected with HIV-LucΔ*env*/Sindbis-ST6LL. Virus was

normalized for p24 antigen (300 pg/ml). Three days postinfection, cells were lysed and analyzed for luciferase activity according to the manufacture's protocol (Promega). As a control, cells preincubated with increasing amounts of ST6LL antibodies were also infected with HIV-LucΔ*env*/VSV and HIV-LucΔ*env*/Sindbis ZZ pseudotyped vectors. Percentage of infectivity was calculated as the level of luciferase activity relative to the value obtained for pseudotyped VSV vector. As shown, viral infection with pseudotyped Sindbis envelope is inhibited by increasing concentrations of ST6SL and ST6LL scFv. No effect was observed with VSV pseudotyped vectors.

4.4.6 Targeting of pseudotyped lentiviral vectors displaying anti-CCR5 chimeric scFv-Sindbis envelope to primary lymphocytes

To demonstrate that chimeric scFv-Sindbis pseudotyped lentiviral-derived vectors can specifically target a subpopulation of CCR5-positive cells, primary blood mononuclear cells (PBMC) were used. As PBMC consist of both CCR5-negative and CCR5-positive cell populations, this assay is optimal for testing our approach for targeting gene delivery. Briefly, stimulated PBMC cells ($1x10^6$) were transduced overnight with pseudotyped lentiviral-derived vectors expressing the EGFP reporter gene, as described in Materials and Methods. Four days after transduction, cells were stained with rabbit anti-human CCR5 antibody plus rhodamine conjugated anti-rabbit IgG. Cells were then analyzed for CCR5 staining and EGFP expression by flow cytometry (FACS). As the data in Figure 4.8 demonstrate, the lentiviral vector that contains the VSV envelope

transduced both CCR5-negative and CCR5-positive cell populations with an efficiency of 40.6%. In contrast, lentiviral vectors pseudotyped with chimeric scFv-Sindbis resulted in a preferential infection of the CCR5-positive cell population of PBMC. As expected, the level of transduction for the total of cells obtained with Sindbis envelope displaying the ST6LL (1.9%) was higher than that with SindbisST6SL (0.5%). As a control, the lentiviral-derived vector containing the Sindbis ZZ envelope was unable to infect either CCR5-negative or CCR5-positive cells. As a comparison, with VSV pseudotyped lentivirus the transduction levels obtained in CCR5-positive cells with chimeric Sindbis-ST6LL envelope vectors were in average 48.7% of that of the amphotropic envelope lentivirus. This transduction efficiency was similar to that obtained with U87-CD4.CCR5 cells. Therefore, these results indicated that lentiviral-derived pseudotyped with Sindbis envelope glycoproteins containing a single-chain antibody fragment (scFv) against CCR5 chemokine receptor can specifically target a CCR5-expressing primary population of cells.

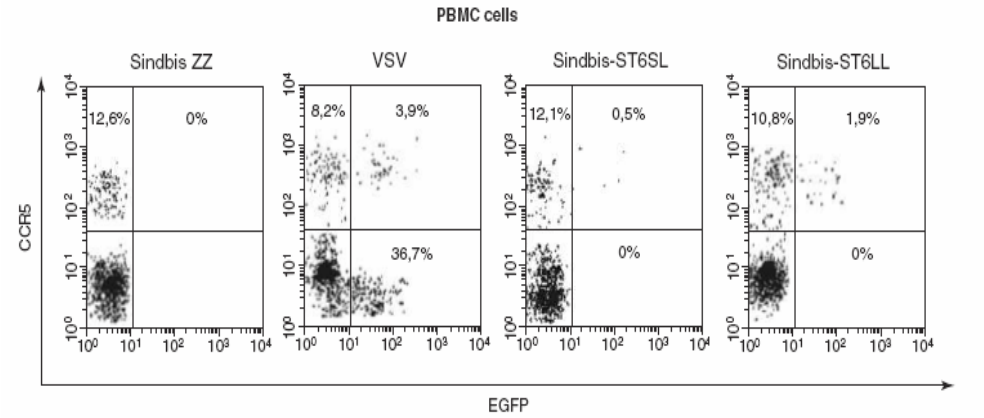

Figure 4.8. ScFv-Sindbis envelope mediates gene transduction of a heterogenous primary cell population by a lentiviral-derived vector. Primary blood mononuclear cells (PBMC) ($1x10^6$ cells) were transduced overnight with pseudotyped lentiviral-derived vectors (1 ml) expressing EGFP, and normalized for p24 antigen as described in Material and Methods. The titers used for transduction were 3.4×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6SL and 7.2×10^5 EGFP transduction units/ml to HIVEGFP/Sindbis-ST6LL. Four days after infection, cells were stained with rabbit anti-human CCR5 antibody plus rhodamine-conjugated anti-rabbit IgG and analyzed for CCR5 staining and EGFP expression by flow cytometry (FACS). The percentage of EGFP-positive cells (viral transduction) in the CCR5 positive population was calculated as the number of events in the upper right quadrant divided by the total number of events in the four quadrants. The percentage of EGFP-positive cells was calculated by dividing the number of events in the lower right quadrant by the total number of events in all quadrants.

4.4 Discussion

A number of potential gene therapy strategies have been identified in the past few years. These strategies include intracellular antibodies, dominant negative viral proteins, intrakines, suicide genes, ribozymes, RNA decoys, antisense messenger RNAs and small interfering RNA (siRNA) molecules [121,131,358]. They represent the most rapidly developing areas in pre-clinical research. Lentiviral vectors are among the most efficient tools for gene delivery into mammalian cells [206]. Nevertheless, some problems need to be solved before this approach becomes routinely adopted in clinic. Two of the most important problems of lentiviral gene delivery systems are the lack of selectivity of the existing vectors to the target cells and the low efficiency of gene transfer.

Recently, many properties of alphavirus vectors have made them a desirable alternative to lentiviral vectors [362]. Alphavirus can be produced at very high titers, show high levels of expression and, unlike retroviruses, the fusogenic protein can fuse to cells independently of the receptor binding protein [208,346,362]. However, as a lytic RNA virus, these vectors are not suitable for applications requiring stable transduction [208]. Within this context, Morizono *et al* reported an approach to improve lentiviral targeting and viral titer by exploring some properties of the Sindbis virus envelope [208]. In that work a Sindbis envelope pseudotyped lentiviral vector displaying the Fc-binding domain of protein A (ZZ domain) was constructed. When used in

combination with monoclonal antibodies to a specific cell surface antigen, the chimeric envelope provides enrichment in terms of specificity of infection and viral titers were relatively high and stable. Theorically this approach is applicable to any cellular membrane-receptor domain for which specific monoclonal antibodies are available. However, chimeric Sindbis-ZZ pseudotyped lentiviral-derived vectors may not be a suitable strategy for *in vivo* applications where plasma antibodies are present. As monoclonal antibodies are not covalently linked to the Sindbis envelope, a percentage of free-ZZ domains will be available to bind autologous antibodies, resulting in non-specific transduction by the lentiviral vector. Furthermore, some level of *in vivo* competition may be envisioned between plasma antibodies and recombinant monoclonal antibodies used to target the ZZ-domain. Given these considerations there is a higher probability for the chimeric ZZ-Sindbis envelope to non-specifically target other cellular receptors or reduce the transduction efficiency for the target cell. Therefore, to overcome these questions, we attempted to develop a novel pseudotyped lentiviral-derived vector where the Sindbis virus E2 protein was modified to directly encode a single-chain antibody fragment (scFv) against the CCR5 chemokine receptor. The results presented in this report demonstrate that Sindbis pseudotyped vectors that display single-chain antibody fragments against a cell surface receptor are competent for infection. As shown chimeric-Sindbis envelope glycoproteins are expressed at virus surface reflecting a good incorporation. We also show that specific targeting into CCR5-

expressing cells was mediated by the anti-CCR5 scFv display and viral titers were close to 10^6 EGFP transduction units/ml. The two chimeric scFv-Sindbis pseudotyped viral particles could be produced at relatively high titers. However, our data indicate that infection levels obtained with Sindbis envelope displaying the ST6 with a longer linker was consistently higher than that with Sindbis envelope displaying the ST6 with a short linker. Although the precise mechanism for the high infectivity level of Sindbis-ST6LL pseudotyped lentiviral-derived vectors was not determined, it is reasonable to assume that ST6LL scFv fused into the E2 protein of Sindbis envelope is able to undergo a conformational change that allows a better binding to the CCR5 receptor and, consequently, promote an increase in infectivity. An optimal interaction of VH and VL promoted by a flexible long linker may bring together the paratopes involved in CCR5 recognition [359]. Therefore, the affinity of the scFv for the receptor will be higher if both domains are closely localized in space. We hypothesize that a short-linker puts apart VH and VL domains reducing antibody affinity for the antigen and consequently the viral infectivity. It will be interesting in the future to see whether other aminoacid linkers will render chimeric scFv-Sindbis more effective for specific viral delivery. Besides the linker length, other variables may be envisioned to interfere with an optimal transduction by this strategy. An efficient expression and folding of the chimeric scFv-Sindbis envelope at virus surface are indispensable for the success of this strategy. In our case, ST6 was previously developed and optimized for CCR5 recognition in eukaryotic cells [148,220]. Therefore, an optimal expression and

folding of the scFv in mammalian cells will be essential for not disturbing the backbone of Sindbis envelope and the antigen-antibody interactions, which will be essential for an effective viral transduction. In addition, an improved expression and folding of scFv-Sindbis envelope would also facilitate incorporation into lentiviral particles improving the viral titers. Furthermore, the recognition epitope on a membrane antigen by the scFv must be localized in a protein domain that can result in a strong binding affinity. This will be important since differences in viral infectivities may result from different binding affinities of the scFv to the antigen.

In conclusion, we show that the region between aminoacids 71 and 74 of E2 can tolerate the insertion of single-chain antibody fragments. Thus, it should be possible to test whether other single-chain antibody fragments displayed on Sindbis envelope can be used for the development of a large variety of cell specific gene delivery vectors. This strategy once optimized may be considered for research purposes, like the identification of specific cellular receptors. Therefore, the present study can be considered a model strategy for specific viral gene delivery mediated by Sindbis envelope that displays single-chain antibody fragments recognizing specific cellular surface proteins. Furthermore, this strategy has the potential to become a powerful approach for targeting gene delivery in anti-HIV gene therapy due to the important role of CCR5 expression in disease progression.

CHAPTER 5

General Conclusions and Future Perspectives

5. General Conclusions and Future Perspectives

Controlling HIV infection continues to be a major challenge in both underdeveloped and developed nations. Although the drug cocktails used in HAART have markedly changed the profile of progression to AIDS in HIV-infected individuals, drug failures continue to occur as a consequence of viral resistance and other complications arising from a lifelong regimen of chemotherapy. As a result, there is an urgent need to identify and develop new drugs that can be effective against these highly resistant virus isolates. The importance of developing new antiretroviral drugs cannot be overstated. However, that HAART is lifelong and may be associated with cumulative toxicities underscores the need for new approaches. Given the increasing knowledge of mechanisms that allow control of HIV infection, several investigators are focusing their attention on gene therapy, either as a stand-alone approach or as a supplement to pharmacological regimens. The specificity and affinity that can be obtained in intrabodies make these molecules effective modulators of viral or cellular targets. The binding of an intrabody to an intracellular target protein has the potential to block, suppress, alter or even enhance the process mediated by that molecule. Compared to other gene manipulation methods, such as gene knock-out or RNA-based technologies (ribozyme, antisense RNA or RNAi), intrabodies have the unique advantage of targeting proteins in different cellular compartments. Moreover, genes that have very high mRNA-turnover rates are very difficult to target effectively with RNAi, whereas intrabodies, which interact with the proteins directly, are unlikely to be affected by target dynamics. Because intrabodies act at the protein level, they will be of value

to map protein–protein interactions and model drug–target interactions. There is also the intriguing possibility of specifically blocking one function of a multi-epitope target by intrabody binding, while leaving intact the remaining functions or interactions of other domains on that target. Within this context, intracellular antibodies provide a powerful research tool that could potentially have many applications in the field of therapeutic molecules for gene therapy. The strong inhibiting potential of intracellular antibodies in the context of HIV gene therapy was the focus of this dissertation thesis.

In **Chapter 1**, we presented a general introduction to HIV-1 virus biology and HIV/AIDS treatment and describe in detail the intracellular immunization (intrabodies) approach and how it can be explored to neutralize the HIV-1 infection.

In **Chapter 2**, we have explored the intracellular immunization approach by developing rabbit intrabodies to the HIV-1 IN protein. We choose the IN as a potential therapeutic target for our intracellular immunization strategy since IN is absolutely required for a stable and productive infection by HIV-1. IN catalyzes the integration of viral cDNA into the host chromosome and it is highly conserved among HIV-1 clinical isolates. In addition, the reactions catalyzed by IN are unique and no human homologue known of IN exists. We identified five new IN-specific scFv antibodies derived from a rabbit immunized combinatorial scFv Phage-Library. Moreover, we demonstrated that these scFv were able to bind simultaneously to the catalytic and C-terminus domains of IN and block the strand transfer reaction. The identified scFv fragments constitute the first set of anti-IN

antibodies that show such epitope binding characteristics. We believe that combinatorial scFv libraries can be extremely effective to identify intrabodies that can bind simultaneously to different epitope regions, as well reaching targets not easily accessible by conventional antibodies molecules (e.g. small pockets or canyons in viral and infectious disease biomarkers). The results presented also demonstrate that intracellular expression of scFv which bind strongly to the catalytic and C-terminal domains results in resistance to productive HIV-1 infection. This inhibition of HIV-1 replication was observed with scFv localized in either the cytoplasmic or nuclear compartment of the cell. The expression of our anti-IN scFv was shown to specifically neutralize the IN activity prior to integration and, thus it, appears to have an effect on the integration process itself. Further studies are in progress with real time PCR analysis to study in more detail where our intrabodies are interfering in the establishment of the provirus. The results presented in this Chapter also demonstrated that anti-IN intrabodies were able to be incorporated into the viral particles which reduce the RT activity and viral infectivity of virions. We believe that this effect is mainly due to the inhibition of the function of the intravirion IN. However, further studies are in progress to better understand why our intrabodies can block the viral replication during late stages. In summary, the results presented in Chapter 2 showed that combinatorial rabbit scFv phage-libraries can be efficiently used to identify scFv with new epitope binding characteristics. Moreover, the data presented here provide proof-in-principle that rabbit anti-IN intrabodies can be designed to block early and late stages of HIV-1 replication and be used as new tools to study the structure and function of HIV-1 IN due to their epitope binding characteristics.

In **Chapter 3**, we showed for the first time that rabbit VL single-domains have also an enormous value as intracellular antigen recognition units. As already mentioned, the strong inhibiting potential of several VH and VHH intrabodies has been demonstrated in the field of cancer, HIV autoimmune and neurodegenerative diseases. In contrast, the VL-domain has not been extensively studied as an independent source for intrabodies construction. To our knowledge, the work developed by Colby *et al* is the only study that reports the construction of a human VL single-domain as an intrabody [135]. Within this context, we evaluated the potential of rabbit VL single-domains as intrabodies in Chapter 3. The VL scaffold study presented here was derived from an anti-Vif scFv that was recently developed from immunized rabbits to HIV-1 Vif protein and inhibited viral replication. The results obtained demonstrated that the anti-Vif VL single-domain preserve the antigen-binding activity and specificity in the absence of the parent VH domain. In addition, the VL domain was independently folded, stable, highly soluble, monomeric, and expressed at high levels in bacteria. The expression in eukaryotic cells also showed that the rabbit VL single-domain was correctly folded as soluble protein in the reducing environment and could strongly neutralize HIV-1 infectivity. Thus, the VL intrabody was shown to be extremely robust in the absence of the parent VH domain and without any process of protein engineering. Further studies are in progress to use the anti-Vif VL scaffold to construct a large and highly diversified phage-displayed synthetic VL library. This library might be a valuable source of antibodies targeting size-restricted epitopes and antigens in obstructed locations where efficient penetration could be critical for successful treatment. In Chapter 3, we have also shown that the CAT-fusion

system differentiate soluble from insoluble intrabodies when expressed under reducing conditions. Future studies are also in progress to evaluate if this system can be a potentially useful approach for direct *in vivo* screening of soluble and stable intrabodies.

Using HIV-1 as an example, we demonstrate in Chapter 2 and 3 the broad versatility of intrabodies to inhibit different stages of the viral life cycle by targeting the IN and the Vif protein of the virus. However, several potential problems may limit the use of intrabodies in the clinical setting for the treatment of HIV-1 and AIDS. One problem that is shared with some of the gene-based therapies is the immunogenicity that the intrabody could elicit. As previously demonstrated, rabbit antibodies can be converted to humanized antibodies that retain both high specificity and affinity for the antigen [220]. Therefore, the use of humanized antibodies from immunized rabbits should minimize the issue of immune recognition, but this will have to be determined in a human clinical trial. A second potential problem is the development of HIV-1 escape mutants, previously mentioned. A way to minimize the development of HIV-1 escape mutants would be to choose conserved epitopes needed for virus survival or to have an intrabody that could bind simultaneously to more than one epitope in the target antigen. Since, our anti-IN scFv intrabodies binds simultaneously to three epitope regions in the IN protein, the development of HIV-1 escape mutants should be minimized. An alternative approach is the use of combination targets, two intrabodies targeting different target/stages of the viral life cycle. For example, a bicistronic expression vector, capable of expressing two different

genes simultaneously, would allow different combinations of intrabodies to be tested for their combined or synergistic inhibition of HIV-1 replication. In our case, escape mutants should be minimized if a vector that could express simultaneously anti-IN and anti-Vif intrabodies is used. A third problem, also shared with other gene-based therapies, is the efficiency of gene delivery. The development of a gene-transfer system capable of delivering the intrabody gene into a sufficient number of a specific cell type is needed. Currently, lentivirus vectors are the main gene delivery tool used to obtain high transduction efficiency and long-term expression of intrabodies. However, one of the main challenges faced by lentiviral gene delivery systems is the development of vectors that can efficiently target specific cell types.

In **Chapter 4,** we presented a study to generate lentiviral-derived particles with specificity of gene delivery for CCR5-expressing cells. For this purpose, we developed a novel Sindbis pseudotyped lentiviral vector where the Sindbis receptor binding envelope protein was modified to directly encode a scFv against the CCR5 chemokine receptor. Targeting into specific cells was mediated by the anti-CCR5 scFv display and viral titers were close to 10^6 EGFP transduction units/ml. Our data demonstrate that the length of the peptide linker that connects the heavy chain and light chain of anti-CCR5 scFv significantly affects the efficiency of infection. Infection levels obtained with Sindbis envelope displaying a scFv with a longer linker were consistently higher than that with Sindbis envelope displaying a scFv with a short linker. The present study can be a model strategy for specific gene

delivery mediated by lentiviral vectors pseudotyped with Sindbis envelope displaying scFv that recognize specific cellular surface proteins.

In conclusion, the results from this thesis provide proof-in-principle that rabbit anti-IN and anti-Vif intrabodies may be potentially useful agents for HIV-1 gene therapy approaches. Furthermore, we also show that the gene delivery strategy presented herein has the potential to become a powerful approach for targeting gene delivery in anti-HIV gene therapy due to the important role of CCR5 expression in disease progression. Furthermore, using HIV-1 as an infection model, the intracellular immunization technology may also be applied to many other suitable infectious diseases. Such opportunities can be usefully expanded to other viral infections such as HIV-2, herpes and hepatitis group viruses. It seems certain that within the next decade gene therapy for both the treatment and prevention of infectious diseases will be an encouraging much needed alternative therapy.

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