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Synthetic antibodies targeting HIV-1 infectivity

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Tese orientada pelo Professor Doutor João Gonçalves

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RESUMO

A Síndrome da Imunodeficiência Adquirida (SIDA) representa hoje um dos principais problemas de Saúde Pública a nível mundial, tendo contraído a doença mais de 60 milhões de pessoas em todo o mundo desde a sua descoberta em 1981, um terço das quais faleceram subsequentemente.

Apesar dos grandes progressos realizados no tratamento da SIDA, especialmente desde a introdução do regime terapêutico HAART em 1996, as alternativas terapêuticas actuamente disponíveis não permitem erradicar completamente o VIH-1 do organismo, o que resulta em toxicidade a longo prazo e leva eventualmente à emergência de estirpes de VIH-1 resistentes à terapêutica disponivel. Estes problemas levam à procura e desenvolvimento de novos fármacos activos contra o VIH, nomeadamente contra as estirpes de VIH resistentes aos fármacos actualmente utilizados. Entre os novos fármacos actualmente disponíveis encontra-se uma nova classe, os inibidores de entrada.

O processo de fusão do VIH-1 é mediado pelas proteínas Env (gp120 e gp41) e inicia-se através da ligação da gp120 ao receptor celular CD4. A interacção gp120-CD4 dá origem a uma alteração conformacional na *loop* V3 da gp120 que expõe o epitopo de ligação ao receptor de quimiocinas, o que por sua vez induz alterações conformacionais na gp41 que culminam na conformação activa do péptido de fusão (conformação fusogénica). Portanto, a interação gp120-CD4 é fundamental para o processo de fusão vírus-célula.

Igualmente, uma descoberta recente identificou na gp120 uma região conformacionalmente invariável que se sobrepõe parcialmente ao local de ligação ao CD4, esta região corresponde à zona de ligação do anticorpo b12 e possui a particularidade que se encontrar constitutivamente exposta no domínio exterior da gp120. Esta região encontra-se envolvida na ligação metaestável ao CD4, antes de ocorrer o rearanjo necessário para a existência de uma ligação estável. Este é por conseguinte um local de vulnerabilidade, relacionado com um requisito funcional para existência de uma associação eficiente com o CD4.

O anticorpo b12 é um dos poucos anticorpos monoclonais humanos conhecidos que pode eficientemente neutralizar uma ampla gama de isolados primários de VIH-1 *in vitro* e pode proteger contra o desafio viral *in vivo*.

O objetivo deste trabalho foi a avaliação da sequência alvo do anticorpo neutralizante b12 ou o epítopo de ligação ao CD4 fora do contexto da glicoproteína gp120. Para tal procedeu-se à construção de anticorpos de domínio, por meio de "grafting" da sequência alvo do anticorpo neutralizante b12 ou o epítopo de ligação ao CD4 num dos CDR's, CDR1 ou CDR3 de um anticorpo de coelho de único domínio V_L,.altamente estável. O potencial das construções V_LB12 e V_LCD4 foi testado para ligação ao CD4 por ELISA e por Citometria de Fluxo. A Citometria de Fluxo foi efectuada recorrendo a células HEK293T, HeLa-P4 e Jurkat, ambas as construções apresentam ligação ao receptor CD4, e o V_LB12 CDR1, em particular, e parece ter uma ligação ao CD4 de elevada afinidade. A partir dos resultados de Citometria de Fluxo e dos rendimentos obtidos para as purificações as construções V_LB12 CDR1 e V_LCD4 CDR3 foram selecionados para análise posterior. Pretendeu-se também caracterizar o local de ligação das construções V_LB12 CDR1 e V_LCD4 CDR3 no receptor CD4, nomeadamente identificar qual o domínio do CD4 ao qual se ligam os anticorpos V_LB12 e V_LCD4. Para tal, realizaram-se ensaios de ELISA utilizando como antigénios que o receptor celular humano CD4 na sua forma solúvel, quer uma construção em que os domínios 1 e 2 do CD4 humano foram colocados em fusão com uma IgG2 humana na qual as regiões variáveis das cadeias leves e das cadeias pesadas foram substituidas pelos domínios 1 e 2 do CD4 humano dando origem a uma proteína de fusão designada CD4-IgG2. Dos ensaios de ELISA foi possivel concluir que ambos os anticorpos V_LB12 e V_LCD4 se ligam ou ao domínio 1 ou ao domínio 2 do CD4 humano. Para identificar especificamente a qual dos domínios se ligavam o V_LB12 e o V_LCD4, foram realizadas experiências em que células HEK293T foram transfectadas com CD4 humano (hCD4), CD4 de murganho (mCD4) ou com CD4 humano em que o domínio 1 foi substituido pelo domínio 1 de murganho (hCD4mD1). Estas células transfectadas com os diferentes receptores celulares CD4 foram submetidas a ensaios de Citometria de Fluxo para avaliação da ligação dos anticorpos V_LB12 e V_LCD4 aos diferentes receptores celulares CD4 e concluiu-se que o V_LB12 se liga ao domínio 2 e que o V_LCD4 se liga ao domínio

1 do CD4 humano. Realizaram-se também ensaios de ELISa em que se verificou que os epitopos reconhecidos pelo V_LB12 e pelo V_LCD4 são ambos conformacionais.

A fim de avaliar a capacidade do V_LB12 e do V_LCD4 na inibição da infecção por VIH-1, foram realizados ensaios de inibição com o clone molecular NL4-3 do VIH-1 (subtipo B) e com os subtipos J e H de isolados primários de VIH-1. Os ensaios de inibição foram realizados em células TZM-bl num ensaio de um ciclo único de infecção. O V_LB12 foi capaz de inibir o VIH-1_{NL4-3} com um IC_{50} = 3,89 μM e o V_LCD4 foi capaz de inibir o HIV-1_{NL4-3} com um IC_{50} = 6,57 μM. Realizaram-se igualmente ensaios de inibição com o T20 para o qual se obteve um IC_{50} = 0,0694 μM.Quanto aos isolados primários, o V_LB12 foi capaz de inibir o VIH-1 subtipo H com um IC_{50} = 3,97 μM e o VIH-1 subtipo J com um IC_{50} = 3,86 μM, mas o V_LCD4 não foi capaz de inibir os subtipo J e H de isolados primários de VIH-1. Para o T20 obteve-se um IC_{50} = 1,33 nanoM para o subtipo H do VIH-1 e para o subtipo J do VIH-1 obteve-se um IC_{50} = 0,42 nanoM.

Estes resultados indicam que o V_LB12 apresenta um amplo potencial de inibição ao contrário do V_LCD4 que não foi capaz de inibir qualquer um dos isolados primários dos subtipo não B.

Devido à elevada afinidade de ligação ao receptor CD4 apresentada pelo V_LB12, uma das abordagens desta Tese teve por objectivo avaliar o potencial de um novo sistema de entrega de moleculas para terapia génica em que se utilizaram nanopartículas revestidas com V_LB12 para avaliar a entrega de biomoléculas a células que expressam o receptor CD4.

Foram testados dois tipos diferentes de formulações de nanopartículas, de quitosano e de PEI, para a entrega do plasmídeo FUGW-dsRed em células Jurkat. Ambas as formulações de nanopartículas foram bem sucedidos na entrega do plasmídeo FUGW-dsRed às células Jurkat, conforme determinado por imunofluorescência.

A formulação de nanopartículas de quitosano provou globalmente ser mais eficaz, devido à reduzida toxicidade celular. A quantidade ideal de V_LB12 adsorvido às nanopartículas deu origem a um aumento de 16% da população dsREd positiva

fluorescente com um direccionamento das nanopartículas para o receptor alvo (CD4) devido à presença do V_LB12.

Numa abordagem distintadas anteriores, pretendeu-se avaliar as propriedades antigénicas do V_LB12 para utilização numa formulação de vacina. Deste modo, a imunidade das mucosas foi testada utilizando o V_LB12 encapsulado em nanopartículas de quitosano, por comparação com a via subcutânea, utilizando como adjuvante alumínio e realizando as mesmas formulações sem adjuvante. A resposta de IgG total específico nas amostras de soro foi avaliada por ELISA utilizando como antigénio o V_LB12. Foram também avaliadas as respostas Th1 ou Th2 através do racio dos antigénios específicos IgG1-IgG2 nas amostras de soro.

Para avaliar o potencial antigénico do V_LB12 como vacina, foram realizados ensaios de inibição de VIH-1 usando os soros dos diferentes grupos de murganhos, aos quais foi administrado cada uma das formulações. Os ensaios de inibição foram realizados em células TZM-bl, com uma MOI de 0,1, num ensaio de um ciclo único de infecção. Apesar de os grupos vacinados apresentarem títulos de IgG específico elevados, nenhum dos soros obtidos apresentou efeito inibitório sobre o VIH-1_{NL4-3}. Confirmou-se por ELISA que os soros obtidos não reconheciam a gp120 o que indica que a *framework* do V_L é altamente imunogénica, não tendo sido obtida uma proporção de anticorpos dirigidos contra a região alvo b12 suficiente para se obter um efeito inibitório nos ensaios de inibição do VIH-1_{NL4-3}.

Palavras-chave: VIH-1; gp120; anticorpos de domínio único V_L; inibidores de entrada; vacina.

ABSTRACT

The HIV-1 entry process is mediated by the Env proteins, and begins with the binding of gp120 to the CD4 cellular receptor. A recent discovery has identified in gp120 a conformationally invariant surface that overlaps a distinct subset of the CD4-binding site, which is a binding site for the b12 natural antibody.

The goal of this work was to evaluate both the b12 target sequence and the CD4 binding epitope outside of the gp120 context and to evaluate the potential for CD4 binding of these two epitopes individually. This was done through grafting of the b12 target sequence or CD4 binding epitope into either CDR1 or CDR3 of a highly stable V_L antibody. The potential of the V_LB12 and V_LCD4 constructs was tested for CD4 binding by ELISA assay and Flow Citometry analysis; both graftings present binding to the CD4 receptor and V_LB12, in particular.

In order to evaluate the V_LB12 and V_LCD4 ability to inhibit HIV-1 infection, inhibition assays were performed with the HIV-1_{NL4-3} subtype B molecular clone and with HIV-1 subtypes J and H primary isolates. V_LB12 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM and V_LCD4 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 6,57 μM. As for the HIV-1 primary isolates, V_LB12 was able to inhibit subtype H with an IC_{50} = 3,97 μM and subtype J with an IC_{50} = 3,86 μM but V_LCD4 was not able to inhibit either subtype J or subtype H. These results indicate that V_LB12 presents potentially a broad inhibition spectrum as opposed to V_LCD4 that was not able to inhibit either of the non subtype B primary isolates.

Due to the V_LB12 high binding affinity to the CD4 receptor the potential of V_LB12 coated nanoparticles as a new therapeutic delivery system was evaluated. Chitosan and PEI nanoparticle formulations were tested, for the delivery of FUGW-dsRed plasmid to Jurkat cells. Both nanoparticle formulations were successful in delivery of specific CD4 targeted FUGW-dsRed as determined by immunofluorescence. Chitosan nanoparticle formulation proved to be more effective due to the lower cell toxicity. The optimal amount of V_LB12 adsorbed to the nanoparticles gave a 16 % positive dsREd fluorescent population with specific V_LB12 targeting.

To evaluate the vaccine antigen properties, of V_LB12 the mucosal immunity was tested by using encapsulated V_LB12 in nanoparticles of chitosan in comparison with subcutaneous routes with aluminium adjuvant and without adjuvant. Th1 or Th2 responses were evaluated by the ratio of antigen-specific IgG2a-IgG1 in the serum samples. HIV-1 neutralization assays using serum from different groups to evaluate the V_LB12 as potential vaccine antigen were performed but the serum presented no inhibitory effect on HIV-1_{NL4-3}.

Keywords: HIV-1, single domain antibody, entry inhibitor, nanoparticles, vaccine.

LIST OF ACRONYMS AND ABBREVIATIONS

Reagents and Techniques

ABTS	2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt
CPRG	ChloroPhenolRed- β -D-Galactopyranoside
CS	Chitosan
CS/DS	Chitosan/Deoxycholate
DMEM	Dulbecco's modified Eagle's Medium
ECL	Enhanced ChemiLuminescence reagent
ELISA	Enzyme-Linked ImunoSorbent Assay
FBS	Fetal Bovine Serum
MOI	Multiplicity Of Infection
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PEI	Polyethylenimine
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
HRP	HorseRadish Peroxidase

General

ADCC	antibody-dependent cell-mediated cytotoxicity
AIDS	Acquired Immune Deficiency Syndrome
APOBEC3G	apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
ART	Antiretroviral Therapy
ARV	Anti Retroviral
CA	Capsid
CCR5	CC Chemokine Receptor 5
CD4	Cluster of Differentiation 4
CD4bs	CD4 binding site
CD4i	CD4 induced
cDNA	complementary DNA
CDR	Complementarity Determining Region
CDRH	complementarity determining region of antibody heavy chains

CDRH3	third complementarity determining region of antibody heavy chains
CDRL	complementarity determining region of antibody light chains
CDRL1	first complementarity determining region of antibody light chains
CDRL3	third complementarity determining region of antibody light chains
C_H	Constant Heavy Chain
C_L	Constant Light Chain
CXCR4	CXC Chemokine Receptor 4
DNA	Deoxyribo Nucleic Acid
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
Env	Envelop polyprotein
ER	Endoplasmic Reticulum
ESCRT	endosomal sorting complex required for transport
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FcRn	neonatal Fc receptor
FR	framework region
Fv	Fraction variable
Gag	Group specific antigen polyprotein
h	hour
HAART	Highly Active Antiretroviral Therapy
hcAbs	heavy chain antibodies
hCD4	Human Cluster of Differentiation 4
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1
HIV-2	Human Immunodeficiency Virus Type 2
HMG-1(Y)	High Mobility Group protein I(Y)
hmAbs	Human monoclonal antibodies
HR1	Heptad Repeat sequence 1
HR2	Heptad Repeat sequence 2
hTRIM5 α	human TRIM5 α
Ig	Immunoglobulin
IL-1	InterLeukine 1
IL-7	InterLeukine 7

IN	Integrase
kDa	kiloDalton
LBD	lipid-binding domain
LTR	Long Terminal Repeats
MA	Matrix
MALT	Mucosa Associated Lymphoid Tissue
MHC	Major histocompatibility complex
min	minute
ml	millilitre
mM	miliMolar
MPER	membrane-proximal external region
mRNA	messenger RNA
nanoM	nanoMolar
NC	Nucleocapsid
Nef	Negative regulator factor
NES	Nuclear Export Signal
ng	nanogram
NLS	Nuclear Localization Signal
nm	nanometer
ºC	Degrees Celsius
PBD	Pocket-Binding Domain
PBS	Primer Binding Site
PIC	PreIntegration Complex
POL	Polimerase
PR	Protease
Rev	Regulator of Expression of viral proteins
RNA	RiboNucleic Acid
RNAPol II	RNA Polymerase II
RNase H	RiboNuclease enzyme H
RRE	Rev Responsive Element
RT	Reverse Transcriptase
RTC	Reverse Transcription Complex
scFv	Single chain Fv
SD	Standard deviation

SIV	Simian Immunodeficiency Virus
SU	Surface
TAR	TransActivation Response
Tat	Transcriptional transactivator protein
TM	transmembrane
TNF- α	Tumor Necrosis Factor-alpha
TRIM5 α	Tripartite Motif Protein 5 alpha
tRNA	transference RNA
U3	Unique 3´ sequence
U5	Unique 5´sequence
V_H	Variable Heavy Chain
VHH	Variable domain of camelid heavy chain antibody
Vif	Viral Infectivity Factor
V_L	Variable Light Chain
VNAR	Variable domain of the shark new antigen receptor
Vpr	Viral protein R
Vpu	Viral Protein U
μg	microgram
μM	microMolar

Amino acids

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

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Introduction

Introduction

1 General Introduction

The Acquired Immune Deficiency Syndrome (AIDS) is now a major public health problem worldwide, having contracted the disease more than 60 million people since its discovery in 1981, one third of whom subsequently died.[1,106]

The etiological agents responsible for AIDS are the human immunodeficiency virus type-1 and type-2 (HIV-1 and HIV-2). The first agent to be isolated was HIV-1[21,113] in 1983. In 1986 HIV-2 was identified,[69,70] having similar biological properties to those of HIV-1, but a greater genetic homology with Simian Immunodeficiency Virus than with HIV-1. This translates into a significant difference in some of its molecular and antigenic properties.

HIV-1 is more virulent and has higher transmission rates than HIV-2, which explains why HIV-1 is responsible for a worldwide pandemic and HIV-2 accounts for more localized epidemics, particularly in countries of West Africa and in some European countries such as Portugal.[69,70] Only HIV-1 will be studied in this Thesis.

HIV-1 has the ability to infect cells that have a specific receptor, cluster of differentiation 4 (CD4), and chemokine co-receptors, especially CCR5 and CXCR4, which are mainly on the surface of $CD4^+$ T lymphocytes.[26,162] Other cells that express these receptors and likely to be susceptible to HIV-1 are, for example, cells of the mononuclear phagocytic system, particularly monocytes, macrophages, B lymphocytes and dendritic cells.

The cell tropism of HIV suggests that once infection is established it will be very difficult to eliminate the virus from the body. This leads to progressive destruction of the immune system, leading to AIDS, with its multiple opportunistic infections, and/or complications such as neurological disorders and cancer. With this type of epidemiological characteristics it is imperative a commitment to a preventative therapy or acting in the early stages of infection.[106,323]

Despite the stunning advances in the treatment of AIDS, especially since the introduction of highly active antiretroviral therapy (HAART) in 1996, current therapy cannot completely eradicate HIV-1 from the body, resulting in long-term toxicity and eventually leading to the emergence of drug-resistant HIV-1 strains. These problems prompt the search for potent new drugs that are active against drug-resistant viral strains. Among these drugs appears a new class, the entry inhibitors.

The monoclonal antibody therapy has had an important development in recent years particularly with regard to new therapeutic approaches for treating HIV infection and AIDS. This form of therapy can be complementary and add to the effectiveness of the existing drugs. Studies with "natural" antibodies (hmAbs) that neutralize the virus by binding to conserved epitopes of gp120 or gp41 proteins have shown a half-life of 50 to 100 times higher than fusion inhibitor peptides, showing no toxicity associated with pharmacological anti-HIV drug therapy.[17,171,239,266,320]

This type of strategy is a priority in the activities of the Unit of Retrovirus and Associated Infections (URIA) of the Faculty of Pharmacy of Lisbon, particularly in identifying new forms of biological therapies against HIV-1, and specifically the identification of recombinant antibodies against various therapeutic targets, whose large-scale production is easily optimized. Thus, it will be presented and discussed in this Thesis a new way to inhibit the infectivity of HIV-1 using synthetic recombinant antibodies with inhibitory capacity of the entry process. Moreover the potential for other therapeutic applications was also explored for one of these molecules, in particular as part of a new therapeutic delivery system and also as a vaccine antigen.

1.1 Human Immunodeficiency Virus type 1

1.1.1 Genetic structure

HIV-1 belongs to the genus *Lentivirus* and family *Retroviridae*. These are enveloped viruses with a nucleocapsid that resembles a truncated cone. They have a diploid positive-strand RNA genome which replicates via a double strand DNA intermediate (reverse transcription), being afterwards integrated in the host genome in the form of a provirus. These features are common to all retroviruses.[222,322,344]

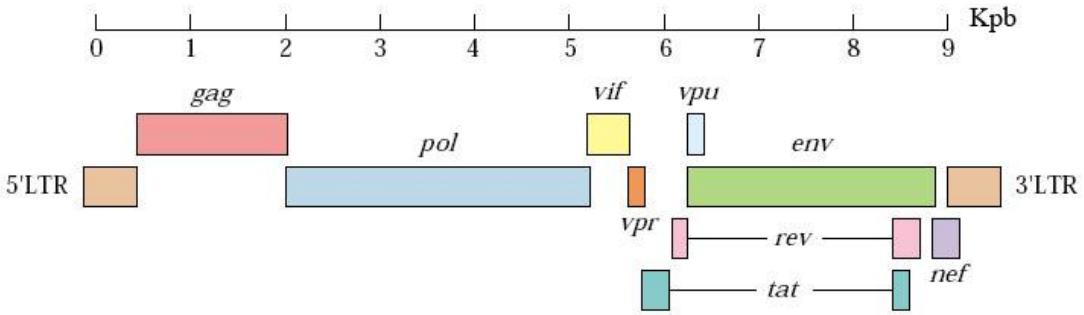

Figure 1.1– Genetic organization of HIV-1.
(Reprinted with permission from: W.H. Freeman and Company, copyright (2006)).[176]

The genome of HIV-1 is composed of three essential genes, *gag*, *pol* and *env*, the regulatory genes *tat* and *rev*, four accessory genes, *nef*, *vif*, *vpr* and *vpu*, and two identical sequences, called long terminal repeats (LTRs) flanking the genome.[222,322,323,352]

The *gag* and *pol* genes are translated into long precursor polyproteins (Figure 1.1). The *gag* gene codes for a precursor polyprotein of 55 kDa (p55) that is subsequently cleaved by the action of the viral protease giving rise to the major internal structural proteins, designated matrix (p17, MA), capsid (p24, CA), nucleocapsid p7 (NC) and p6. After cleavage the Gag-Pol precursor polyprotein gives rise to the protease (p10, PR), reverse transcriptase (p66/p51, RT) and integrase (p31, IN) .[322]

The product of the *env* gene is translated in the form of a precursor of 160 kDa (gp160) that is subsequently cleaved generating the two subunits of the viral envelope, the surface glycoprotein (gp120, SU) and the transmembrane glycoprotein (gp41, TM).[93]

The *tat* and *rev* regulatory genes give rise, respectively, to the Tat (p14) and Rev (p19) proteins. The Tat protein is a trans-activator of transcription that increases the processivity of RNA polymerase II. The Rev protein is the regulator of expression that inhibits the splicing of viral RNAs and is also involved in the translocation to the cytoplasm of RNAs that did not undergo splicing.[322,352]

The down-regulating factor (Nef), the viral infectivity factor (Vif), viral protein R (Vpr) and protein U (Vpu) are all small accessory proteins encoded respectively by genes *nef*, *vif*, *vpr* and *vpu*, and each play more than one function in the replication cycle of HIV-1.[322,323]

The Vif protein averts the lethal threat of deamination, precluding the packaging of APOBEC3G (A3G) into assembling virions by mediating its proteasomal degradation.[238] Vpr functions early in the viral life cycle, in the transport of the PIC to the nucleus. Vpr has several other critical functions including activation of HIV-1 LTR transcription, cell-cycle arrest due to DCAF-1 binding, and both direct and indirect contributions to T-cell dysfunction. [165,233] Vpu enhances virus budding and degrades cellular CD4.[37] Nef also has similar functions, and appears to be required for disease induction in vivo.[108]

The LTR regions are important for replication and transcription of viral RNA. These sequences are divided into three parts: U3 (derived from a unique sequence of the 3' region of the viral RNA), R and U5 (derived from a unique sequence of 5' region of the viral RNA). The LTR sequences include promoters, enhancer elements and other genomic sequences used to connect to different cellular transcription factors.[222]

1.1.2 Morphology

HIV-1 is a virus whose virions have an icosahedral structure of about 100 nm in diameter. The mature virion consists of an outer layer, called the viral envelope, which derives from the host cell membrane and is acquired during their release (budding) of the cell. The viral envelope is formed by a lipid bilayer impregnated with some human proteins, such as Major Histocompatibility Complex (MHC) proteins, and viral

glycoproteins gp120 and gp41 that form a trimer of heterodimers and whose function is to mediate the entry process allowing the virus to infect target cells.[327]

The coating of the inner viral envelope is the matrix protein (MA). In the center there is a nucleo-shaped truncated cone, formed by p24 (CA), within which there are two copies of the viral genome. The single-stranded RNA copies associate with one another by base-pairing near its 5 'end. Each of these molecules is coupled with a transfer RNA (tRNA lysine) which acts as an oligonucleotide to initiate reverse transcription of viral RNA. The viral RNA forms a ribonucleoprotein complex with NC, p6 and a few molecules of IN, PR and RT. Nef, Vif and Vpr are also incorporated in the viral particle (Figure 1.2).[72,176,327]

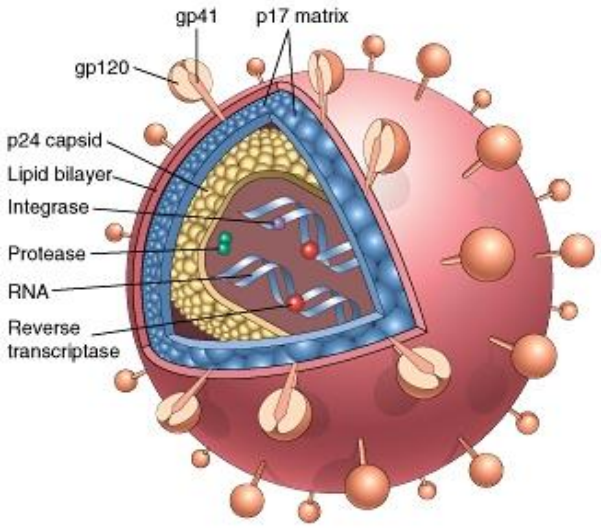

Figure 1.2 - Schematic representation of the HIV-1 virion.
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1.1.3 Replication Cycle

HIV-1 has a typical retroviral life cycle, divided into two phases: the early phase and the late phase. 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 (see Figure 1.3).[322]

The first step in HIV-1 infection is the Env mediated direct fusion of the viral membrane with the plasma membrane of the host cells. The Env glycoproteins[62,89,114,355] are organized into trimeric spikes,[342] anchored to the viral membrane by the gp41 transmembrane protein. The Env glycoprotein gp120, forms surface trimeric spikes, which are associated by noncovalent interactions with each subunit of the also trimeric, and normally hidden, gp41.[169]

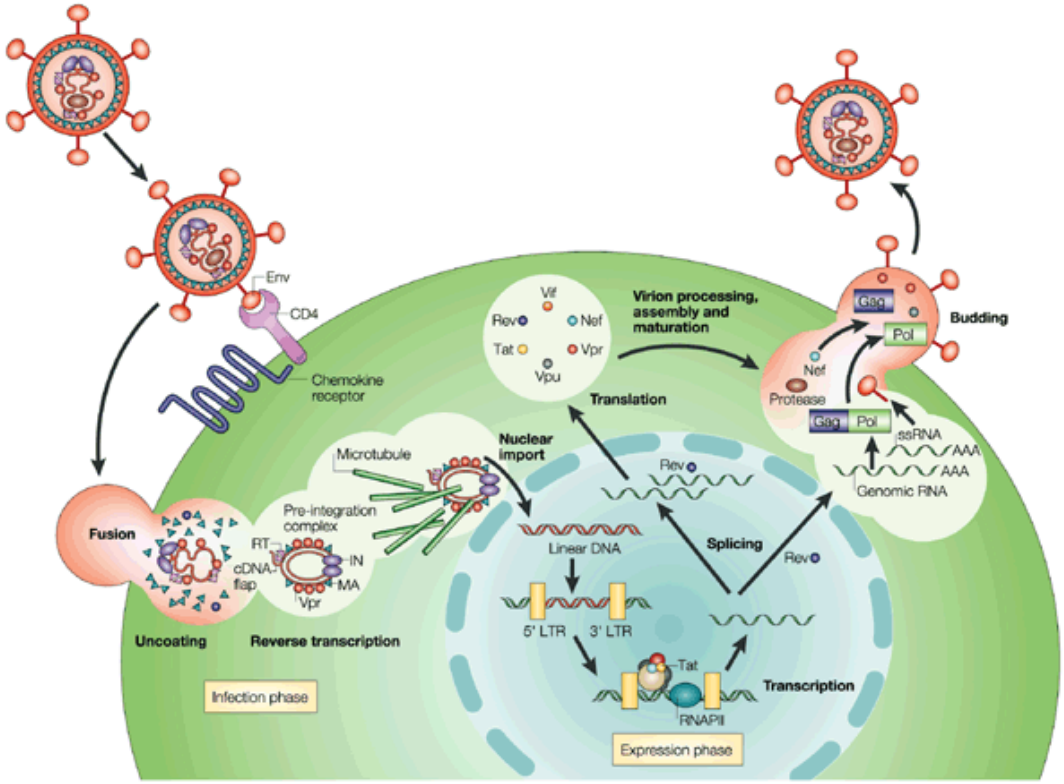


Figure 1.3 - Steps involved in HIV-1 replication.
(Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Immunol, copyright (2004).) [364]

The fusion process begins with the binding of gp120 to the CD4 cellular receptor. The binding of gp120 to CD4 induces conformational changes in gp120, exposing the chemokine receptor binding site and promoting the interaction between gp120 and the chemokine receptor, usually CXCR4 or CCR5. This interaction subsequently induces conformational changes in gp41 that result in an active conformation of the fusion peptide (fusogenic conformation) promoting the fusion of the cellular and viral membranes. [16,237,277,285,322,369] Membrane fusion is followed by the release and uncoating of the HIV capsid into the cytoplasm of the host cell. Completion of uncoating leads to the formation of the reverse transcription complex (RTC), composed of viral RNA, reverse transcriptase, integrase, $tRNA^{lys3}$, MA, nucleocapsid (p7), Vpr (Viral protein R)

and several host proteins.[111,317,327] Reverse transcription is performed by RT, which copies the viral RNA template into a complementary double strand DNA. To initiate the synthesis of viral DNA, a oligonucleotide of cellular origin is used, $tRNA^{lys3}$. The binding site of the lysine tRNA is designated primer binding site (PBS) and is located in the 5 'end of the unspliced viral RNA.[163,193] The process of synthesis also depends on NC, MA and the accessory protein Vif.[322]

Once synthesized, the viral DNA is transported to the nucleus as part of a preintegration complex (PIC), containing the viral proteins IN, MA, RT and Vpr and the cellular protein High Mobility Group protein I(Y) (HMG-I (Y)).[317,322] The integration of HIV DNA into the host cell chromossome, giving rise to the provirus, is promoted by the catalytic activity of IN.[111,190,317,322]

The provirus thus becomes an integral part of the host cell genome and is treated by the cell as a cellular gene. After integration, the provirus may either remain latent (post-integration latency) or become transcriptionally active. The provirus acts as a template for the synthesis of viral RNA that can be subsequently used as genomic RNA for virion progeny or translated into viral proteins.[322] HIV-1 transcription is controlled by the inducible viral promoter located at U3 of 5´LTR.[300]

The transcription of the viral DNA is mediated by cellular RNA polymerase II and involves regulatory sequences in the region of the 5'- LTR U3 viral promoter, which functions as an activator of transcription, since it has binding sites for several cellular transcription factors.[317,322,327]

The late phase of the replication cycle begins with the synthesis of spliced and unspliced mRNAs that are transported out of the nucleus to be translated. Transcription from the HIV-1 LTR leads to the generation of three major classes of viral RNAs:[264]

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;

3) small (1.7 to 2.0 kb), multiply spliced mRNAs, which are translated into Rev, Tat, and Nef.

Initially, only short spliced RNAs are translated and these code for proteins Tat, Rev and Nef.[322,327] Since most cellular mRNAs are fully spliced before their transport out of the nucleus, the need for unspliced and partially spliced RNAs in the cytoplasm has been overcome through the evolution of viral protein, Rev (for "regulator of expression of viral proteins"), and a *cis*-acting RNA element, the Rev responsive element (RRE). The RRE is a large (250 nucleotide), highly structured RNA element that is located in the *env* gene and is present in all unspliced and partially spliced HIV-1 RNAs. [111,255]

The basal transcriptional activity from the HIV LTR is very low and RNA synthesis is greatly increased (by more than two logs) when the transcriptional transactivator protein Tat is present.[111,149] When sufficient amounts of Rev protein are produced, Rev binds to the the Rev-responsive element (RRE) of the unspliced or single-spliced RNAs, leading to the formation of a protein complex that interacts with the cellular nuclear export machinery, consequently promoting unpliced or partially spliced RNA transport out of the nucleus, for translation in the cytoplasm.[111,338]

Following the synthesis of the full complement of viral proteins, the assembly process begins. The major player in virus assembly is the Gag precursor polyprotein, $Pr55^{Gag}$.[115,149] The Gag polyprotein is synthesized in the ribosomes from unspliced RNAs and a translation frameshift leads to the formation of smaller amounts of Gag-Pol precursor proteins.[322] Gag polyprotein is the major responsible for the assembly of new immature viral particles. This protein contains determinants that target it to the plasma membrane, bind the membrane itself, promote Gag-Gag interactions, encapsidate the viral RNA genome, associate with the viral Env glycoproteins, and stimulate budding from the cell.[30,41,111]

The assembly of lentiviruses, including HIV-1, takes place at the plasma membrane of the infected cell. During assembly, Gag is targeted to the plasma membrane. The anchored Gag leads to the induction of Gag multimerization and subsequently incorporation of the viral genomic RNA, Env glycoproteins and Gag-pol precursor into the viral particle.[41,111,322]

The MA domain is responsible for the targeting of Gag to the plasma membrane and for the incorporation of gp120-gp41 complexes and Gag-Pol precursor into the viral particle, whereas the C-terminal domain of CA (CA-CTD) and NC are responsible for Gag multimerization.[111] In addition,NC as part of the Gag polyprotein, specifically binds the genomic RNA via tight interactions between its zinc-finger motifs and the Psi packaging signal located in the 5' leader sequence. These specific NC–RNA molecular interactions are thought to promote genomic RNA dimerization leading the RNA genome for packaging into the virions.[86,236] Viral enzymes, accessory viral proteins, the cellular tRNAlys3 primer and cellular proteins also associate to the immature core for incorporation into the viral particle.[3,30,149] Subsequently, the immature core associated to the plasma membrane suffers budding through the plasma membrane. The P6 protein helps in this process by recruiting the Endosomal Sorting Complex Required for Transport (ESCRT), facilitating fission of virions from the plasma membrane to the extracellular medium.[82,221,310]

In contrast to all the other viral proteins, the Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum (ER) where CD4 molecules are also present. To prevent the premature binding of Env to CD4, the CD4 molecules are targeted for removal by Vpu and Vpu signals their degradation *via* the ubiquitin-proteasome pathway.[36,322] The binding of Env to CD4 in the ER would prevent the translocation of Env to the cell membrane and the formation of fully functional gp120-gp41 trimers.[36,322] Gp160 is translated from the single spliced *env* mRNA and suffers posttranslational modifications in the ER and in the Golgi apparatus. During trafficking through the Golgi, gp160 is cleaved by a host cell protease generating the mature gp120 and gp41 proteins that subsequently form trimeric non-convalent complexes (gp120-gp41). These complexes are transported to the cell surface via the secretory pathway and gp41 protein anchors the complexes in the cell membrane for virus assembly.[36,322]

New viral particles can be produced and released through the cell membrane (budding), which after suffering a process of maturation become infectious, and can infect new cells. However, when integrated into the host's genetic material, there is the possibility of HIV remaining dormant for many years.[322]

1.2 The fusion process: a crucial step in HIV infection

To initiate a new infection, mature virus particles need to encounter a potential target cell that expresses the appropriate receptor structures. In the case of HIV, these are the CD4 molecule found primarily on T lymphocytes, macrophages and dendritic cells. Although the virus binds to CD4 on the cell surface, this interaction alone is not sufficient for entry and productive infection. Expression of other cell-surface molecules, designated co-receptors, is required for HIV-1 infection.[176]

The entry of HIV-1 into a target cell involves three distinct stages: binding of gp120 to CD4, binding of gp120 to a co-receptor, and gp41-mediated fusion of the viral and host membranes.

The discovery that CXCR4 and CCR5 serve as co-receptors for HIV-1 on T cells and macrophages, respectively, explained why some strains of HIV-1 preferentially infect T cells (T-tropic strains) while others prefer macrophages (M-tropic strains). A T-tropic strain uses CXCR4, while the M-tropic strains use CCR5.[176]

As previously mentioned the fusion process begins with the binding of gp120 to the CD4 cellular receptor. CD4 binding results in a major reorganization of the Env trimer, causing an outward rotation and displacement of each gp120 monomer. [195]

Since the first step in HIV infection involves both cellular and viral proteins, to understand the process it is necessary to identify all the proteins involved and the part each protein represents in this crucial step of the virus life cycle.

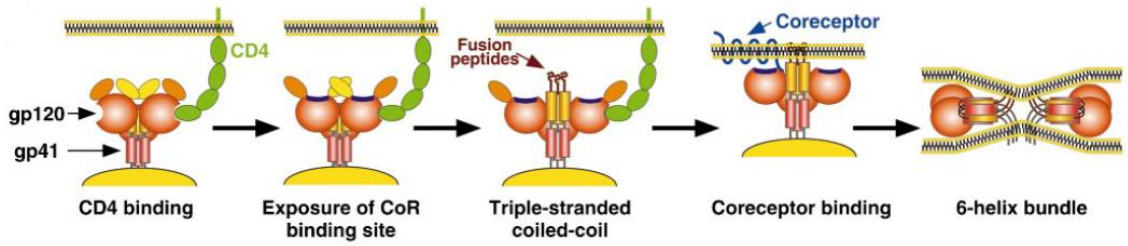


Figure 1.4 – Simplified schematic representation of the current model for the fusion mechanism between HIV-1 and the target cell.
(Adapted with permission from ©Dooms et al., 2000. Originally published in The Journal of Cell Biology. 151:9–13).[93]

1.2.1 The CD4 receptor – targets of opportunity

Cluster determinant 4 (CD4) is a transmembrane glycoprotein of 58 kDa, composed of four immunoglobulin-like extracellular domains spanning 370 amino acids, a transmembrane segment of 25 amino acids and a cytoplasmic tail of 38 amino acids at the C-terminal end (Figure 1.5).[36,201] The other three domains are less closely related to Ig molecules at the level of primary structure but fold similarly to Ig family domains, confirming that CD4 is a member of the Immunoglobulin like superfamily.[68,201,202,349] Post-translational modifications of CD4 include the formation of disulfide bonds which stabilize the D1, D2, and D4 domains and the addition of two N-linked glycans between D3 and D4.[201]

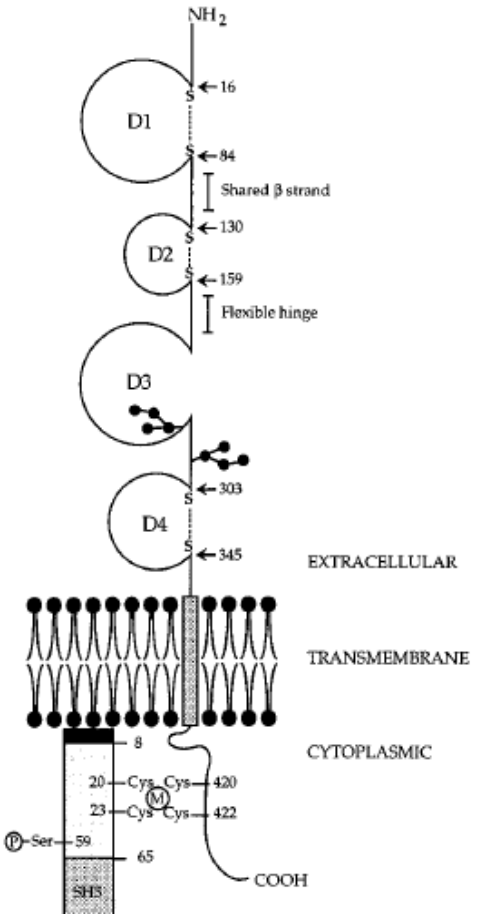


Figure 1.5 - Schematic representation of the CD4 molecule.
The major structural features of the CD4 molecule are shown, including the D1-to-D4 immunoglobulin-like domains and the two N-glycosylation sites (●——●). The cysteine residues involved in disulfide bonds (S– – –S) are indicated by arrows together with their location in the amino acid sequence. The T-lymphocyte-specific protein tyrosine kinase p56*^{lck}* is also partially depicted (Reprinted with permission from American Society for Microbiology, copyright (1995)).[36]

As an important component of the immune system, CD4 functions as a co-receptor of the T cell receptor (TCR) on the surface of $CD4^+$ T cells for stronger association with the class II major histocompatibility complex (MHC II) on antigen-presenting cells (APCs). This association is sufficient to trigger T-cell signaling transduction resulting in activation of the $CD4^+$ T cells.[64] The CD4 molecule uses its D_1 domain to interact with the β_2-domain of MHC class II molecules.[53,167]

More importantly, epitope mapping of CD4 substitution mutants with MAbs showed that a small region, containing amino acids 41 to 52 of human CD4 D1, was involved in gp120 binding.[288] Despite extensive homology with human CD4, mouse CD4 (L3T4) cannot bind gp120.[36]

Other studies, performed with substitution of nonconserved amino acids from the mouse CD4 D1 domain onto human CD4 showed that residues 38 to 57, located in a region analogous to an Ig light-chain variable domain (CDR2), were indispensable for gp120 binding.[71,185] Studies, in which CD4 mutants were selected through loss of reactivity with MAbs against different CD4 epitopes, showed that binding of gp120, as measured by syncytium formation, required residues 42 to 49 in the CDR2 domain.[250]

Finally, with the resolution of the atomic structure of the D1 and D2 domains of CD4 [282,335] it was possible to assess that four charged residues (Lys-29, Lys-35, Lys-46, and Arg-59) and one hydrophobic phenylalanine at position 43 were essential for gp120 binding.[66,214,321] These five amino acids are predicted to form a hydrophobic pocket by holding the four charged amino acids around the hydrophobic phenylalanine residue, a structure that may be involved in direct contact with gp120.[214,283]

Several studies have demonstrated that only aminoacids located in the D1 domain of hCD4 are required for gp120 binding.[183,282] Interestingly, a monoclonal antibody that blocks HIV-1 entry by binding to D2 domain of human CD4 has been identified.[88,181,301] This antibody has the commercial designation of Ibalizumab and is a humanized IgG4 monoclonal antibody that was engineered from its mouse progenitor (5A8) by grafting the mouse complementary-determining region (CDR) onto a human IgG4 construct. [301]

This shows that in spite of the fact that only the D1 domain of hCD4 is required for gp120 binding, antibodies that bind the D2 domain can impair the CD4-gp120

interactions and effectively blocking HIV-1 entry into the target cell.[112] On the contrary, antibodies that bind the D3 domain of hCD4, like the OKT4 clone,[59,116] do not block HIV-1 entry.[269]

To date only antibodies that target D1 or D2 domains of hCD4 have been found to possess the ability to block HIV-1 entry into the target cell, as these domains appear to be the ideal targets for an antibody based HIV-1 inhibition strategy that targets the CD4 receptor.

1.2.2 Viral surface glycoprotein (gp120)

Env is a heterodimer of a transmembrane glycoprotein (gp41) and a surface glycoprotein (gp120) and forms trimers on the surface of the viral membrane. There is, however, evidence that other envelope species may also be present on the surface of HIV-1.[242] Although trimers may likely represent the functional envelope spike, both functional and nonfunctional forms of the envelope may be present on the virion surface. These nonfunctional envelope entities may be monomers, dimers, or tetramers and could arise as the result of the dissociation of functional gp120-gp41 complexes, perhaps causing gp120 to be shed from the viral surface, or inefficient trimerization of the spike in the Golgi.[46,244,356]

CD4 binding results in a major reorganization of the Env trimer, causing an outward rotation and displacement of each gp120 monomer. This appears to be coupled with a rearrangement of the gp41 region along the central axis of the trimer, leading to closer contact between the viral and target cell membranes.[195]

In its native state, gp120 is composed of two distinct regions: an inner domain, involved in interactions with gp41 and the formation of trimeric envelope spikes, and an outer domain that forms a large part of the exposed surface of the spikes and is extensively glycosylated (see Figure 1.6 and Figure 1.7).[187,241]

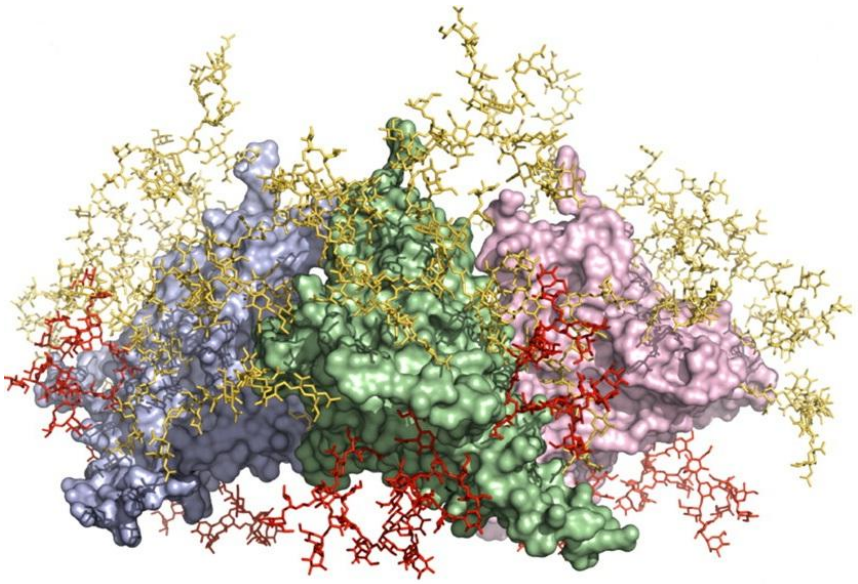


Figure 1.6 - Model of the gp120 component of the trimeric HIV-1 envelope spike based on the structure of core gp120, with three gp120 monomers shown in purple, green, and pink. Carbohydrate chains are shown in yellow.(Reprinted with permission, Copyright (2010) National Academy of Sciences, USA).[95]

The gp120 protein contains five variable regions (V1-V5)[213,304,348] and five conserved regions (C1 –C5). The conserved domains contribute to the core of gp120 (inner domain), while the variable domains (and numerous N-linked glycosylation sites) are located near the surface of the molecule (outer domain).[315]

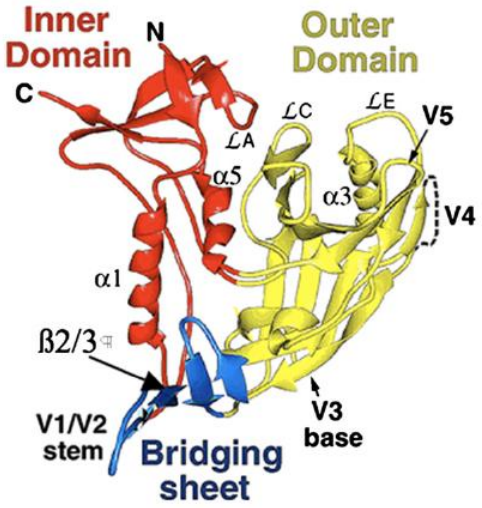


Figure 1.7 - Core gp120 showing the inner domain, outer domain, and bridging sheet (Reprinted with permission from Elsevier science, copyright (2010)).[315]

The gp120 C1 and C5 regions are considered to be the main areas on gp120 for contact with gp41, since these regions are accessible to antibody on monomeric gp120 but not on gp120-gp41 complexes.[132,218,220] Major segments of the C2, C3, and C4 regions were proposed to form a buried, relatively hydrophobic core within the gp120 molecule. This gp120 core harbors several discontinuous neutralizing antibody epitopes that overlap the binding sites for CD4, and for the coreceptor.[218,219,235,254] In contrast to the conserved regions, the variable regions (in particular, V1, V2, and V3) are predicted to be well exposed on the surface of monomeric gp120.[218,241] Deletion of V1/V2 and V3 generally increases the binding affinity of antibodies to epitopes that overlap the binding sites for CD4 and the coreceptor, which suggests that these variable regions may shield conserved epitopes from efficient antibody recognition.[54,311,354,357] Regarding the V4 and V5 variable regions, no definitive role has been ascribed; although deletion of the V4 region has been shown to disrupt gp160 folding,[254,357] V4 also seems to tolerate insertion of foreign antibody epitopes which increase virus neutralization efficiency.[270,337]

The gp120-CD4 interaction induces a conformational change in the V3 loop of gp120 exposing an envelope epitope that binds a chemokine receptor, inducing conformational changes in gp41 that result in an active conformation of the fusion peptide (fusogenic conformation).[277,369]

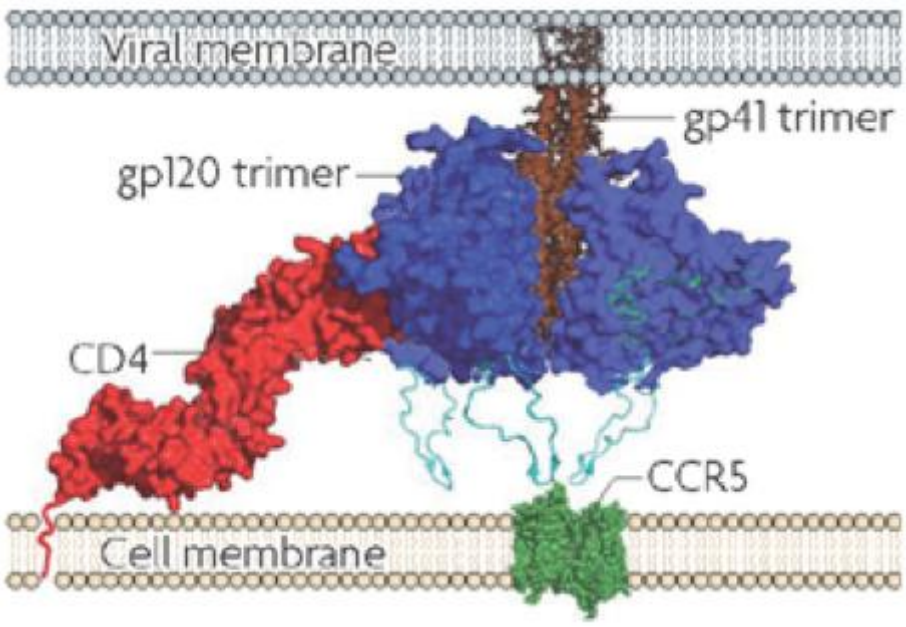


Figure 1.8 – Tridimensional representation of the interaction between CD4 and gp120. (Reprinted by permission from Macmillan Publishers Ltd: Nat Rev Microbiol., copyright (2008)). [154]

Binding of CD4 to gp120 (Figure 1.8) induces significant conformational changes that result in the formation of a third domain termed the bridging sheet. This domain consists of two pairs of anti-parallel β-sheets that link the inner and outer domains, and plays a major role in interacting with the viral co-receptors.[273]

1.2.2.1 The CD4 binding site

The binding site for CD4 on the liganded gp120 structure is formed by the interface between the inner domain, bridging sheet, and outer domain.[183,353] At the center of this interface lies a hydrophobic cavity that has been dubbed the Phe43 cavity.[183]

However, most of the CD4 contact residues are located on the outer domain of the liganded HIV-1 gp120 structures and form a contiguous binding region. The location of these conserved residues likely minimizes their immediate recognition by antibodies, while preserving the ability to contact CD4. The CD4 binding site (CD4bs) is not coherently present on the unliganded structure. It indeed seems likely that gp120 transiently samples conformations that are reflective of the liganded structure; upon interaction with CD4, the gp120 structure is locked in the bound conformation.[242]

1.2.2.2 The co-receptor binding site

The region that is important for the interaction with the β-chemokine receptor CCR5 has been mapped to residues in the bridging sheet and near the V3 stem.[272,274] Only one amino acid change is necessary for co-receptor switch from CCR5 to CXCR4. These residues are closer together on the liganded HIV-1 gp120 structure, than in the predicted unliganded gp120 structure.[63,336] These differences are consistent with the notion that CD4 binding is required to lock these areas into a contiguous binding site. The fact that the coreceptor site is not presented until after CD4 binding suggests that the site may be susceptible to antibody recognition.[242]

1.2.3 Viral transmembrane protein gp41

The viral transmembrane protein gp41 promotes HIV-1 entry by mediating the fusion of viral and cellular membranes. The primary structure of gp41 contains an amino-terminal domain (ectodomain), a single transmembrane domain (TM) and a cytoplasmic tail (Cyto).[114,277,369]

The gp41 ectodomain consists of an amino-terminal fusion peptide and two segments of hydrophobic heptad repeat (HR) sequences.[98] The fusion peptide is approximately 20 amino-acid in lenght, rich in glycine and hydrophobic residues and is believed to insert into the target cell membrane following receptor activation of Env.[277,285]

The heptad repeat sequences are designated first heptad repeat (HR1) and second heptad repeat (HR2), these are segments with 40 to 60 amino acids and are found immediately after the fusion peptide (HR1) and preceding the gp41 transmembrane domain (HR2). [120,277,369]

In its native conformation the core of the isolated gp41 ectodomain is a bundle of six α-helices derived from three gp41 ectodomains. The N-peptides (HR1) form a central, three-stranded coiled coil. The C-peptides (HR2) pack as α-helices in an antiparallel direction into hydrophobic grooves formed at the outer interface of two N-peptides.[114]

When gp41 is in its native conformation most of its extracellular amino-terminal domain is "inaccessible" due to the presence of the gp120 surface protein. The gp41 structural changes that result in the fusogenic conformation start with the extension of gp41, exposing the HR1 and HR2 regions and enabling the insertion of the fusion peptide into the target cell. Afterwards, this intermediary structure collapses, forming the trimer of heterodimers, or six-helix bundle, and the fusion between the viral and cellular membranes occurs.[277,369]

The fusion mechanism (Figure 1.4) is not yet fully understood, it is still unclear if the membrane fusion occurs before, concurrent with, or after gp41 collapses into the six-helix bundle. But it is clear that both the binding to CD4 by gp120 and the gp41 fusion-mediated structural changes are crucial to the fusion process. [98,114,369]

1.3 Antiretroviral therapy for treatment of HIV-1 infection

Over the past decades, the growing knowledge of the mechanisms of HIV-1 infection, led to the development of antiretroviral drugs that until recently were restricted to reverse transcriptase inhibitors and protease inhibitors. The reverse transcriptase inhibitors are divided into two groups: nucleoside analogues, which act as alternative substrates, being incorporated by reverse transcriptase in the nascent DNA chain, interrupting the polymerization reaction, and non-nucleosides, which inhibit the functioning of the enzyme by other mechanisms.[222]

Protease inhibitors block the morphogenesis of the virion, inhibiting the cleavage of Gag polyproteins and Gag-Pol. The evolution of antiretroviral therapy has led to the development of a treatment regimen called HAART (Highly Active Antiretroviral Therapy) which is the combination of three or more drugs of the following antiretroviral classes: reverse transcriptase inhibitors, protease inhibitors and more recently an entry inhibitor. The use of combination HAART can increase the therapeutic effectiveness but cannot eradicate HIV-1 from the body which results in long-term toxicity and inevitably leads to the emergence of resistant strains, as well as being associated with a high level of severe side effects.[222,249,306] These problems lead to the demand and development of new drugs against HIV-1, especially against HIV-1 strains resistant to currently used drugs. There are several compounds in development and in clinical or preclinical trials, acting at various stages of the life cycle of HIV-1: the virus adhesion to the cell, the binding of gp120 to the CD4 receptor, gp120 binding to the co-receptor, fusion of the viral envelope with the cell membrane, formation of the viral particle, reverse transcription, nuclear import of the PIC, integration of proviral DNA, among others.[4,104,151,253,263,267,298]

From the demand for new therapeutic approaches emerged a new class of drugs, the HIV-1 entry inhibitors (see Section 0). These compounds have a mechanism of action different from other classes of antiviral drugs since they act outside the $CD4^+$ T cells in order to prevent HIV-1 from infecting the cell.[256,277,316]

Due to the characteristics and nature of HIV-1 infection, including the ability to establish reservoirs in various cell lines, progressive and irreversible destruction of the immune system associated with an increased prevalence of strains resistant to drug therapy, one of the priority areas in the development of new drugs has been the development of new molecules, which prevent viral entry into cells, including peptides and antibodies that prevent the entry process.

Target	Drug class	Substance (abbreviation, commercial name®)
CCR5	Entry inhibitors	Maraviroc (UK-427 857, Selzentry®)
gp41 (N-HR)	Fusion inhibitors	Enfuvirtide (T20, Fuzeon®)
IN	Integrase inhibitors	Raltegravir (MK-0518, Isentress®)
RT	NRTI	Abacavir (ABC, Ziagen®), Didanonise (ddI, Videx®), Emtricitabine (FTC, Emtriva®), Stavudine (d4T, Zerit®), Lamivudine (3TC, Epivir®), Tenofovir (DF, Viread®), Zalcitabine (ddC, Hivid®), Zidovudine (AZT, Retrovir®)
RT	NNRTI	Delavirdine (DLV, Rescriptor®), Efavirenz (EFV, Sustiva®), Etravirine (TMC125, Intelence®), Nevirapine (NVP, Viramune®)
PR	PI	Amprenavir (AMP, Agenerase®), Atazanavir (ATZ, Reyataz®), Darunavir (TMC114, Prezista®), Fosamprenavir (GW-433908, Lexiva®), Indinavir (IDV, Crixivan®), Lopinavir combined with Ritonavir (ABT-378, Kaletra®), Nelfinavir (NFV, Viracept®), Ritonavir (RTV, Norvir®), Saquinavir (SQV, Fortovase®, Invirase®), Tipranavir (TPV, Aptivus®)

Figure 1.9 - Approved anti-retroviral drugs for the treatment of HIV-1 infection. (Reprinted with permission from Elsevier science, copyright (2010)) [315]

1.4 Entry inhibitors

Drugs that block HIV-1 entry are collectively known as entry inhibitors, but comprise a complex group of drugs with multiple mechanisms of action. Entry inhibitors can target the gp120-CD4 interaction, coreceptor binding or the fusion process (Figure 1.10).

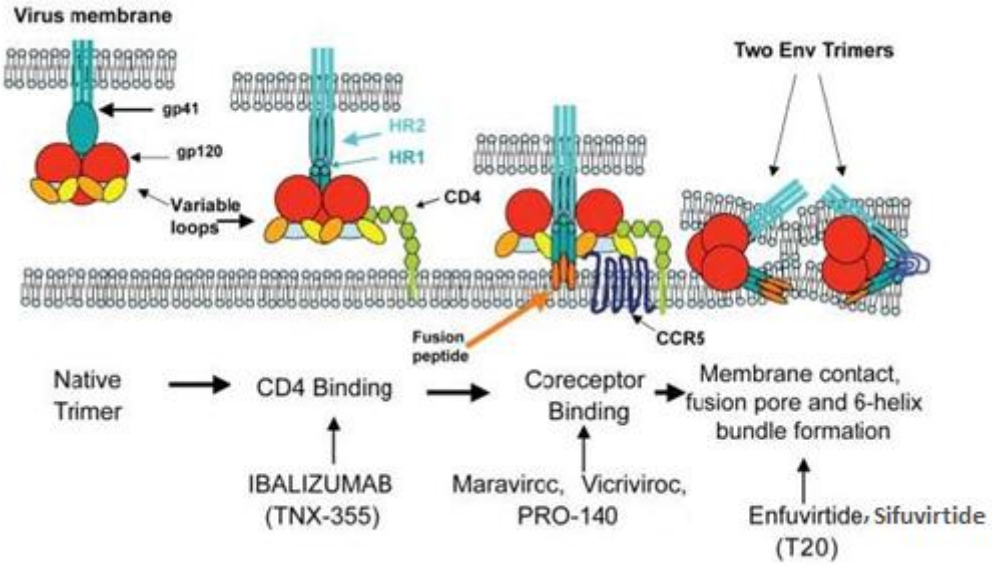


Figure 1.10 - Simplified schematic representation of the current model for the fusion mechanism between HIV-1 and the target cell, and its inhibition by entry inhibitors. (Adapted with permission from Biomed Central, Copyright (2009)). [211]

1.4.1 Inhibitors of gp120-CD4 interaction

Several strategies have been pursued in order to block the interaction between gp120 and CD4. So far none has resulted in a clinically useful anti-HIV drug. One of the first strategies was the development of recombinant soluble CD4 (rsCD4) molecules, which function as molecular decoys inhibiting the ability of gp120 to attach to cell-associated CD4. Despite good activity *in vitro* against lab-adapted HIV-1 strains, *in vivo* the levels of recombinant sCD4 were too low to inhibit primary isolates.[78,316] Other attempts were made, using variations that mimic the CD4 receptor, like a tetravalent CD4-IgG2 fusion protein, comprising human IgG2 in which the Fv portions of both heavy and light chains have been replaced by the V1 and V2 domains of human CD4 (PRO-542).[7,363] PRO-542 presented only modest reductions in HIV-1 viremia in phase I and II clinical trials and it was not persued.[180]

Another entry inhibitor, BMS-488043[105,180,204,281,315] (a follow-up molecule from BMS-378806 with improved pharmacokinetic properties) is a small-molecule that binds with great affinity to a specific region within the CD4 binding pocket of gp120 and seems to prevent the CD4-induced conformational changes in gp120.[194,200] It has strong antiviral activity against HIV-1 subtype B but is less active against other subtypes and inactive against HIV-2 [124,200] and the development of this molecule stopped at phase II trials.

Ibalizumab (TNX-355) is an anti-CD4 monoclonal antibody that binds to the D2 domain of CD4.[217] It acts as a post-attachment inhibitor such that instead of preventing gp120-CD4 binding it seems to decrease the flexibility of CD4 and affects gp120 dissociation.[180]

1.4.2 CCR5 antagonists

CCR5 antagonists' development has been actively pursued, since the Δ32-CCR5 mutation confers resistance to HIV-1 infection in homozygous individuals (or delayed rates of disease progression in heterozygous patients) without significant clinical impact. This has encouraged different approaches of pharmacological blockade of the gp120-CCR5 interaction in an effort to inhibit HIV infection.[316] CCR5 antagonists can be divided in three groups according to the size of the molecule: large molecules, such as the PRO-140, an anti-CCR5 monoclonal antibody; medium size molecules, e.g. AOP-RANTES and PSC-RANTES, derivatives of RANTES, a CCR5 natural ligand; and small-molecules, like TAK-779, Maraviroc and Vicriviroc.[40,316] PRO-140 is a strong inhibitor of HIV-1 B and non-B subtypes and is currently on phase II clinical trials and will be further discussed in Section 1.6.[40,105]

Vicriviroc (SCH-D) is an orally bioavailable second-generation compound (based in a previous molecule, SCH-C) highly active against a large spectrum of HIV-1 primary isolates, that made it's way to phase III clinical trials. However, Vicriviroc didn't achieve the primary efficacy endpoint of these studies and further development of Vicriviroc was suspended.[105,309,316]

Of all these, Maraviroc is the only coreceptor antagonist approved for clinical use in HIV infection. Maraviroc acts as a functional antagonist of CCR5. It inhibits the binding of the CCR5 natural ligands (like, MIP-1α, MIP-1β and RANTES), blocks chemokine-induced signal transducing events and once bound to CCR5 it does not trigger the release of intracellular calcium and fails to induce CCR5 internalization.[96] Maraviroc interacts with residues lining a cavity formed by the by the 2, 3, 6 and 7 transmembrane helices of CCR5 ECLs.[57] Maraviroc is only active against viruses using exclusively the CCR5 coreceptor, which limits its therapeutic usefulness. [96]

1.4.3 CXCR4 antagonists

The development of CXCR4 inhibitors has proceeded more slowly than that of the CCR5 antagonists. Probably because, unlike for CCR5, there are no known naturally occurring mutations that lead to the absence of CXCR4. So, while there are numerous CCR5 antagonists with very different structures, the number of CXCR4 antagonists available is more reduced and their structure is similar to AMD3100, one of the first small molecules of this group to enter in clinical trials.[105,134,296] Despite strong activity against X4 strains *in vitro*, the clinical development of AMD3100 was discontinued due to cardiac abnormalities.[133,134] Subsequent studies resulted in the identification of AMD070, a compound orally bioavailable with similar antiviral activity to AM3100.[307]

However, this molecule is no longer being developed due to results of abnormal liver histology in preclinical studies. Toxicities of these two compounds have raised the concern of the long-term safety of targeting CXCR4, since this coreceptor seems to be important for multiple physiological processes.[181,316] Like SDF-1, the CXCR4 natural ligand, these antagonists are positively charged and of basic nature. They bind strongly to the negatively charged surface of the coreceptor, hampering its interaction with the gp120 glycoprotein.[97] Like for CCR5 antagonists, the blockade of CXCR4 results in the shift from X4 to R5 phenotype in HIV-1 primary isolates.[103]

1.4.4 Entry inhibitors

Enfuvirtide (T-20) was the first entry inhibitor to be used in antiretroviral therapy and currently no other entry inhibitor has entered the market. It is a 36 amino acid peptide that binds to the HR-1 region of the ectodomain of gp41, preventing the transition to a conformation critical for the fusion process, the trimer of harpins conformation.[277,313] C34 (34 amino acids) is a linear peptide that mimics the HR2 sequence and inhibits virus entry by binding to the HR1 core, exposed at prehairpin intermediate state of gp41, thereby blocking the subsequent formation of the six-helix bundle structure and viral fusion.[99,216] Both T-20 and C34 inhibit virus-cell fusion and cell-to-cell contact at the nanomolar range.[61,346,347] Although C34 displays stronger antiviral activity than T-20, the poor solubility of C34 under physiological conditions hindered its potential as a drug candidate.[52]

Unlike, T-20 and C34, second generation peptides were developed based on the consensus sequences of not only HIV-1, but also HIV-2 and SIV strains. T-1249 is a representative second generation peptide with 39 amino acids that contains the three regions: the pocket-binding domain (PBD), the HR core (3HR) and the lipid-binding domain (LBD) and has potent activity against both HIV-1 and HIV-2.[102] However, the clinical development of T-1249 was halted after phase I/II clinical trials apparently due to formulation issues, probably due to the production cost and reduced delivery efficiency.[205]

Sifuvirtide, a peptide based in C34, is a good example of the third generation entry inhibitors.[131] Sifuvirtide is now under development and showed promising results in phase II clinical studies being active against a broad range of HIV-1 isolates, including T-20- resistant strains. Although Sifuvirtide shows a better pharmacokinetic profile than T-20, it is still administered as a subcutaneous injection. [131]

Enfuvirtide was seen as a therapeutic alternative, particularly for the treatment of patients experiencing treatment failure, and despite the high cost of production was rapidly introduced in the market.[256,257,281,313]Shortly after the beginning of its clinical use cases of resistance to Enfuvirtide were identified.[256,257] The main mechanism of resistance to Enfuvirtide is the appearance of mutations in the HR-1 region of gp41, particularly in a field of 10 amino acids located between residues 36 to

45.[212,256,257,277,365] Enfuvirtide also presents problems in terms of bioavailability, derivatives of their biophysical properties and removal of many existing mechanisms in the human body. It is susceptible to proteolysis, it is immunogenic, and its structure allows for it to connect strongly to hydrophobic surfaces, especially to cell membranes. These factors contribute to a significant decrease in the levels of Enfuvirtide available to inhibit HIV-1 entry into cells and are probably responsible for the rapid emergence of resistant viruses.[277] Despite the rapid emergence of enfuvirtide resistance, it continues to represent an important step in developing new therapeutic strategies and their use in synergy with other antiretrovirals in many cases continues to be an alternative therapy, which emphasizes the need for development of new entry inhibitors.

1.5 Antibodies

1.5.1 The immune system and the origin of antibodies

Antibodies are produced by B lymphocytes during a humoral immune response and play a key role in the immune system. The primary function of the antibody is to bind to the antigen. This link may in some cases have a direct effect of a bacterial toxin to inactivate or prevent the entry of a virus in a cell. On the other hand, the antibody lacks the ability to kill or eliminate an antigen, so it needs to use additional features.

These additional features include effector functions, such as activating the complement system or interaction with a variety of cells such as macrophages and auxiliary T lymphocytes.[174,177] Due to its specific binding to the antigen (an antibody is able to distinguish between two proteins that differ in only one amino acid), high affinity and also due to the fact that these are highly stable molecules and easily manipulated by genetic engineering, antibodies have been used in recent decades in various therapeutic and diagnostic applications.[172,175] The ability to generate human antibodies that are not accessible by conventional monoclonal or polyclonal approaches has led to the development of antibody engineering. The selection of synthetic antibodies using techniques such as phage display or ribosome display led to the discovery of molecules which have a high potential for use as an alternative to conventional pharmacological therapy.[39,123,126,127,142,172]

1.5.2 Structure and genetic organization of the antibodies

1.5.2.1 Antibody Structure

Antibodies or immunoglobulins are composed of one or more monomer units. Each monomeric structure consists of a constant region (Fc – Fragment crystallizable) which determines the effector function and two antigen-binding fragments (Fab - Fragment antigen binding) that include the variable region (Fv) which determines the specificity (Figure 1.11).[39,177]

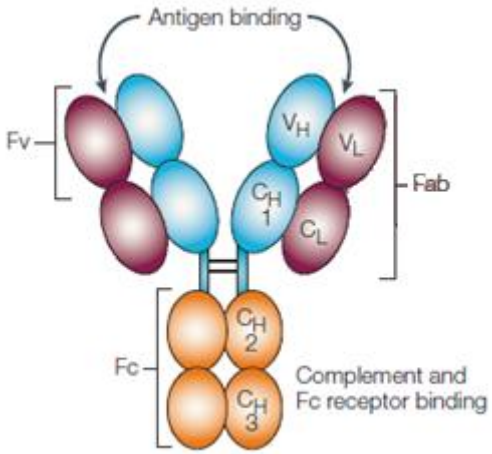


Figure 1.11- Modular structure of immunoglobulins. (Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Drug Discovery, copyright (2003)).[39]

Each monomer unit has, in turn, a common structure of four peptide chains. This structure is composed of two light chains (L) with an identical molecular weight of approximately 25 kDa and two heavy chains (H) with an identical molecular weight of 50 to 70 kDa (Figure 1.12).[177]

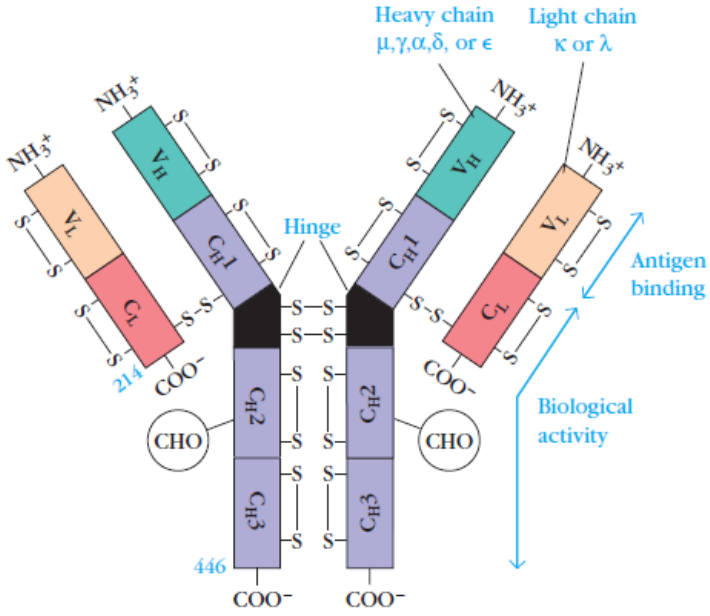


Figure 1.12- Schematic diagram of the structure of immunoglobulin G.
The sequencing of immunoglobulins has shown that these sequences are composed of about 110 amino acids, called domains, and these amino acid sequences are either highly conserved (C domains) while others are variable (V domains). (Reprinted with permission from: W.H. Freeman and Company, copyright (2006)).[177]

Through the analysis of conserved sequences it could be verified that there are two types of light chains (L), designated κ and λ, and there are five different types of heavy chains (H), γ, μ, α, δ and ε.[39,177]

The light chains are composed of an amino-terminal variable domain (V_L) and a constant domain (C_L). On the other hand, the heavy chains are composed of an amino-terminal variable domain (V_H) and three constant domains (C_H1, C_H2 and C_H3).[130] The variable domains of light chains and heavy chains form the antigen binding region (Fab). Despite being designated variable domains, they do not have a homogeneous variability, since within the variable domains there are three segments called complementarity determining regions (CDR) 1, 2 and 3, which are highly variable, giving a greater degree of definition to the diversity and specificity of the antibody binding. The CDRs are in turn located between four very conserved segments, termed framework region (FR).

The Fc region is distinct for each class of immunoglobulins, each composed of a class of different types of heavy chain: γ (IgG), μ (IgM), α (IgA), δ (IgD) and ε (IgE). The Fc region is glycosylated and contains sites of interaction with effector molecules, such as the C1 complex of complement and a variety of Fc receptors, including FcRn, as well as molecules that determine the biodistribution of the antibody.[39,177] The heavy chains are linked covalently in the Hinge region and light chains are in turn linked to the heavy chains by covalent bonds, also established by disulfide bridges and non-covalent bonds, such as hydrogen bonds.[177] Each form, or isotype, of light chain can combine with any heavy chain isotype. However, in the same immunoglobulin molecule there is only one isotype either for the light chains, or for the heavy chains. Thus, each light chain is associated with the amino-terminal region of the heavy chain and form a unity Fab-Fc The unit consists of the carboxy-terminal halves of both heavy chains.[38]

1.5.3 Antibodies - Mechanisms of *in vivo* action

The therapeutic methods based on the use of antibodies, aim to remove or neutralize a pathogenic infection or the pathogenic agent itself, for example bacterial, viral, or the case of oncological targets.[39]

The antibodies used for therapeutic purposes have three principal modes of action:[39]

- blocking the action of specific molecules;
- recognizing specific molecules as targets;
- functioning as signaling molecules.

The blocking activity of antibodies used for therapeutic purposes is achieved by preventing cytokines, growth factors or other cellular mediators to reach their target receptors. This blockage can be achieved through antibody binding to the target molecule or its receptor.[39]

Target recognition involves the targeting of antibodies to specific populations of cells. The antibodies can be genetically modified to carry specific segments of effector molecules such as enzymes, toxins, radionuclides, cytokines or even DNA molecules, to the target cells where the effector molecule can exert its function (eg, radionuclides can selectively eliminate cancer cells).[39]

The effector functions of natural antibodies are associated with binding to the Fc receptor or binding to complement proteins and induction of complement-dependent cytotoxicity (CDC).[39] Depending on the therapeutic strategy used, antibodies designed to recognize specific targets may or may not maintain their effector functions.[39,138,160]

The signaling effect of antibodies is attributed to the induction of cross-linking of receptors that are in turn linked to mediators of cell division or programmed cell death, or targeting them to specific receptors in order to act as antagonists for the activation of specific cell populations.[39]

Another approach is the use of antibodies as means to deliver DNA or to deliver antigens to certain immune cells that will process them and will present the antigens to T cells, in order to activate a specific immune response against that antigen.[39,160]

1.5.4 Production of polyclonal, monoclonal and recombinant antibodies

Due to the potential use of antibodies as a low cytotoxicity, highly specific targeted therapy, various methods have been developed to obtain and produce antibodies with predefined specificities.[39]

Most antigens have multiple epitopes which means that they induce the proliferation and differentiation of various B cell clones, each originating from a B cell that recognizes a particular epitope. That is, the resulting serum is composed of a mixture of antibodies, each specific to an epitope - a policlonal serum.[177]

A polyclonal antibody response speeds up the target location, phagocytosis and complement-mediated lysis of antigen, and increases the protection that is generated by the immune system *in vivo*.[38,39]

For most *in vitro* uses, whether for diagnostic, therapeutics or research, it is more advantageous to use monoclonal antibodies derived from a single clone and specific to only one epitope.[38,39]

In 1975 Georges Kohler and Cesar Milstein developed a method of preparing monoclonal antibodies through the fusion of a normal activated antibody producing B cell with a myeloid cell, which gave rise to a hybrid cell called a hybridoma, which had the growth properties of myeloid cells (immortalization) and excreted the antibody produced by the B cell. This method called mouse hybridoma technology was an important step in antibody technology and led to the emergence of monoclonal antibodies for therapeutic use.[39,177]

Monoclonal antibodies from mice have proved to be of limited use for therapeutic purposes due to low residence times in serum, inability to activate human effector functions and production of human anti-mouse antibodies (HAMA response).[39,340] In order to try to reduce the immunogenicity of mouse antibodies, genetic engineering was used, taking into account the existing knowledge on the structure of antibodies, to produce chimeric antibodies with human Fc regions and mouse Fv regions.[39,340] While chimeric antibodies did not generate such an exacerbated immune response as mouse monoclonal antibodies, there continued to be a human anti-chimeric response (HACAs),

so it was still necessary to minimize the mouse component present in the antibodies.[39,160] With the mapping of the complementarity determining regions (CDR), the mouse component could be restricted solely to the CDRs responsible for binding to the antigen. This was achieved by grafting the mouse CDRs into the human variable domain framework.[39,160]

The ability to manipulate the antibodies in order to maximize the human component while maintaining the properties of binding to the antigen provenient from other species, allowed for the clinical use of antibodies. The isolation of genes encoding human variable regions and their expression in *Escherichia coli* led to the emergence of techniques such as phage display technology which is still the most widely used method for selecting and producing antibodies from fully human variable domains.[29,39,207,299]

1.5.5 Recombinant antibody fragments

Recombinant antibodies that have been used in phage display technology, or other recombinant antibodies technologies, can be Fab (fragment antigen binding), scFv (single-chain variable fragment) or dAbs (single domain antibody). They have many advantages compared to immunoglobulins since:[172]

• there is no binding / activation of the Fc receptor, which reduces its immunogenicity;

• the rate of tissue penetration is higher;

• Fab, scFv or dAbs antibodies can be produced quickly in prokaryotes, including in *E. coli*.

The Fab segments consist of V_H-C_H and V_L-C_L linked by disulfide bridges, whereas scFv are composed only by V_L and V_H regions joined by a polypeptide linker, usually with a sequence of 15 amino acids (GGGGSGGGGSGGGGS) whose function is to increase the efficiency of folding and expression.[6,19,100,207]

A dAb is either the variable domain of an antibody heavy chain (V_H domain)[339] or the variable domain of an antibody light chain (V_L domain)[248]. Each dAb therefore contains three of the six naturally occurring complementarity determining regions (CDRs) from

an antibody. Although it might seem surprising that three CDR regions are sufficient to confer antigen-binding specificity and high affinity, evolution itself has arrived at that very same solution in camelids[125] but also in sharks[94,308], which produce antibodies comprising only a heavy chain (see Figure 1.13). The antigen binding site of these antibodies consists of a single unpaired variable domain (designated VHH for camelid and VNAR for shark).[138]

Currently, almost all FDA approved therapeutic antibodies (except ReoPro, Lucentis and Cimzia which are Fabs), and the vast majority of those in clinical trials are full-size antibodies mostly in IgG1 format of about 150 kDa size.[90,91]

A fundamental problem for IgG format antibodies is their poor penetration into tissues (e.g., solid tumors) and poor or absent binding to regions on the surface of some molecules (e.g., on the HIV envelope glycoprotein) which due to structural features are fully accessible only by molecules of smaller size. Therefore, in recent years much work has been aimed at developing novel antibody scaffolds of much smaller size and high stability[90,138]

From the recombinant antibody scaffolds available the single chain (scFv) and the single domain (VHH, V_H, and VNAR) formats are the most widespread for both research and industrial applications but their full potential is still to be reached.[168,343]

After camelid VHH and shark VNAR domains began to be studied, it was found that both display long surface loops, often larger than for conventional murine and human antibodies. VNAR and VHH domains have the ability to penetrate cavities in large antigens and bind to cryptic (immune-evasive) antigens. Unlike mouse V_H domains, camelid VHH and shark VNAR are in general soluble. This lead to the development and increased research of dAbs, either naturally occurring like camelid VHH and shark VNAR but also dAbs obtained through recombinant antibody technology from conventional antibodies (see Section 1.5.5.1). [6,11,138]

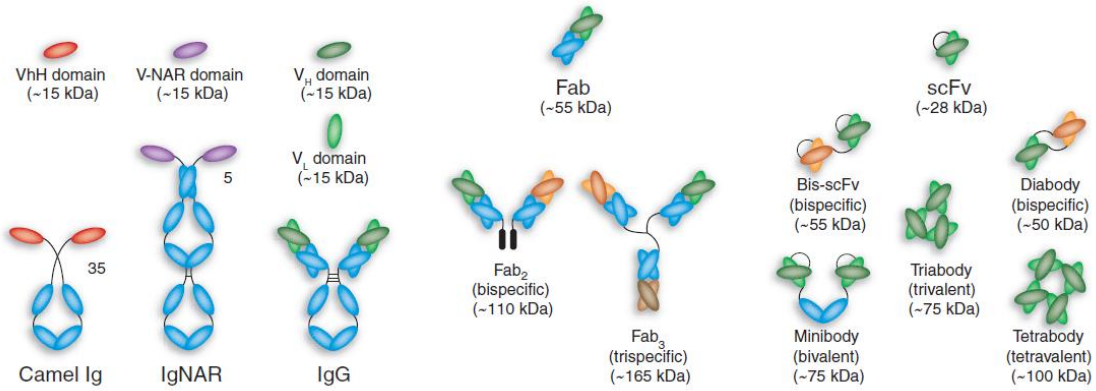


Figure 1.13 – Schematic representation of different antibody formats.
(Reprinted by permission from Macmillan Publishers Ltd: Nat. Biotechnol., copyright (2005)). [138]

The choice of recombinant antibody format depends crucially on the intended therapeutic use, although in some cases the choice is also based on avidity and stability issues.

1.5.5.1 Single domain antibodies

Most naturally occurring antibodies are composed of two heavy and two light chains. Both chains contribute to the antigen-binding site which is usually flat or concave. In addition to these conventional antibodies, llamas, other camelids, and sharks also produce antibodies composed only of heavy chains.[94,125,308] VHH and VNAR are easily produced as recombinant proteins, designated dAbs or nanobodies.[343]

The CDR3 region of these heavy chain antibodies (hcAbs) possesses the extraordinary capacity to form long fingerlike extensions that can extend into cavities on antigens, e.g., the active site crevice of enzymes, like lysozyme.[83] The CDR3 of VHHs and VNARs is often much longer than that of conventional V_H domains, e.g., 24 and 18 residues in the case of anti-lysozyme dAbs versus 7 residues in the mouse V_H.[128,350] The extended CDR3 is often, but not always, stabilized by an additional disulfide bond connecting the CDR3 to the adjacent CDR1 loop (common in camel and shark dAbs) or to the CDR2. [343] The CDR3 of the heavy chains is the one that displays a higher degree of variability in naturally occurring antibodies[178] and because of that is often used for comparison of length variation between species. Furthermore, third complementarity

determining region of antibody heavy chains (CDRH3) are unique in defining the fine specificities of antibodies, in the sense that any given amino acid sequence of human CDRH3 correlates with a defined specificity, and this is also true for most non human sequences.[75,152,350]

For instances sequences of human CDRH3 vary from 2 to 28 amino acids residues, but vary less extensively in other species [75,350] As an example, mouse antibodies show CDRH3 loops ranging from 1–19 residues.[75] Common in camel and shark dAbs are CDRH3 loops with 20 or more aminoacids.[80,128]

The length of the CDRs is important because it has been correlated with the type of antigens that they recognize, for instances longer CDRH3 loops forming planar or concave surfaces tend to recognize large antigens, while shorter loops favor small antigens; i.e. a long CDRH3 implies a flat (or protruding) antigen combining site, while short loops favor pockets or grooves. Haptens are bound by a cavity between the CDR loops, and peptides and DNA antigens by groove–like structures.[75,231]

Regarding length distributions in all species for the light chains, we have for the first complementarity determining region of antibody light chains (CDRL1) long loops (16 and 17 residues) that are two to three times more abundant in peptide binders than in other classes. For CDRL3 longer loops are seen almost exclusively in viral binders.[75]

The dAbs have a variety of uses, ranging from simple research tools as diagnostic reagents to highly refined biopharmaceutical drugs.[138] They can be readily cloned into various formats by fusion to other proteins or peptides, thereby tailoring their utility for certain diagnostic and/or therapeutic applications. For example, fusion to a fluorescent protein yields a fluorescent probe (also designated chromobody or fluobody) suitable for tracking the target antigen in cells.[234,280] Tandem cloning of two identical dAbs connected by a linker peptide yields a bivalent reagent with higher avidity for the antigen, amongst many other applications.[284,343]

The dAbs are usually generated by PCR cloning of the V-domain repertoire from blood, lymph node, or spleen cDNA obtained from immunized animals into a phage display vector, such as pComb3X.[19] Antigen-specific dAbs are commonly selected by panning phage libraries on immobilized antigen,[14,142] e.g., antigen coated onto the plastic surface

of an ELISA plate, biotinylated antigens immobilized on Streptavidin beads, or membrane proteins expressed on the surface of cells. Several laboratories have also constructed semi-synthetic libraries by cassette-mutagenesis of the CDR regions. The latter offers the advantage of selecting antibodies against toxic or difficult to express antigens. However, dAbs derived from such non-immune libraries often show lower affinities for their antigen than dAbs derived from animals that have received several immunizations.[128,141,252] The high affinity of dAbs from immune libraries is attributed to the natural selection of variant dAbs during clonal expansion of B-cells in the lymphoid organs of the immunized animals. The affinity of dAbs from non-immune libraries can often be improved by mimicking this strategy in vitro, i.e., by site directed mutagenesis of the CDR regions and further rounds of panning on immobilized antigen under conditions of increased stringency.[14,123,160,207]

Recombinantly expressed dAbs display several advantages as compared to conventional antibodies and to the scFv derived from the variable-domains of conventional antibodies. Their high thermal stability, high refolding capacity, and good tissue penetration in vivo make dAbs ideally suited for various biotechnological and therapeutic applications (as previously mentioned). Moreover, dAbs can be readily cloned into various formats by fusion to other proteins or peptides, thereby tailoring their utility for certain diagnostic and/or therapeutic applications inaccessible to conventional antibody formats.[138,284]

Recombinant antibody scaffolds are based on various human and non-human molecules of high stability.[33,138,166] Of those the dAbs are one of the most promising as therapeutic molecules.[140,284] Firstly, the size (12–15 kDa) of the dAbs is about an order of magnitude smaller than the size of an IgG1 (about 150 kDa) and the small size leads to relatively good penetration into tissues and the ability to bind into cavities or active sites of protein targets which may not be accessible to full size antibodies.[80,138] This could be particularly important for the development of therapeutics against rapidly mutating viruses, e.g., HIV. Because these viruses have evolved in humans to escape naturally occurring antibodies of large size, some of their surface regions which are critical for the viral life cycle may be vulnerable for targeting by molecules of smaller size including dAbs.

Another important feature of the dAbs is that they can be relatively easy engineered to further increase their stability. For example, some dAbs with increased stability could be taken orally or delivered via the pulmonary route or may even penetrate the blood-brain barrier, and retain activity even after being subjected to harsh conditions, such as freeze-drying or heat denaturation. In addition, dAbs are typically monomeric, of high solubility and do not significantly aggregate or can be engineered to reduce aggregation. Their half-life in the circulation can be relatively easy to adjust from minutes or hours to weeks. The dAbs for therapeutical applications are in most cases human molecules, but can also be humanized molecules, which decreases the likelihood of undesirable immune responses. [67,81,128,139,343]

Regarding production issues, unlike conventional antibodies, domain antibodies are well expressed in bacterial, yeast and mammalian cell systems. Finally, the small size of dAbs allows for higher molar quantities per gram of product, which should provide a significant increase in potency per dose and reduction in overall manufacturing cost.[34,85,141,170]

1.6 Antibody response to HIV-1

The antibody response to HIV-1 *in vivo* is directed against several viral proteins. However, essentially all neutralizing antibodies are directed toward the viral envelope spike, in particular the surface glycoprotein gp120,[245,356] which is anchored to the viral surface by gp41.[356] Until recently only four broadly neutralizing antibodies had been identified (Figure 1.14), B12, and 2G12 that target gp120 and 4E10 and 2F5 that target the membrane-proximal external region (MPER) of gp41. In 2007 another broadly neutralizing antibody that targets the gp41 (Z13e1)[226] had been identified. Between 2009 and 2010 four new broadly neutralizing antibodies that target gp120 (PG9, PG16, VRC01 and HJ16) [76,334,351] were identified and in 2011 17 new broadly neutralizing antibodies were also described that target gp120.[333]

In the context of antibody response to HIV-1, it is also worth mentioning the CD4 receptor targeted antibodies, in particular Ibalizumab, which is not naturally occurring but shows great promise for therapeutic use.[217,218]

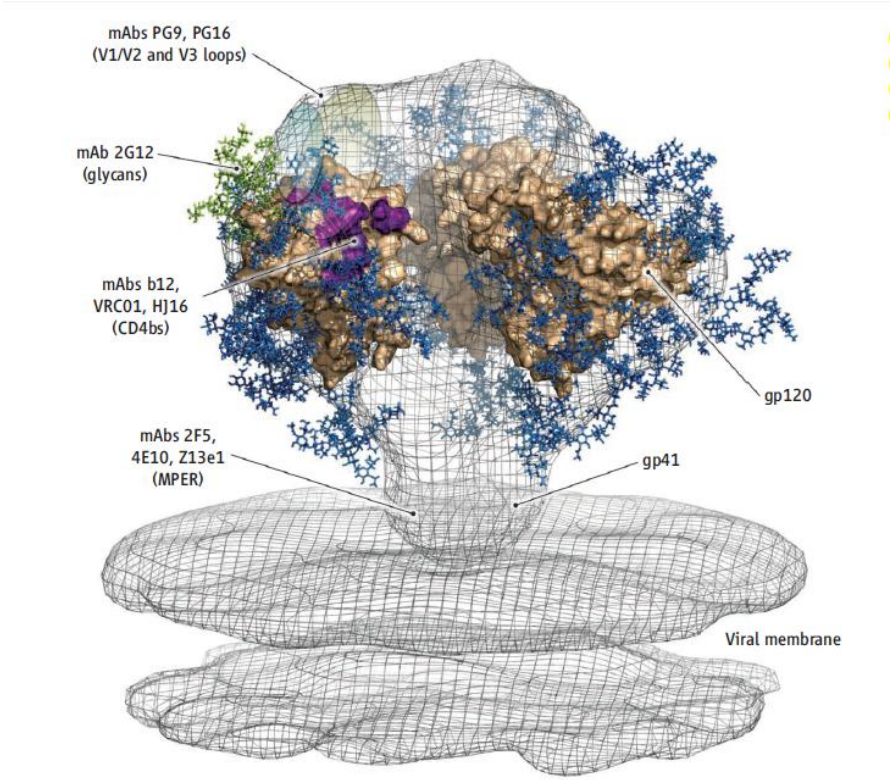


Figure 1.14 – Tridimensional model of gp120. Location of ENV neutralizing monoclonal antibodies is depicted.
The model is derived from [292] with permission from Wolters Kluwer Health–Lippincott Williams and Wilkins; from [332] with permission from Elsevier; and from [195] with permission from Macmillan Publishers Ltd./Nature Publishing Group.[49]

1.6.1 Gp120 CD4bs Antibodies: the b12, VRC01 and HJ16 antibodies.

The b12-IgG1, is a recombinant neutralizing antibody derived from a phage-display library that recognizes the CD4-binding site (CD4bs) on gp120.[289]

The antibody b12, which binds to an epitope that overlaps the CD4bs on gp120,[17,275] has been studied extensively with regard to its antiviral activity both *in vitro* and *in vivo*. In a comprehensive analysis involving a panel of 90 viruses, the antibody reached a 50% inhibitory concentration (IC_{50}) with approximately half of the viruses tested.[32] Although gp120 is the primary target of neutralizing antibodies elicited during natural infection[23], most gp120-reactive antibodies are ineffective at neutralizing primary HIV-1 isolates.[362] As such, b12 was until recently the most potently and broadly neutralizing anti-gp120 antibody. *In vivo* studies using macaque models of HIV infection have shown that b12 can also protect animals against viral challenge.[76,246,328,333,334,351]

To gain insight into how b12 is able to neutralize broadly, its structure was determined and, at the time, docked onto the CD4-liganded structure of gp120.[287]

The crystal structure of b12 was originally solved as an intact human IgG1 molecule.[287] The most prominent feature of the antibody combining site is a heavy-chain complementarity-determining region 3 (CDRH3) that extends directly out from the surface of the antibody like a finger (Figure 1.15). Computerdocking and mutagenesis studies on gp120 and b12 have been used to argue that the finger probes the recessed CD4 binding site of gp120.[243,287,367] The CDRH3 is 18 aa long, which is exceptionally long, compared with those typically found in well studied mouse mAbs to protein antigens (average CDRH3 length of about 10 residues). It also is relatively long, compared with the average in anti-protein human antibody, reported to be about 13 residues.[74] This unusually long CDRH3 is responsible for binding to the recessed CD4 binding site on gp120.[362]

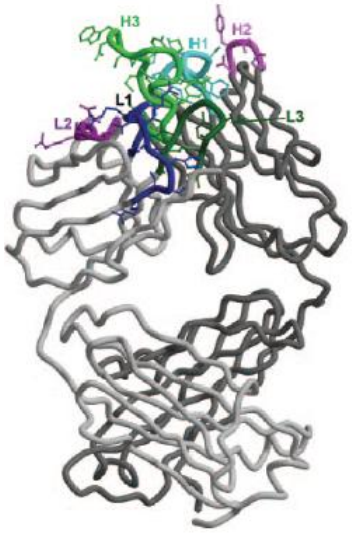


Figure 1.15 - The structure of the b12 monoclonal antibody.
The structure of Fab b12 as described it has been is shown with the CDRs presented in colors and labeled as belonging to the heavy (H) or light (L) chain. The long HCDR3 (H3) is clearly visible as an extended finger-like structure. (Reprinted with permission, Copyright (2005) National Academy of Sciences, USA).[48]

More recently, the b12 binding epitope on gp120 was identified and it was found that b12 binds a conformationally invariant surface that overlaps a distinct subset of the CD4 binding site.[362] This b12 binding epitope is constitutively exposed at the gp120 outer domain and is involved in the metastable attachment of CD4, before the gp120 rearrangement required for stable engagement (see Figure 1.16).[362]

Notably, only the heavy chain of b12 interacted with gp120, with each of the three heavy-chain CDRs making extensive contact. Three b12 residues (Asn 31, Tyr 53 and Trp 100) from each of the heavy-chain CDRs are depicted in stick representation. Together, these three residues combine to form 40% of the b12 contact surface. They can be seen gripping the CD4-binding loop, the central focus of the b12 interaction with gp120 (Figure 1.16). Although the heavy-chain-only usage is quite unusual, the surface areas of interaction were in the range typical for antibody–protein interfaces correlating with the strong binding ability of b12.[275,312,362] Antibody heavy-chain-only interactions are rare, although heavy chain interactions predominate in a number of viral Env–antibody complexes, including those from SARS coronavirus and influenza virus haemagglutinin.[107,258 362]

When comparing the CD4 cell receptor and b12 interaction with the CD4-binding loop, one finds that these interactions are quite different. The b12 antibody uses all three of its CDR heavy-chain loops to grasp virtually all surface-exposed portions of the loop. In contrast, CD4 only binds to one side of the loop, making anti-parallel hydrogen bonds between CD4- and gp120-main-chain atoms. [362]

The b12 antibody takes advantage of the backbone reactivity of the outer domain of gp120, forming six direct and four water mediated hydrogen bonds with the main-chain atoms of two parallel loops—the CD4-binding loop and the outer domain exit loop. [362]

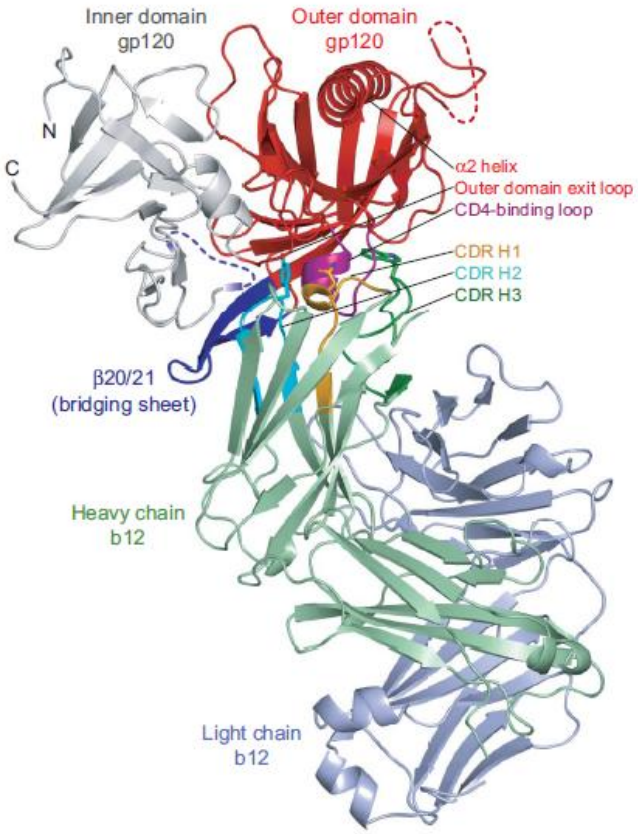


Figure 1.16 - Structure of b12 in complex with an HIV-1 gp120 core.
Polypeptide chains are depicted in ribbon representation, with disordered regions as dashed lines. The gp120 inner domain is grey, and the outer domain is red, except for the CD4-binding loop, which is purple. The strands and associated loops, which in the CD4-bound conformation correspond to the bridging sheet, are blue. The b12 light chain is blue–grey and the b12 heavy chain is green, with associated CDRs highlighted in orange (H1), cyan (H2) and dark green (H3). (Reprinted by permission from Macmillan Publishers Ltd: Nature, copyright (2007)). [362]

Due to the unusual characteristic of the b12 heavy chain only interaction with gp120, there was some concern was that b12 might be a unique broadly neutralizing antibody to the CD4bs, and the CD4bs thus an almost intractable vaccine target. [49]

Although the mAbs described to date, are able to recognize monomeric gp120, most of them cannot do so in the context of gp120 organized in Env spikes on the viral surface. It appears that from the antibodies described until 2010 only b12 is able to gain access to the CD4 binding site on the Env spike, indicating a strong selection pressures on HIV to conceal this crucial and conserved site from antibody recognition.[109,275,290] The recent identification of several broadly neutralizing antibodies to the CD4bs from different HIV-infected individuals[76,351] lays the concerns regarding the lack of broadly neutralizing antibodies against the CD4bs to rest and promotes interest in designing immunogens to elicit antibodies to the CD4bs.[49]

In 2010 two new broadly neutralizing antibodies that target the CD4bs were identified, the HJ16 and the VR01 antibodies. The HJ16 recognizes a novel epitope proximal to the CD4 binding site on gp120 and selectively neutralized a multi-clade panel of Tier-2 HIV-1 pseudoviruses, and demonstrated reactivity that was comparable in breadth, but distinct in neutralization specificity, to that of the b12 antibody.[76] The VRC01, was identified in a screening where a panel of broadly neutralizing sera was evaluated for the presence of antibodies that could preferentially bind to the CD4bs of gp120. This antibody was found to neutralize ~90% of nearly 200 viruses of different HIV clades tested.[351]

More recently, in 2011 a study where the neutralizing antibody repertoires of four HIV infected donors were probed with remarkably broad and potent neutralizing responses and 17 new monoclonal antibodies that neutralize broadly across clades were rescued. Many of the newly identified PGT monoclonal antibodies are almost tenfold more potent than the recently described PG9, PG16 (see Section 1.6.2) and VRC01 broadly neutralizing monoclonal antibodies and 100-fold more potent than the original prototype HIV broadly neutralizing monoclonal antibodies. The epitopes recognized by the PGT monoclonal antibodies were identified by competition ELISA assays. Most of the epitopes recognized by the PGT monoclonal antibodies were in proximity to or contiguous with V3, and the epitopes recognized by the remaining PGT antibodies bind the gp120 polypeptide backbone (gp120 glycan shield). [333]

1.6.2 Gp120 V2/V3 Antibodies: the PG9/PG16 antibodies.

The PG9 and PG16 are antibodies with exceptional neutralizing breadth and potency that were identified through a large-scale direct functional screen of B cells from a donor with a high broadly neutralizing antibody response.[334]

These antibodies were found to recognize residues that are primarily located in conserved parts of V2 and V3 loops of gp120 with broad isolate reactivity.[334] The PG9 and PG16 are somatic variants and seem to recognize the same or overlapping epitopes. Like for the CD4bs broadly neutralizing antibodies, PG9 and PG16 do not recognize monomeric gp120, and in fact it was demonstrated that the epitope or overlapping epitopes targeted by these antibodies is preferentially expressed on trimeric HIV Env.[49,334]

1.6.3 Antibodies against gp120 glycans: 2G12

2G12 is an antibody that recognizes glycans on the surface of gp120. It recognizes the dense carbohydrate shield that protects the protein from antibody recognition.[48,242]

It has been shown that 2G12 does indeed recognize glycans exclusively and a number of oligomannose candidates were identified.[291] 2G12 has the unique ability to recognize the oligomannose structure on the gp120 surface, because this antibody assembles into a previously uncharacterized structure in which the two Fabs of the IgG assemble into an interlocked V_H domain-swapped dimer – a dimeric configuration. The extraordinary configuration of this antibody provides an extended surface consisting of two classical binding sites that is responsible for its broad reactivity.[48,291] In fact, 2G12 has potent *in vitro* and *in vivo* efficacy, but unfortunately it is unable to neutralize viruses from clade C.[32,318,320]

1.6.4 MPER antibodies : 2F5, 4E10 and Z13e1

The 2F5 and 4E10 are antibodies that recognize the membrane-proximal external region (MPER) of gp41. Although most of the surface of gp41 appears to be occluded from antibody binding on Env spikes, a region close to the viral membrane, the MPER,[366] has some accessibility to the neutralizing human mAbs 2F5 and 4E10.[223,224,366]

Current models suggest that MPER access is achieved after receptor engagement and during formation of the putative transitional fusion intermediate. It has also been suggested that the transitional intermediate may be required to fully form the MPER neutralizing epitopes into the structurally defined "antibody bound" conformations.[60,366]

Some evidence has emerged that the epitopes of these two mAbs are accessible, and possibly more so, after CD4 binding to gp120.[31] Both mAbs appear to recognize linear gp41 epitopes in that they bind with relatively high affinity to short peptides corresponding to cognate gp41 sequences, and their neutralizing activity can be effectively inhibited by such peptides. 2F5 and 4E10 may bind directly to free virus or they may neutralize virus during the process of receptor-triggered entry.[60]

A third Ab, Fab Z13, had previously been mapped to an epitope that overlaps those of 2F5 and 4E10 but only weakly neutralizes a limited set of primary isolates. Because the epitope of Z13 at least partially overlaps with those of both 2F5 and 4E10, an attempt to improve its affinity for gp41 would possibly also improve its neutralization potency.[366]

Random mutations were introduced into the CDR L3 of Fab Z13 in a phage display library,[19,123,172] and high-affinity variants of Z13 were selected. From that a new high-affinity Fab, Z13e1, was identified. Although its binding site overlaps with those of both 2F5 and 4E10, inspite of its increased potency when compared to the original Z13 Fab, the Z13e1 has only moderate neutralization potency when compared to either 2F5 or 4E10. Those remain to this date the more potent broadly neutralizing antibodies targeting gp41.[226]

1.6.5 CD4 receptor antibodies: Ibalizumab

Ibalizumab, also known as TNX-355, is a non-immunosuppressive, humanized IgG4, monoclonal antibody that blocks HIV-1 entry by binding to domain 2 of human CD4.[40,88,148]

Ibalizumab was engineered from its mouse progenitor (5A8) by grafting the mouse complementary-determining region (CDR) onto a human IgG4 construct.[35,42] The IgG4 isotype was chosen to minimize the chances for CD4 T-cell depletion by antibody- and complement-dependent cytotoxicity, mediated by binding to Fc receptors. Ibalizumab or 5A8 block CD4-dependent virus entry and inhibit a broad spectrum of both laboratory-adapted and clinical HIV-1 isolates, including CCR5-tropic and CXCR4-tropic strains from multiple subtypes.[301]

Ibalizumab is currently on phase III clinical trials.

1.6.6 CD4-Induced (CD4i) Antibodies

CD4i antibodies recognize the coreceptor binding site on gp120. Similarly to the trimeric Env, the coreceptor site on monomeric gp120 is revealed by CD4 ligation, when it is also then recognized by a set of CD4i antibodies.

In a stunning demonstration of convergent evolution, a number of CD4i antibodies have acquired post-translational modifications in the form of sulfated tyrosines on their CDRH3 regions; these tyrosines, in some cases, are important in gp120 binding.[65] The CD4i antibodies fall into two classes, those with long CDRH3s and those with short CDRH3s. Only for a subgroup of the long CDRH3 antibodies does tyrosine sulfation contribute to antigen binding. Another remarkable feature of CD4i antibodies is the fact that their neutralizing activity, as antibody fragments, has an inverse relationship between molecular size and neutralization, i.e., single-chain Fv > Fab > IgG in neutralizing potency. Thus, it appears that during infection, the virus exposes the coreceptor site sufficiently to allow access by CCR5 and antibody fragments but not by the physiologically relevant IgG molecule.[48,320]

1.7 Vaccines

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent (antigen) that is composed of fragments or by the totality of a disease-causing microorganism, and is often made from weakened or killed forms of the microorganism or its toxins. The antigen stimulates the body's immune system to recognize it as foreign, by stimulating antibody production or cellular immunity against the pathogen but being “designed” to be incapable of causing severe infection.

Vaccines can be prophylactic (e.g. to prevent or ameliorate the effects of a future infection by any natural or "wild" pathogen), or therapeutic (e.g. vaccines against cancer).

In the case of the AIDS vaccine several approaches have been attempted over the years, focusing mainly on developing a safe prophylactic strategy as discussed in section 1.7.1

1.7.1 AIDS vaccine: Present status and future possibilities

Although intensive anti-retroviral therapy has been able to slow or halt expansion of the epidemic in some industrialized countries, these treatments are poorly accessible in developing countries. Development of a preventive vaccine for HIV is the best hope of controlling the AIDS pandemic worldwide. HIV has, however, proved a difficult pathogen to vaccinate against. This is largely because HIV has a very high mutation rate and can escape immune responses. Recent discoveries in HIV entry and pathogenesis, and promising studies of candidate vaccines in animal models have provided reasons to hope that developing a safe and effective AIDS vaccine is possible.[228]

The first Phase I trial of an HIV vaccine was conducted in the USA in 1987. Since then, more than 30 candidate vaccines have been tested in over 80 Phase I/II clinical trials, involving more than 10 000 healthy human volunteers.[118,158,345]

Initially the efforts on developing an HIV-1 vaccine were focused on antibody responses elicited by subunit protein (subunit vaccines),[27] but were later switched to T-cell responses elicited by a range of viral proteins.[44,189,210]

Early studies reported preliminary data that HIV-specific $CD4^+$ T helper cell responses were more common in HIV-1 infected individuals who controlled viremia in the absence of ART, and that ART given during acute HIV-1 infection could restore HIV-1-specific $CD4^+$ T cell proliferative responses.[279] These data documented the importance of $CD4^+$ helper T cells in controlling HIV-infection and also suggested that HIV-specific T helper responses would be desirable in a vaccine.[203,206,240,278]

Studies of SIV in animal experiments and studies in humans suggest that CTLs are important for control of viral replication, in both acute and chronic disease, suggesting that cellular immunity impacts HIV-1 infection and is likely to play a major role in clinical outcome.[9,79,122,206]

Due to the complexity of the interaction between HIV-1 and the human immune system, in order to achive immune protection, multiple vaccine concepts are being explored in parallel.

1.7.1.1 Live Attenuated Vaccines

The finding that nef-deleted mutants of SIV could elicit protection against challenge with pathogenic SIV in rhesus macaques triggered the development of a live attenuated HIV vaccine approach.[79] Further research showed that the SIV Δnef mutants, however, establish a lifelong, persistent low grade infection that does not protect the vaccinated monkeys against superinfection with wild-type virus, and also is pathogenic in most vaccinated adult monkeys, given enough time.[136,143] In addition, the attenuated virus still may cause AIDS when administered orally to infant monkeys.[136] Further attempts to introduce additional deletions or mutations can continue to attenuate the virus but at the expense of its protective efficacy. Mostly because of safety concerns, this approach was therefore discontinued.[137]

1.7.1.2 Subunit Vaccines

A subunit HIV vaccine was developed based on monomeric gp120 added with aluminium (VaxGen). Two different studies were conducted; in one of them the vaccine used a mixture of two subtype B gp120s as the immunogen, and in the other using a mixture of a subtype E (CRF_AE) and a subtype B gp120s (AIDSVAX® gp120 B/E). None of these studies showed a statistically significant reduction of HIV infection and showed no efficacy in Phase III trials.[158,208]

The same subtype E/B gp120 vaccine was used for booster immunizations in a prime-boost Phase III trial which was launched in late 2003 in Thailand and uses for priming a recombinant canarypox virus (ALVAC) that expresses CRF_AE gp120 and subtype B Gag, Pol and Nef antigens (ALVAC-AIDSVAX B/E) this vaccine is also designated RV144.[15,158] The RV144 combination treatment's efficacy ranged from 26.1 to 31.4%, which although far from optimal still constitutes a positive result.[271]

Other approaches aimed at eliciting HIV neutralizing antibodies based on the Env proteins, have been develop over the years, initially these were based on monomeric gp160.[24,92] These Env subunit vaccines were shown to induce neutralizing antibodies that were mostly directed at the hypervariable V3 loop in gp120 and were able to protect chimpanzees against challenge with a homologous or near-homologous HIV-1 strain, but not against challenge with a distant virus strain.[28,117]

Since the first attempts to develop an Env subunit vaccine, several other approaches have been develop, these have included:

- trimeric gp140 molecules (gp120 + the ectodomain of gp41) with a deletion of the hypervariable V2 loop in order to expose the neutralization epitopes overlapping the CD4-binding site;[20,302,303]

- oligomeric gp140 molecules covalently coupled to synthetic mimics of the CD4 receptor that should expose neutralization epitopes overlapping the coreceptor (CCR5 or CXCR4)-binding site;[84,110]

- gp120/gp41 trimers internally stabilized by disulfide bond formation (SOS proteins) which should elicit both neutralizing and fusion-blocking antibodies.[22]

Another strategy that seems promising is the development of subunit vaccines based on the accessory protein Tat, in the hope that immune responses directed at antigens that are expressed very early in the virus replication cycle might lead to containment of infection. And also because anti-Tat antibodies have been correlated with reduced viremia and slow progression to AIDS.[265,359] Tat is believed to contribute to viral pathogenesis and immunosuppression by inducing apoptosis in uninfected lymphocytes.[118,191]

The native Tat protein was tested in a Phase I clinical study and shown to be well tolerated and immunogenic in both HIV naive and HIV-infected individuals and is currently in a Phase II clinical trial.[23,101] A Tat–Nef fusion protein that was combined with a gp120 subunit vaccine was found to prevent the development of disease in rhesus macaques following challenge with SHIV89.6P.[331]

The same Tat–Nef fusion protein/gp120 combination was tested in Phase I trials[119] and shown to induce strong and persistant $CD4^+$ T-cell responses in human volunteers when combined with various adjuvants.[188] HIV Tat vaccines might therefore show some promise specially when combined with other HIV antigens.[55,56]

1.7.1.3 Live Recombinant Vaccines

Rather than attempting to elicit a protective antibody response, recent HIV vaccine approaches have aimed to induce a T-cell response, especially a $CD8^+$ CTL response, whose role in control of virus load and evolution of disease has been well documented in the monkey model.[5,209] $CD8^+$ T-cell secrete antiviral cytokines (IFN-γ), virus entry-blocking β-chemokines, and other factors that have been correlated with protection against SIV infection in the monkey model, as well as associated to asymptomatic HIV-1 infection in humans and slower disease protection in HIV-2-infected patients.[118]

Vaccines that stimulate the T-cell immune response are however not expected to protect against infection, but rather to control its course and reduce viral loads, in an attempt to prevent or at least delay the occurrence of symptoms.[118]

Several prime-boost strategies involving priming with a DNA vaccine followed by boosting with a live recombinant vector-based vaccine have been tested in monkeys against challenge with a lethal dose of simian–human immunodeficiency virus (SHIV) that causes AIDS-like illness in the animals. These strategies resulted in reduction in virus load but failed in protection against SIV challenge.[129,182]

A number of recombinant vaccines also have been tested in Phase I/II trials in humans, including plasmid DNA and poxvirus vectors (MVA, fowlpox or canarypox viruses) expressing a variety of HIV antigens, such as Gag, Env, Pol and Nef. [25,147,251,314]

Replication-defective adenovirus type 5 (Ad5) was the base for another HIV-1 vaccine, a recombinant Ad5-gag/pol/nef vaccine (Merck). Phase I clinical trial of Ad5 recombinants induced cell-mediated immune responses against HIV Gag peptides in most healthy adult volunteers with baseline Ad5 antibody titers <200, but the response was dampened in those volunteers with titers >200.[261] The vaccine reached Phase II trials but in individuals devoid of pre-existing Ad5 neutralizing antibody, an equal number of HIV-1 infections was recorded in the vaccinated and placebo groups. In volunteers with pre-existing immunity, however, the number of HIV-1 infections in the vaccinated group was significantly higher than in the placebo group. This led to the interruption of the trial and the cessation of all Ad5-based vaccine trials.[73,199,297]

In general, T-cell vaccines for HIV-1 have not elicited the same levels of responses in humans as they elicited in nonhuman primates. Also a variety of other vectors have been used.[118]

1.7.1.4 Other Vaccinal Approaches

Other vaccinal approaches are under development like the induction of persistent HIV Gag-specific $CD8^+$ CTL responses, multi-epitopic combinations of peptides, fusion proteins and long lipopeptides also are at an early stage of clinical development, either alone or in prime-boost combinations with live vector-based recombinant vaccines. Lipopeptides whose sequence corresponds to that of CTL epitopes-rich regions in the Gag and Nef viral proteins have undergone Phase II trials in the USA and in France (NIAID/ANRS) and many others.[118]

Another important aspect to be aware of for the development of a successful AIDS vaccine is the type of vaccine formulation. A critical goal of HIV vaccination is the induction of mucosal humoral immune responses. Since nanoparticles are an important tool for antigen delivery and subsequent induction of cellular and humoral immune responses, nanoparticle encapsulation can be a crucial step for the development of AIDS vaccines (see Chapter 5).[12,135,156,358]

1.8 Aims

Given the characteristics and nature of HIV infection, the ability to establish reservoirs in various cell lines, progressive and irreversible destruction of the immune system, associated with an increased prevalence of strains resistant to drug therapy, one of the priority areas in the development of new drugs has been the development of new molecules which prevent viral entry into cells, including peptides and antibodies that prevent the entry process.

The objective of the work developed and presented in this Thesis was to evaluate both the b12 target sequence and the CD4 binding epitope outside of the gp120 context and to explore their potential for CD4 targeting in different therapeutic applications.

For that we used a novel approach. Here we used the knowledge of a recent discovery that identified in gp120 a conformationally invariant surface that overlaps a distinct subset of the CD4-binding site, which is the binding site for the b12 natural antibody. We wanted to use both gp120 sequences, the CD4 binding epitope and the b12 target sequence, to construct single domain antibodies for independent epitope assessment and to evaluate their ability to bind to the hCD4 receptor.

The strategy developed involved grafting of the b12 target sequence or CD4 binding epitope into either CDR1 or CDR3 of a highly stable rabbit single domain V_L antibody. This grafting process involved the selection of the more stable constructs, their expression and development of high yield purification protocols.

After validating that these constructs can bind specifically to the hCD4 receptor we wanted to further explore their potential in three different perspectives:

1) as HIV-1 entry inhibitors;

2) as a new therapeutic delivery system using dAb coated nanoparticles;

3) and V_LB12 in particular, as a vaccine antigen.

One of the most innovative characteristics of this work is the potential of obtaining a uniquely versatile molecule, the V_LB12 construct and the ability to distinguish two independent regions for binding to the CD4 cell receptor within the gp120 CD4bs region each binding to a different CD4 domain.

The V_LB12 construct has the potential of simultaneously being used as a therapeutic molecule that inhibits HIV-1 infection by binding to the CD4 cell receptor and preventing HIV from entering the target cell. Furthermore, the V_LB12 construct also has the potential to be used as a vaccine antigen, since this b12 epitope is the target of a natural broadly neutralizing antibody.

The V_LCD4 construct also shows potential for being used as an entry inhibitor molecule that inhibits HIV-1 infection by binding to the CD4 cell receptor, but since this gp120 is subjected to constant conformational masking its usefulness may be limited.

Furthermore, the use of gp120 sequences and simply grafting those into an antibody and in that manner develop a strategy to prevent the virus from entering the cell, is ironic at best. Successfully using a piece of the virus to fight the virus itself maybe one of Nature's ironies.

A new approach for CD4 targeting – grafted single domain antibodies

2 A new approach for CD4 targeting – grafted single domain antibodies

2.1 Introduction

Although gp120 is the primary target of neutralizing antibodies elicited during natural infection,[262] most gp120-reactive antibodies are ineffective at neutralizing primary HIV-1 isolates. Only two gp120-reactive antibodies (b12, 2G12) with effective neutralization activity against diverse primary HIV-1 isolates had been identified until 2009 (see Section 1.6).[46,47,319,320,356]

A recent discovery has identified in gp120 a conformationally invariant surface that overlaps a distinct subset of the CD4-binding site, which is a binding site for the b12 natural antibody. [362] This surface is constitutively exposed at the gp120 outer domain and is involved in the metastable attachment of CD4, before the gp120 rearrangement required for stable engagement.[362]

The contact surfaces of CD4 and b12 on gp120 have considerable overlap (see Figure 2.1). However, b12 is able to latch onto this outer domain surface with high affinity, without additional gp120 conformational changes. The b12 binding site remains accessible to b12 binding since it is not subject to the gp120 conformational changes that occur in most of the outer domain (conformational masking), this allows b12 to bind and neutralize primary isolates. In this manner, b12 uses the functionally conserved initial contact site for CD4 on gp120 to effectively neutralize HIV-1. [362]

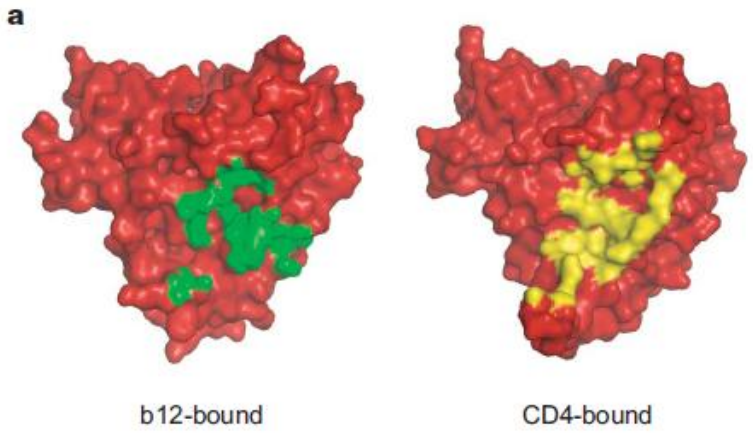


Figure 2.1 – Model of the contact surfaces of CD4 and b12 on gp120.
Molecular surface of gp120 in red, with the b12- contact surface in green (left) and the CD4-contact surface in yellow (right).(Reprinted by permission from Macmillan Publishers Ltd: Nature, copyright (2007)).[362]

The identification of the gp120 epitope targeted by the b12 antibody and the fact that this epitope partially overlaps the CD4-binding loop has prompted us to graft this gp120 epitopes, the CD4 and the b12 binding epitopes, into the CDRs of a highly stable V_L domain antibody (Figure 2.2).[121]

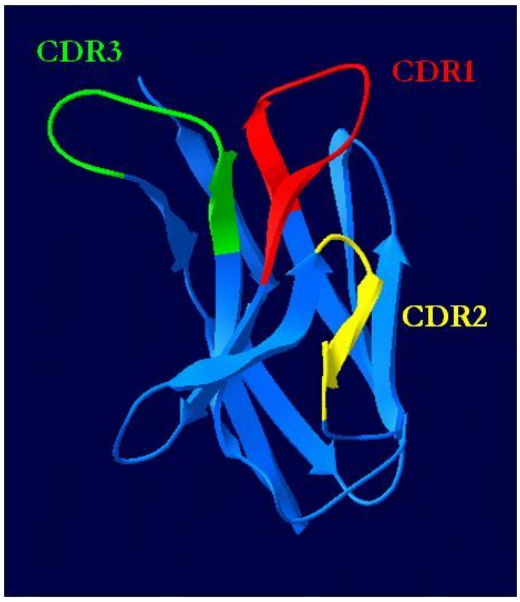


Figure 2.2 – Predicted structure of the single domain V_L antibody.
The model for this structure was performed by us (J Gonçalves Laboratory). The single domain V_L antibody is derived from the 4BL anti-Vif single chain antibody. [121]

In this Chapter we wanted to evaluate the ability of both the CD4 binding epitope and the b12 target sequence to be stably grafted in the CDRs of a single domain V_L antibody in order to explore the potential of both gp120 epitopes individually, outside the whole gp120 context.

The reports available on the literature indicate that the gp120 conformation resulting of the binding to the CD4 receptor or the binding to the b12 antibody result in a gp120 conformation where both contact sequences present considerable spatial overlap as demonstrated on Figure 2.1. This prompted us to design the V_L grafting system in order to obtain stable protein expression while maintaining the functionality of both gp120 target sequences. In order to evaluate the functionality of both gp120 target sequences the recognition of the human CD4 receptor and the characterization of the target epitopes also needed to be evaluated.

2.2 Materials and methods

2.2.1 Construction of recombinant single domain antibodies, dAbs

The recombinant antibodies developed in this work, were constructed through grafting of the b12 target sequence or CD4 binding epitope (Table 2.1) into either CDR1 or CDR3 of a highly stable single domain V_L antibody.

Table 2.1– Coding sequences used for CDR grafting of gp120 epitopes.

Sequence	Coding sequence
B12 target sequence	T R D G G N S N N E S E I F R P G G G D M R D
CD4 binding epitope	S S G G D P E I V T

The original CDR1 or CDR3 sequences of the V_L antibody were removed, and the gp120 b12 target epitope or CD4 binding epitope were inserted in their places, using an overlap PCR. The amplification product was subsequently cloned into the pComb3X vector using the Sfi I restriction enzyme.[19] All the constructs were confirmed by DNA sequencing.

Table 2.2 - Primers used to clone the b12 target sequence, the CD4 binding epitope sequence, or both sequences in the CDRs of the V_L antibody.

Primer name	Sequence (5' – 3')
RSC -F	GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC
VL-R	GGAGGACCAGGAGGAGCCTGNCCGGCCT
VL-CD4-CDR1-F	AGCAGTGGAGGCGACCCAGAAATAGTAACATGGTATCAGCAG
VL-CD4-CDR1-R	TCTGGGTCGCCTCCACTGCTGCAATTGATGGTGACTGTGCCC
VL-CD4-CDR3-F	AGCAGTGGAGGCGACCCAGAAATAGTAACATTTGCTTTCGGCGGAGGG
VL-CD4-CDR3-R	TCTGGGTCGCCTCCACTGCTACAGTAGTAAGTGGCAGCATCGGC
VL-OL-CDR1-F	ACAAGAGACGGAGGAAACAGCAACAACGAAAGCGAAATATTCAGACCA GGAGGCGGAGACATGCGCGACTGGTATCAGCAG
VL-OL-CDR1-R	GTTGCTGTTTCCTCCGTCTCTTGTGCAATTGATGGTGACTGTGCCC
VL-OL-CDR3-F	ACAAGAGACGGAGGAAACAGCAACAACGAAAGCGAAATATTCAGACCA GGAGGCGGAGACATGCGCGACTTTGCTTTCGGCGGAGGG
VL-OL-CDR3-R	GTTGCTGTTTCCTCCGTCTCTTGTACAGTAGTAAGTGGCAGCATCGGC

2.2.2 Cloning of the CD4 sequence in the CDR1 of the V_L antibody

For the cloning of the CD4 binding epitope sequence in the CDR1 of the V_L antibody, two rounds of PCR were performed. The first round of PCR was composed of two independent PCR amplifications (see Figure 2.3).

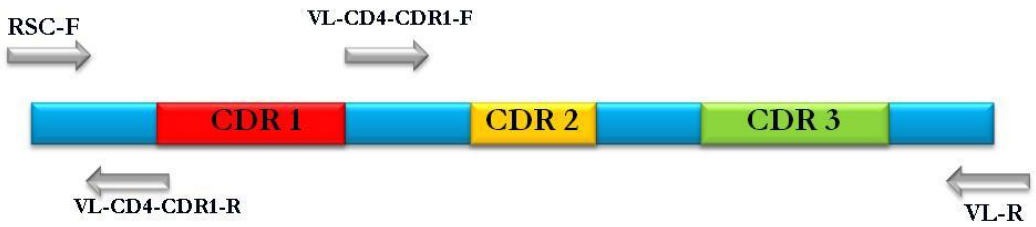


Figure 2.3 – 1st round of PCR for grafting of b12 target epitope or CD4 binding epitope.
PCR 1 is performed with primers RSC-F and VL-CD4-CDR1-R. This PCR amplifies the N-terminal region of the V_L antibody up to the beginning of CDR1 (not including any of the original CDR1 nucleotides). The Primer VL-CD4-CDR1-R includes a tail that codes for part of the CD4 binding epitope sequence. PCR 2 was performed with primers VL-CD4-CDR1-F and VL-R. The Primer VL-CD4-CDR1-F includes a tail that codes for the remaining nucleotides of the CD4 binding epitope sequence. Both CD4 epitope coding primers (VL-CD4-CDR1-F and VL-CD4-CDR1-R) possess overlapping sequences that allow for PCR1 and PCR2 amplification products to anneal on the 2nd round of PCR and serve as one "unified" template.

PCR1 amplified the N terminal part of the protein, up to the beginning of CDR1 (not including any of the original CDR1 nucleotides). PCR 1 was performed with primers RSC –F and VL-CD4-CDR1-R (see Table 2.2). The Primer VL-CD4-CDR1-R includes a tail that codes for part of the CD4 binding epitope sequence. PCR 2 amplified the C terminal part of the protein, from the end of CDR1 (not including any of the original CDR1 nucleotides). PCR 2 was performed with primers VL-CD4-CDR1-F and VL-R. The Primer VL-CD4-CDR1-F includes a tail that codes for the remaining nucleotides of the CD4 binding epitope sequence. Both CD4 epitope coding primers (VL-CD4-CDR1-F and VL-CD4-CDR1-R) possess overlapping sequences that allow for PCR1 and PCR2 amplification products to anneal on the 2nd round of PCR and serve as one "unified" template.

The second round of PCR, uses both of the amplified DNAs from PCR 1 and PCR2 as a unique template. The PCR is performed with the flanking primers, RSC-F and VL-R. The DNA of the original V_L antibody was used as the template for both PCR1 and

PCR2. All the PCRs were performed with the phusion DNA polymerase (Finnzymes, Finland).

2.2.3 Cloning of the CD4 sequence in the CDR3 of the V_L antibody

The protocol used for the cloning of the CD4 binding epitope sequence in the CDR3 of the V_L antibody is identical to the protocol described in 2.1.1. PCR1 was performed with primers RSC-F and VL-CD4-CDR3-R and PCR2 was performed with primers VL-CD4-CDR3-F and VL-R. The second round of PCR was identical to 2.2.2.

2.2.4 Cloning of the b12 sequence in the CDR1 of the V_L antibody

The protocol used for the cloning of the B12 binding epitope sequence in the CDR1 of the V_L antibody is identical to the protocol described in 2.2.2. PCR1 was performed with primers RSC-F and VL-OL-CDR1-R and PCR2 was performed with primers VL-OL-CDR1-F and VL-R. The second round of PCR was identical to 2.2.2.

2.2.5 Cloning of the b12 sequence in the CDR3 of the V_L antibody

The protocol used for the cloning of the B12 binding epitope sequence in the CDR3 of the V_L antibody is identical to the protocol described in 2.2.2. PCR1 was performed with primers RSC-F and VL-OL-CDR3-R and PCR2 was performed with primers VL-OL-CDR3-F and VL-R. The second round of PCR was identical to 2.2.2.

2.2.6 Cloning of the CD4 sequence in the CDR3 of the V_LB12(CDR1) antibody

The V_LB12(CDR1)-CD4(CDR3) was constructed using as template the V_LB12(CDR1) and the remaining cloning protocol was as described in 2.2.2.

All the V_LB12 or V_LCD4 constructs were cloned initially in the *Escherichia coli* (*E. coli)* expression vector pComb3X[19] and transformed in the *E. coli* Top10 strain (Invitrogen, UK).

2.2.7 Expression and purification of V_LB12 or V_LCD4 antibodies cloned in pComb3X

To express and purify selected V_LB12 or V_LCD4 antibodies, phagemid DNA was transformed into non-suppressor *E. coli* strain TOP10. The dAbs optimal expression conditions were determined by Western Blot analysis for all the constructs.

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 1 mM IPTG and growth was continued for either 6 or 16 hours, depending on the construct. After induction, bacteria were harvested by centrifugation (6,000 × *g*, 4°C, and 15 min), resuspended in 100 ml equilibration buffer (20 mM NaH_2PO_4, 500 mM NaCl, 30 mM imidazole (pH 7.4)), supplemented with protease inhibitors (Roche Applied Science, Mannheim Germany)), and lysed by sonication or French cell press. Cell debris were removed by centrifugation (6,000 × *g*, 4° C, 30 min), and the supernatant was filtered through a 0.45 μm syringe filter.

All chromatographic steps were performed at 4 ºC. dAb extracts were purified by nickel NTA affinity chromatography in a HisTrap HP column (GE Healthcare, UK) using the C-terminal His tag. Bound proteins were eluted with a linear imidazole gradient (0-500 mM imidazole in 20 mM NaH_2PO_4, 0.5 M NaCl (pH 7.4)). The appropriate fractions from affinity chromatography were pooled, and dialyzed against HEPES buffer (20 mM HEPES, 100 mM NaCl, 0,1 mM EDTA, 5 % glycerol (pH 7.4)) using Slide-A-Lyzer Dialysis Cassettes with a 3,5 K MWCO (Pierce, Rockford, USA) at 4ºC and according to the manufacturer's instructions.

2.2.8 Expression and purification of V_LB12 or V_LCD4 antibodies cloned in pET28a

To express high levels of V_LB12 or V_LCD4 antibodies (single domain antibodies, dAbs), the selected constructs were amplified by PCR using the primers listed on Table 2.3.

Table 2.3 - Primers used to amplify V_L B12 or V_L CD4 for cloning in the pET28a vector.

Primer name	Sequence (5' – 3')
Primer VL-F	CTC GCT AGC GAG CTC GTG CTG ACC CAG
Primer pComb3 Xho-R	CCG CTC GAG TCA TTA AGA AGC GTA GTC CGG AAC GTC

The amplification product was subsequently cloned into the pET28a vector Novagen, Germany) using the Nhe I and Xho I restriction enzymes.[19] All the constructs were confirmed by DNA sequencing.

Selected clones were transformed into the *E. coli* Tuner (DE3) strain (Novagen, Germany). The dAb optimal expression conditions have been previously determined for the V_L framework[286] and all the CDR grafting variations were expressed and purified in these conditions.

A fresh colony of each clone was grown at 37º C overnight in LB medium containing 100 μg/ml of ampicillin. A 10 ml sample of cells was used to inoculate 1 liter of LB medium containing 100 μg/ml of ampicillin. Cells were grown at 37º C until A_{600nm}= 0.4, induced by the addition of 0,2 mM IPTG and growth was continued for 4 hours.

After the 4 h expression, bacteria were harvested by centrifugation (10,000 × *g*, 4°C, and 15 min), resuspended in 40 ml buffer A(50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5mM $CaCl_2$ (pH 8,0)), and lysed by sonication for 20 min. Cell pellet/insoluble fraction was collected by centrifugation (10,000 × *g*, 4° C, 30 min), resuspended in 20 ml buffer A (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5mM $CaCl_2$ (pH 8,0)), and lysed by sonication for 10 min. Cell pellet/insoluble fraction was recollected by centrifugation (10,000 × *g*, 4° C, 20 min), resuspended in 20 ml buffer B (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5mM $CaCl_2$, 1mM β-mercaptoethanol,

2M urea (pH 8,0)), and lysed by sonication for 20 min. Cell pellet/insoluble fraction was once more recovered by centrifugation (10,000 × *g*, 4° C, 30 min) an resuspended in 25 ml buffer C (50 mM HEPES, 1 M NaCl, 10 mM imidazole, 5mM $CaCl_2$, 1mM β-mercaptoethanol, 6 M urea (pH 8,0)).

The protein pellet ressuspended in buffer C is subjected to a denaturation/solubilization step overnight at 4 ºC in a vertical rotator (stuart rotator, Dynalab). The solubilized protein (in buffer C) was submitted to centrifugation (12,000 × *g*, 4° C, 60 min) for removal of remaining cell debris and unsolubilized protein. The solubilized protein solution (dAb) was filtered through a 0,45 μm syringe filter.

The dAb was applied to a Ni-NTA His GraviTrapTM (GE Healthcare, New York, USA) and purified according to the manufacturer's instructions for purification under denaturing conditions. dAb was eluted in 2 x 3 ml buffer D (50 mM HEPES, 1 M NaCl, 500 mM imidazole, 5mM $CaCl_2$, 1mM β-mercaptoethanol, 6 M urea (pH 8,0)).

The dAb refolding/buffer exchange was performed in Disposable PD-10 Desalting Columns (GE Healthcare, UK) according to the manufacturer's instructions. The dAb protein was refolded in 2 x 7 ml buffer HEPES (20 mM HEPES, 100 mM NaCl, 0,1 mM EDTA, 5 % glycerol (pH 8,0)).

All the dAbs expression and purification conditions were confirmed by coomassie staining for all the constructs.

2.2.9 Western blot

Protein separation was performed according to the method of Laemmli in 12 % polyacrylamide gels (SDS-PAGE). Once separated, the proteins were transferred to onto nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK), blotted with anti-HA-HRP, High Affinity antibody (clone 3F10, Roche Applied Science, Mannheim Germany) and developed using the ECL (GE Healthcare, UK). Protein concentration was quantified by Bradford colorimetric assay (BioRad, CA, USA).

2.2.10 Coomassie staining

Protein separation was performed according to the method of Laemmli in 12 % polyacrylamide gels (SDS-PAGE). Following electrophoresis, the gel was placed in staining solution (40% methanol, 10% acetic acid, 0,025% Coomassie Brilliant Blue R-250[*]). The gel was incubated for 1 hour to overnight in the staining solution. The gel was destained with several changes of destain solution (30% methanol, 10% acetic acid) until the background is transparent. All staining destaining steps were done on a rotary shaker with gentle mixing.

2.2.11 Enzyme-linked immunosorbent assay (ELISA) to evaluate the dAbs binding to human CD4

Soluble human CD4 (obtained through the NIH AIDS Research and Reference Reagent Program, contributor: Progenics) or CD4-IgG2 (obtained through the NIH AIDS Research and Reference Reagent Program, contributor: Progenics) at 500 ng or 250 ng per well (in phosphate-buffered saline, PBS) was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, NY, USA). The plates were then blocked with 3 % bovine serum albumin (BSA) in PBS. The dAbs were diluted with 1% BSA in PBS. Amounts used ranged from 1500 ng - 100 ng per well. dAbs were added to the wells, and incubated for 1 h. The plates were washed with tween 20 (0.05 % in PBS) and incubated with horseradish peroxidase (HRP)-conjugated rat monoclonal anti-HA (Roche, Germany), the α-HA-HRP antibody was used at a 1:1000 dilution in 1% BSA (in PBS). All incubations were performed for 1 h at 37 ºC. The plates were then washed with PBS and developed with an HRP substrate, ABTS[†] solution (citric acid (pH 4.0) with 0,2 % H_2O_2 and read on an Infinite 200 Microplate Reader (Tecan i-control software, version , 1.5.14.0, Tecan, North Carolina, USA) at an optical density (OD) of 405/492 nm.

[*] Which has been filtered through Whatman #1 paper
[†] 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

2.2.12 ELISA assay to evaluate the dAbs binding to b12 mAb

The ELISA assay to evaluate the dAbs binding to b12 mAb, was performed using as antigen either V_LCD4 or V_LB12 at 500 ng per well (in phosphate-buffered saline, PBS), the antigen was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, NY, USA). The plates were then blocked with 3 % bovine serum albumin (BSA) in PBS. The b12 mAb (b12-IgG1, obtained through the NIH AIDS Research and Reference Reagent Program, contributor: Dr. Dennis Burton and Dr. Carlos Barbas III)[18,45,47,276] was diluted with 1% BSA in PBS. Amounts used ranged from 1000 ng - 50 ng per well. The b12 mAb was added to the wells, and incubated for 1 h. The plates were washed with tween 20 (0.05 % in PBS) and incubated with horseradish peroxidase (HRP)-conjugated goat anti-Human IgG (Santa Cruz Biotechnology, Santa Cruz, USA), the α-Human-HRP antibody was used at a 1:1000 dilution in 1% BSA (in PBS). All incubations were performed for 1 h at 37 ºC. The plates were then washed with PBS and developed with an HRP substrate, ABTS solution (citric acid (pH 4.0) with 0,2 % H_2O_2 and read on an Infinite 200 Microplate Reader (Tecan i-control software, version , 1.5.14.0, Tecan, North Carolina, USA) at an optical density (OD) of 405/492 nm.

2.2.13 ELISA assay for epitope characterization

For the epitope characterization ELISA assays were performed using as antigen, native conformation soluble hCD4 or denaturated soluble hCD4. Soluble human CD4 (obtained through the NIH AIDS Research and Reference Reagent Program, contributor: Progenics) was used at 500 ng per well (in phosphate-buffered saline, PBS). Denaturated soluble hCD4 was obtained by submitting the protein to heat denaturation for 10 minutes at 95 ºC. Either native conformation soluble hCD4 or denaturated soluble hCD4 was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, NY, USA). The plates were then blocked with 3 % bovine serum albumin (BSA) in PBS. 3 % BSA (in PBS) was also used and as negative control. The dAbs were diluted with 1% BSA in PBS. Amounts used ranged from 1500 ng - 100 ng per well. dAbs were added to the wells, and incubated for 1 h.

The plates were washed with tween 20 (0.05 % in PBS) and incubated with horseradish peroxidase (HRP)-conjugated rat monoclonal anti-HA (Roche, Germany), the α-HA-HRP antibody was used at a 1:1000 dilution in 1% BSA (in PBS). All incubations were performed for 1 h at 37 ºC. The plates were then washed with PBS and developed with an HRP substrate, ABTS solution (citric acid (pH 4.0) with 0,2 % H_2O_2 and read on an Infinite 200 Microplate Reader (Tecan i-control software, version , 1.5.14.0, Tecan, North Carolina, USA) at an optical density (OD) of 405/492 nm.

2.2.14 Production of HEK293T expressing mCD4, hCD4 or hCD4mD1

Human embryonic kidney 293T (HEK293T) (ATCC, VA, USA) cells were transfected, by calcium phosphate method, with either human CD4 (original plasmid pMX hCD4, Addgene, Cambridge MA, USA)[185] for the purpose of this work the hCD4 was cloned in pcDNA 3.1 (Invitrogen, UK), human CD4 with mouse Domain1 (hCD4mD1) (pEYFP-N1-hCD4mD1, a kind gift from Dr. Alexandra Trkola)[295] or mouse CD4 (mCD4) (pCMV-Sport6-mCD4, a kind gift from Dr. Alexandra Trkola).[295] Briefly, 240 000 HEK293T cells were transfected with one of the plasmids in order to produce HEK293T cells expressing hCD4, hCD4mD1 or mCD4. After 48 h, the transfected cells were stained (see 2.2.15.3) and submitted to the Flow Cytometry assay to identify the hCD4 domain recognized by VLB12 and VLCD4.

2.2.15 Flow cytometry analysis

2.2.15.1 V_LB12 (CDR1) specific binding to the CD4 receptor

For each assay condition, 200 000 cells, either HEK293T or HeLa-P4 (HeLa-CD4-LTR-β-gal, obtained through the NIH AIDS Research and Reference Reagent Program, MD, USA, contributor Dr. Richard Axel) were stained. Cells were detached from the flasks with cell dissociation buffer (Gibco, NY, USA), washed with PBS and incubated with either 10 nmol of V_LB12(CDR1) or 2,5 µl of fluorescein isothiocyanate (FITC) Mouse Anti-Human CD4 (clone RPA-T4; BD Pharmingen, San

Diego, CA) and diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with V_LB12(CDR1) were incubated with 2,5 µl anti-HA-FITC-conjugated (Clone BMG-3F10; Roche, Germany) diluted in 1% BSA (in PBS). Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS).

2.2.15.2 V_LB12 (CDR1), V_LB12 (CDR3) and VL CD4 (CDR3) binding to CD4 in Jurkat cells

For each assay condition, 200 000 Jurkat E6-1 T-cells obtained through the NIH AIDS Research and Reference Reagent Program (MD, USA, contributor Dr. Arthur Weiss). were stained. Cells were washed with PBS and incubated with different concentrations of either V_LB12 (CDR1), V_LB12 (CDR3), V_LCD4 (CDR3) or 2,5 µl of FITC Mouse Anti-Human CD4 and diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with the dAbs were incubated with 2,5 µl anti-HA-FITC diluted in 1% BSA (in PBS). Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS).

2.2.15.3 Flow cytometry assay to identify the hCD4 domain recognized by V_LB12 and V_LCD4

For each assay condition, 200 000 (HEK293T) transfected cells were stained. HEK293T cells were stained 48h after Calcium phosphate transfection of mouse CD4 (mCD4), human CD4 (hCD4) or human CD4 with mouse Domain1 (hCD4mD1). For each assay condition 200 000 transfected cells were washed with PBS and incubated with 10 nmol or 25 nmol of either V_LB12 or V_LCD4 and also with 2,5 µl of FITC Mouse Anti-Human CD4 (α-CD4-FITC), all diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with the dAbs were incubated with 2,5 µl anti-HA-FITC diluted in 1% BSA (in PBS). Cells were washed with PBS. All the cells stained with either V_LB12, V_LCD4 or α-CD4-FITC were again washed with PBS and stained with goat anti-mouse-DyLight 405 (1:5000). Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS).

All flow data were acquired on a BD Calibur (BD Bioscience, Franklin Lakes, NJ USA), 10 000 gated cells were acquired for each assay condition and data analysis was performed using Flowjo software 6.4.7 (BD Biosciences, Franklin Lakes, NJ USA).

2.2.16 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, 2007, San Diego, California, USA, www.graphpad.com) with a level of significance of 5%.

2.3 Results

2.3.1 Construction, expression and purification of the recombinant domain antibodies, dAbs

The recombinant antibodies developed in this work, were constructed through grafting of specific sequences into either CDR1 or CDR3 of a highly stable single domain V_L antibody. The single domain V_L antibody used as a framework in this Thesis was originally cloned in the pComb3X vector. This vector was originally designed for Phage Display and has an N terminal peptide leader sequence that targets the expressed proteins to the periplasm, and also a C terminal 6 His Tag followed by a Hemagglutinin Tag (HA Tag). The peptide leader sequence minimizes the formation of inclusion bodies and allows the recovery of soluble dAbs. The optimal expression conditions were determined by Western Blot for all the dAbs (Table 2.4).

Table 2.4 - Optimal expression conditions for the dAbs cloned in pComb3X. Protein expression was performed at 30 ºC with shaking at 220 rpm.

Protein (dAb)	Expression time (h)	Induction with IPTG (mM)	Cell lysis method
V_L CD4 (CDR1)	4	1,0	French cell press
V_L CD4 (CDR3)	6	0,6	Sonication
V_L B12 (CDR1)	16	1,0	French cell press
V_L B12 (CDR3)	6	1,0	French cell press
V_L B12 (CDR1)CD4(CDR3)	4	1,0	French cell press

From the expression assays it was possible to verify that for the CD4 binding epitope (hereafter designated CD4 loop) the antibody expression was much higher when the loop was placed in the CDR3 that in CDR1. In fact, from 12 positive clones tested for V_LCD4 (CDR1) all presented very low expression (in the soluble fraction) that was undetectable after 4 h, as opposed to V_LCD4 (CDR1) clones that presented much higher expression levels that peaked at 6 hours post induction.

It seemed that the CD4 loop could only be inserted in the CDR3 of the V_L antibody since almost no soluble protein could be recovered from V_LCD4 (CDR1). Either the

protein is insoluble or expression levels are very low. Therefore the only V_L CD4 (CDR3) was selected for further studies.

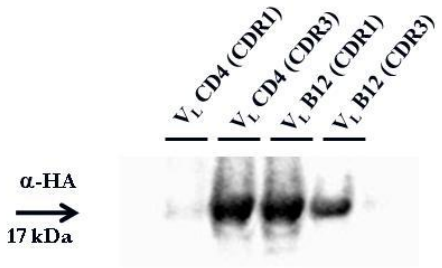


Figure 2.4 – Western blot analysis of the expression levels (soluble fraction) of the dAbs.
Equal amounts of protein were loaded onto the gel. The dAbs detection was performed with α-HA-HRP.

Regarding V_LB12, both constructs V_LB12 (CDR1) and V_LB12 (CDR3) presented fair expression rates in the soluble fraction, with the CDR1 construct only peeking expression 16 hours post induction and the CDR3 construct peeking expression at 6 hours post induction (see Figure 2.5).

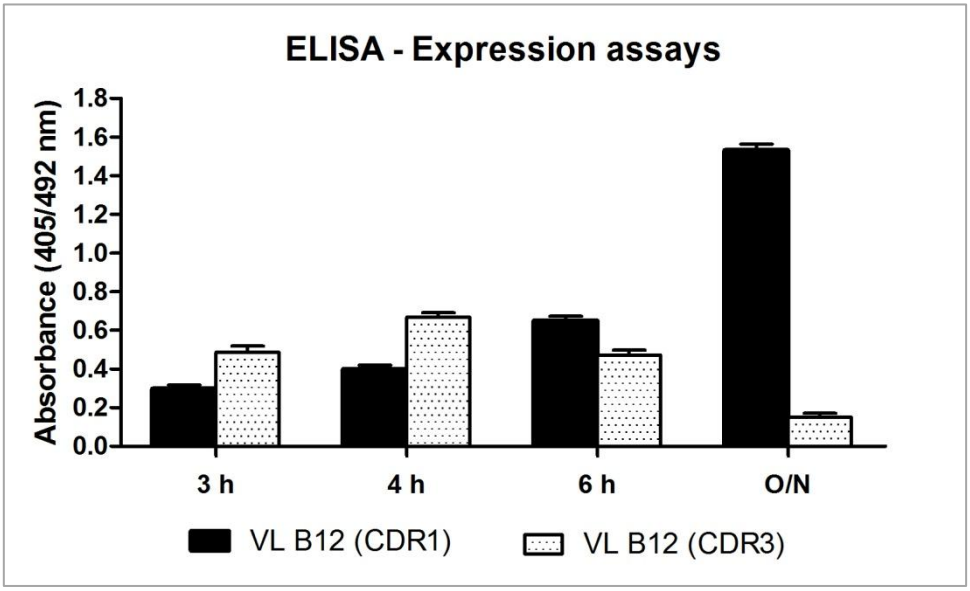


Figure 2.5 - The V_L B12 (CDR1) and V_L B12 (CDR3) protein expression assays were compared by ELISA assay.
Assay was performed using 500 ng per well of protein extract as antigen. 3 % BSA (in PBS) was used for blocking and as negative control. The antibody used for detection was α-HA-HRP (1: 1000) and the colorimetric substrate used was ABTS (0,2 % H_2O_2). Assay was performed in triplets.

After the protein expressions of the single CDR replaced loop were compared and due to the very low expression of soluble V_LCD4 (CDR1), only the V_LB12 (CDR1)CD4(CDR3) construct was developed. As for the V_LCD4 (CDR1) all the V_LB12 (CDR1)CD4(CDR3) clones tested (12) had very low soluble protein expression. This is an indicator that not all CDR combinations will be supported by the V_L framework and that the sequence present in the CDRs plays an important role in antibody stability/solubility and that this is not directly correlated to the size of the CDR sequence (see Table 2.5).

Table 2.5 – Comparison between the CDR1 and CDR3 sequences of the original V_L and the CD4 and b12 epitopes.

Single domain antibody	CDR1 sequence	CDR3 sequence
V_L (original)	**Q A S Q S V Y N N N N L A**	**Q G E F S C V G G D C**
V_LCD4 (CDR1)	**S S G G D P E I V T**	
V_LCD4 (CDR3)		**S S G G D P E I V T**
V_LB12 (CDR1)	**T R D G G N S N N E S E I F R P G G G D M R D**	
V_LB12 (CDR3)		**T R D G G N S N N E S E I F R P G G G D M R D**

After the optimization of the expression conditions it was necessary to purify the proteins. The proteins were purified by affinity chromatography (see Section 2.2.7) with a linear gradient of Imidazol and afterwards were submitted to buffer exchange. The results are shown on Table 2.6.

Table 2.6 - Results of the dAbs purifications per liter of bacteria culture.

dAb	Total amount of dAb	Purity (%)[‡]
V_LCD4 (CDR3)	386 μg	90
V_LB12 (CDR1)	315 μg	20
V_LB12 (CDR3)	92,8 μg	20

[‡] Purity was determined by densitometry using the Chemidoc software version 4.6 (Bio-Rad, USA)

The total amounts of purified protein are very low, in part because the pComb3X vector is not designed for high levels of protein expression; it does not possess a strong promoter like the T7 promoter. This system was designed for soluble expression of recombinant antibodies (mainly for antibody library screening)[100] and the C terminal 6 His tag is followed by an HA tag. This limits the access of the His tag to Ni^{2+} and greatly diminishes the amount of protein recovered from the affinity chromatography.

2.3.2 ELISA assays to evaluate the dAbs binding to human CD4

After purification it was necessary to verify if the constructs could bind to the human CD4 receptor. Preliminary binding assays were performed by ELISA (see 2.2.11) using as antigen soluble human CD4 (Full-length extracellular domain of human CD4). Results showed that both the V_LCD4 and the V_LB12 constructs could bind specifically to hCD4 (see Figure 2.6).

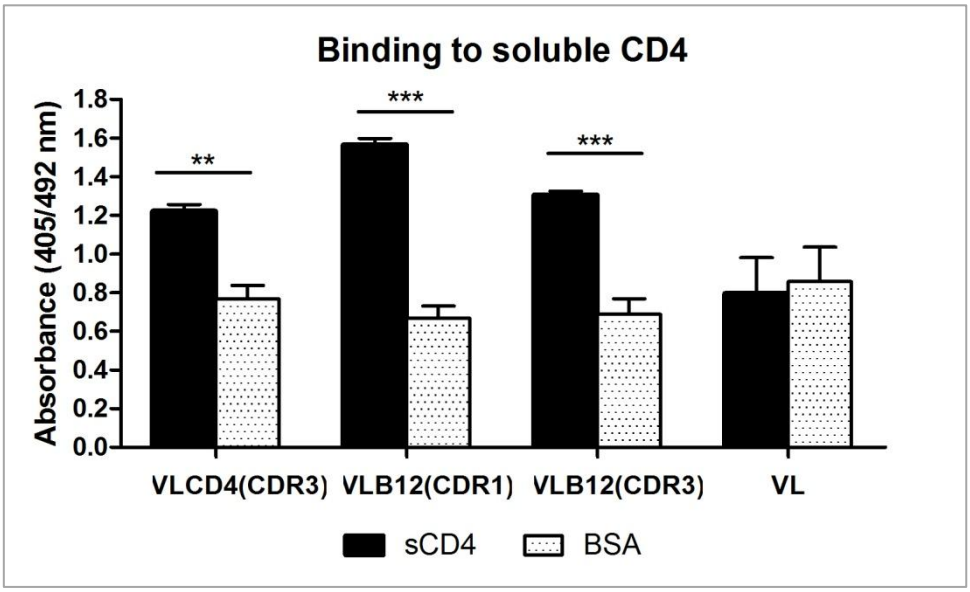


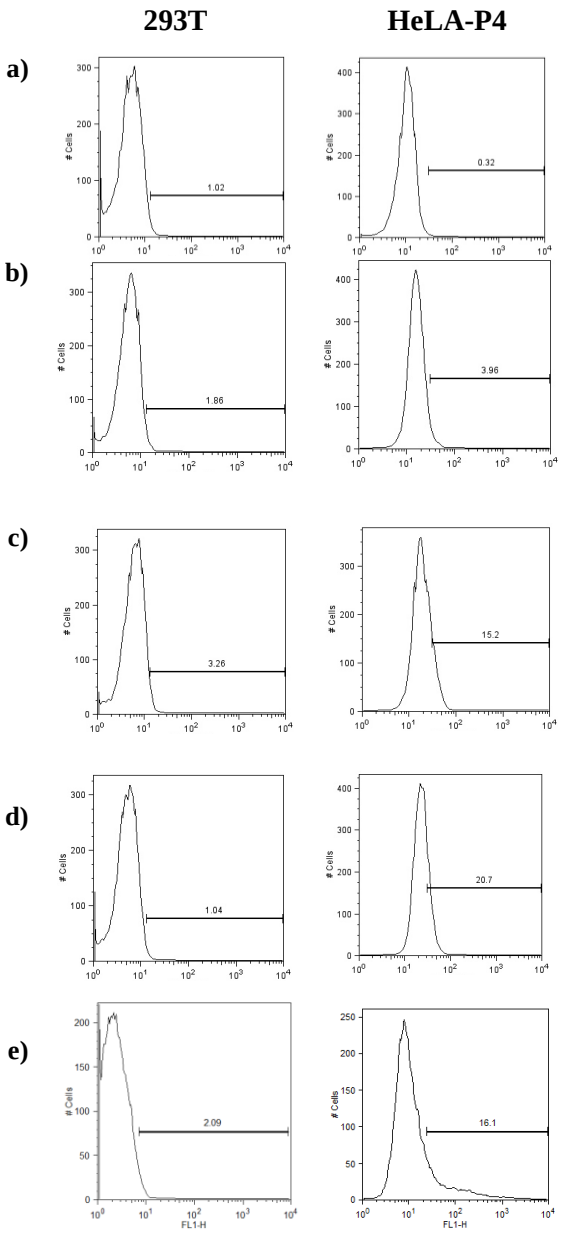
Figure 2.6 - ELISA assays to evaluate the dAbs binding to hCD4.
Assay was performed using 500 ng per well of hCD4 as antigen. 3 % BSA (in PBS) was used for blocking and as negative control. Equal amounts of dAbs were used (500 ng per well). The antibody used to detect the binding of the dAbs was α-HA-HRP (1: 1000) and the colorimetric substract used was ABTS (0,2 % H_2O_2). Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate. ** corresponds to $P < 0{,}001$, * corresponds to $P < 0{,}0001$.**

Both the V_LCD4 and the V_LB12 constructs could bind specifically to hCD4. Surprisingly, although V_L CD4 is the construct that possess the gp120 epitope that, according to the literature[362] binds the CD4 receptor, both the V_LB12 constructs show higher binding to hCD4 that the V_LCD4.

With this results we wanted to see if V_LB12 constructs could in fact bind hCD4 in a "cell context", so we performed FACS assays in HeLa-P4 cells and used as an additional negative control a cell line that does not express the CD4 receptor (293T). The results are discussed in the next section.

2.3.3 Flow cytometry assay to evaluate V_LB12 binding to CD4

a) Cells + IgG-FITC
b) Cells + α-HA-FITC
c) Cells + α-CD4-FITC
d) Cells + 10 nmol V_L B12 (CDR1) + α-HA-FITC
e) Cells + 10 nmol V_L B12 (CDR3) + α-HA-FITC



% of CD4 positive cells

	293T	HeLA-P4
a)	1,02	0,32
b)	1,86	3,96
c)	3,26	15,2
d)	1,04	(20,7-3,96)
e)	2,09	(16,1-3,96)

Figure 2.7 – Flow cytometry assay to evaluate that V_LB12 (CDR1) binds specifically to the CD4 receptor.
For each assay condition, 200 000 cells, either Human embryonic kidney 293T (HEK293T) or HeLa-P4 were stained. Cells were detached from the flasks with cell dissociation buffer (Gibco, NY, USA), washed with PBS and incubated with either 10 nmol of V_LB12(CDR1) or 2,5 µl of fluorescein isothiocyanate (FITC) Mouse Anti-Human CD4 and diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with V_LB12(CDR1) were incubated with 2,5 µl anti-HA-FITC-conjugated diluted in 1% BSA (in PBS). Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS). Shown is one representative experiment out of three.

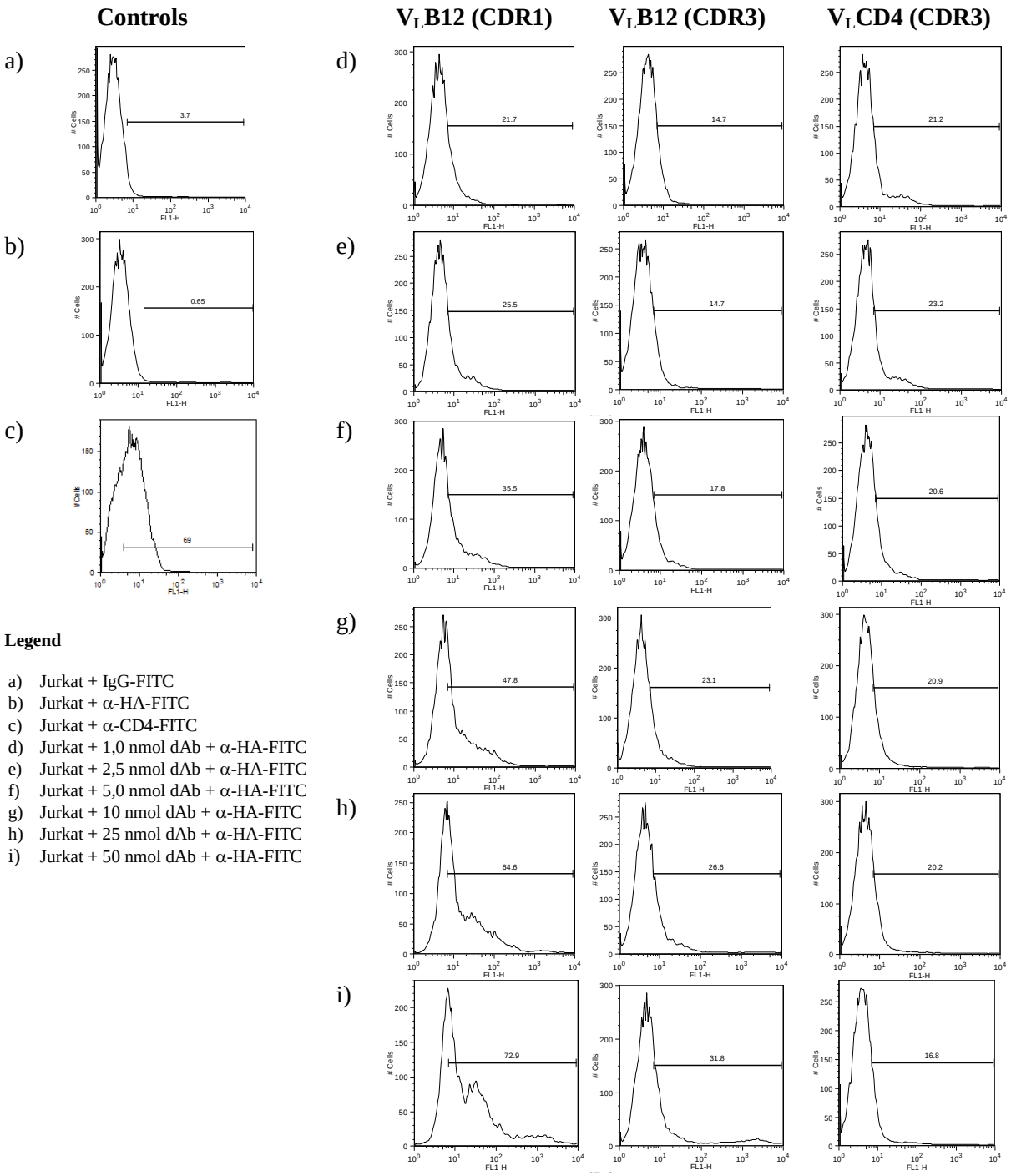
The V_LB12 constructs can in fact bind specifically to hCD4. With the V_LB12 (CDR1) presenting a higher binding ability than V_LB12 (CDR3), and also higher than the commercial α-CD4 at the conditions tested. The actual number of CD4 positive cells was 14,9 % (15,2 – 0,32) for the commercial α-CD4-FITC, 16,7 % (20,7-3,96) for the V_LB12 (CDR1) and 12,1 % (16,1-3,96) for V_LB12 (CDR3).

The V_LB12 (CDR1) is also expressed at higher amounts that the V_LB12 (CDR3) indicating that the structure of the V_L framework supports the B12 loop better in the CDR1 than in the CDR3 and that this loop can bind specifically to the hCD4 receptor integrated in a dAb structure.

Furthermore, the original CDR1 of this rabbit V_L is 13 amino acids long and the CDR3 is 11 amino acids long (see Table 2.5). For most antibodies the CDR3 possesses the bigger loop since the variability is higher for CDR3. But contrary to that, in this rabbit V_L the CDR1 loop is bigger so probably that is related to the fact that the B12 loop, that is 23 amino acids long, is more stable in the CDR1 than in CDR3.

After validating that the B12 loop can bind specifically to the CD4 receptor without the presence of the gp120 CD4 binding epitope, we wanted to compare this V_LB12 results with the V_LCD4, but this time using an immortalized line of T lymphocyte cells, Jurkat cell line.

2.3.4 Flow cytometry assay to evaluate V_LB12 (CDR1), V_LB12 (CDR3) and VL CD4 (CDR3) binding to CD4 in Jurkat cells



Legend

a) Jurkat + IgG-FITC
b) Jurkat + α-HA-FITC
c) Jurkat + α-CD4-FITC
d) Jurkat + 1,0 nmol dAb + α-HA-FITC
e) Jurkat + 2,5 nmol dAb + α-HA-FITC
f) Jurkat + 5,0 nmol dAb + α-HA-FITC
g) Jurkat + 10 nmol dAb + α-HA-FITC
h) Jurkat + 25 nmol dAb + α-HA-FITC
i) Jurkat + 50 nmol dAb + α-HA-FITC

Figure 2.8 - Flow cytometry assay to evaluate V_LB12 (CDR1), V_LB12 (CDR3) and VLCD4 (CDR3) binding to CD4 in Jurkat cells.

For each assay condition, 200 000 Jurkat cells were stained. Cells were washed with PBS and incubated with different concentrations of either V_LB12 (CDR1), V_LB12 (CDR3), VLCD4 (CDR3) or 2,5 µl of FITC Mouse Anti-Human CD4 and diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with the dAbs were incubated with 2,5 µl anti-HA-FITC diluted in 1% BSA (in PBS). Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS). Shown is one representative experiment out of three.

Table 2.7 - % of CD4 positive cells

	Controls	V_LB12 (CDR1)	V_LB12 (CDR3)	V_LCD4 (CDR3)
a)	3,7			
b)	0,65			
c)	69,0			
d)		21,7	14,7	21,2
e)		25,5	14,7	23,2
f)		35,5	17,8	20,6
g)		47,8	23,1	20,9
h)		64,6	26,6	20,2
i)		72,9	31,8	16,8

The percentage of CD4 positive cells for the Flow cytometry assay presented on Figure 2.8 is listed on Table 2.7. The percentage of CD4 positive cells for the three independent Flow cytometry assays are presented in a graphic format (see Figure 2.9).

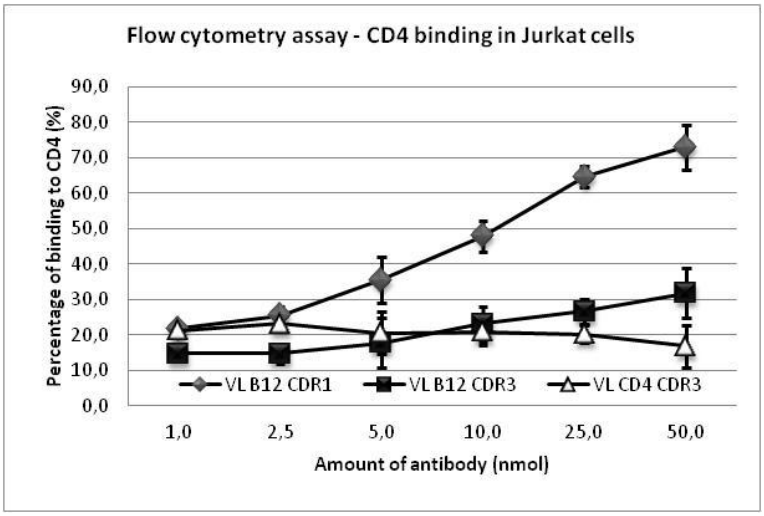


Figure 2.9 - Graphical representation of the percentage of CD4 positive cells obtained for the three independent Flow cytometry assays performed. Single domain Ab amounts range from 1,0 nmol to 50,0 nmol.

Mouse Anti-Human CD4 FITC conjugated (clone RPA-T4) was used as a positive control. The α-CD4-FITC antibody was used to correlate the percentage of positive CD4 Jurkat cells between independent assays.

This results correlate to the results obtained in HeLa-P4 cells, where V_LB12 (CDR1) also presents a higher binding ability than V_LB12 (CDR3). So the V_LB12 (CDR1) construct is not only expressed in higher amounts, and the purification yield is also

higher than for V_LB12 (CDR3) (see Figure 2.5 and Table 2.6), but this also correlates with a higher binding ability do hCD4. The results obtained for binding to soluble hCD4 were consistent between the ELISA assays and the Flow cytometry assays (see Figure 2.6, Figure 2.7 and Figure 2.8) so all subsequent experiments were performed with only one of the V_LB12 constructs, V_LB12 (CDR1).

The range of dAb amounts used in the Flow cytometry assays is limited to 50 nmol, because due to the low concentration of the dAbs obtained after the purification process, the volume of dAbs necessary to stain the cells is so big that higher amounts of dAbs result in higher percentage of cell death and the same percentage of CD4 positive cells (data not shown).

The average number of CD4 positive cells stained with 50 nmol of dAbs was 72,2 % for V_LB12 (CDR1), 30,1 % for V_LB12 (CDR3) and 15,3 % for V_LCD4 (CDR3).

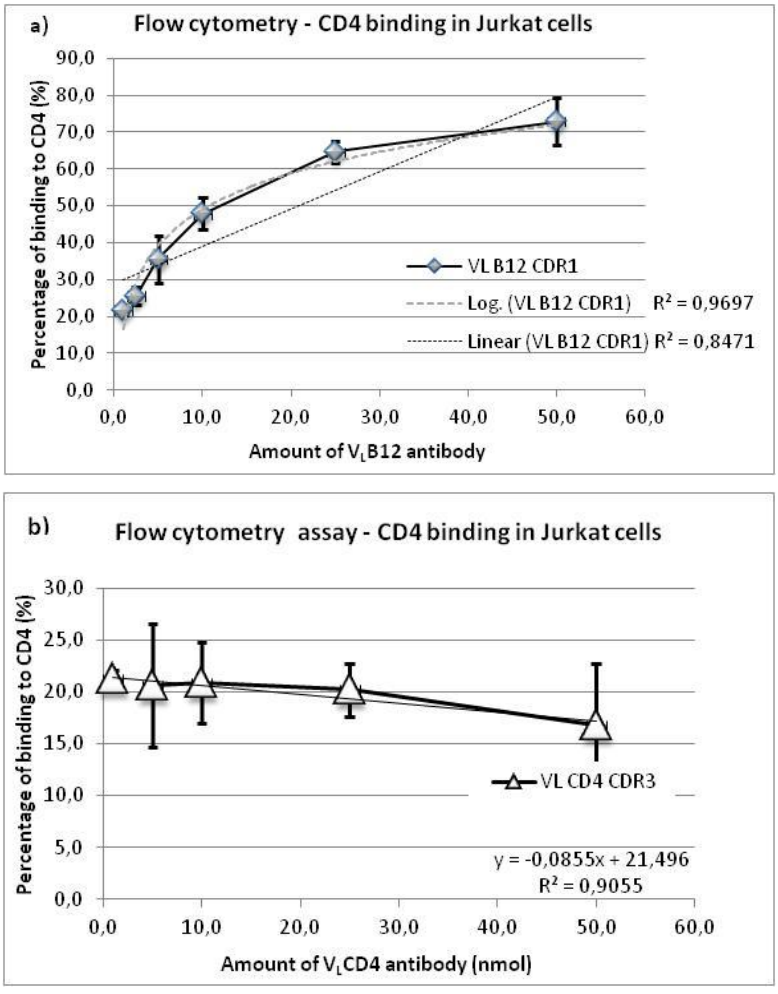


Figure 2.10 - Graphical representation of the percentage of CD4 positive cells obtained for the three independent Flow cytometry assays performed.(continues on the next page)

a) Graphical representation of the percentage of CD4 positive cells stained with V_LB12 (CDR1) showing both linear and logarithmic function adjustments and respective R^2. b) Graphical representation of the percentage of CD4 positive cells stained with V_LCD4 (CDR3) showing both linear and logarithmic function adjustments and respective R^2.

The graphical representation of the percentage of CD4 positive cells obtained for the three independent Flow cytometry assays performed shows an logarithmic (R^2 = 0,9697) increase in the percentage of cells stained with V_LB12 (CDR1) up to 50 nmol as opposed to the percentage of CD4 positive cells stained with V_LCD4 (CDR3) that present a linear fit (R^2 = 0,9055) with a slope of -0,0855, meaning that the percentage of CD4 positive cells is almost constant, with an optimum of 2,5 nmol, afterwich the percentage of CD4 positive cells decreases this if due to the dilution factor previously mentioned.

Form this point forward only V_LB12 (CDR1) and V_LCD4 (CDR3) will be submitted to further study and characterization. These domain antibodies are subsequently designated by the abbreviations, V_LB12 and V_LCD4.

Because the method being used to express and purify the proteins gave very low protein yields. It was necessary to develop an alternate method to express and purify large amounts of the proteins.

2.3.5 Alternate expression and purification method of recombinant domain antibodies, dAbs

The method optimized in our laboratory to produce and purify large amounts of purified V_L proteins (see Section 2.2.8) involves the use of the pET system.

The V_LB12 and V_LCD4 proteins were cloned in the pET28a vector, and four clones from each construct were tested for optimal expression conditions. These dAbs can only be found in the insoluble fraction of the bacteria cell extract (Figure 2.11).

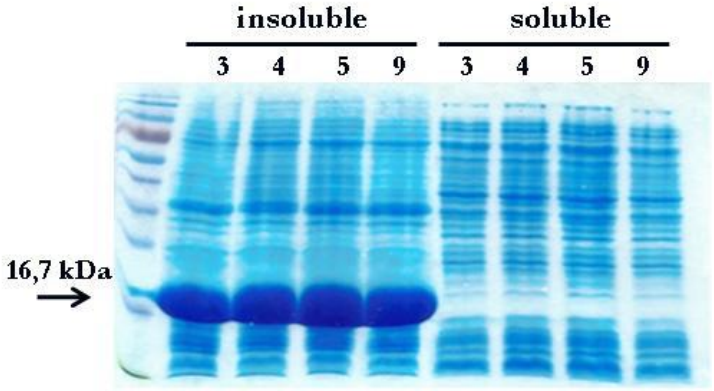


Figure 2.11 - Optimized expression conditions for V_L B12 in the pET-28a vector.
Both the soluble and insoluble fractions were analyzed. *E. coli* Tuner cells were grown at 37º C until A600nm= 0.4, induced by the addition of 0,2 mM IPTG and growth was continued for 4 hours at 30ºC. Bacteria were harvested by centrifugation (6,000 × *g*, 4°C, and 15 min), resuspended in PBS, and lysed by sonication. The proteins in both the soluble and insoluble fractions were separated by SDS-PAGE and stained with coomassie staining solution.

Unlike for the dAbs expressed in pComb3X, the pET-28a vector expresses very large amounts of protein, but all in the insoluble fraction (Figure 2.11). Insoluble proteins require a completely different purification protocol (see Section 2.2.8), so it was necessary to develop a new purification protocol for the purification of the dAbs.

Legend:

1) **Protein Ladder**
2) **Purification step 1**
3) **Purification step 2**
4) **Purification step 3**
5) **Purification step 4 – O/N solubilization**
6) **Affinity chromatography**
7) **Protein refolding/dialysis**

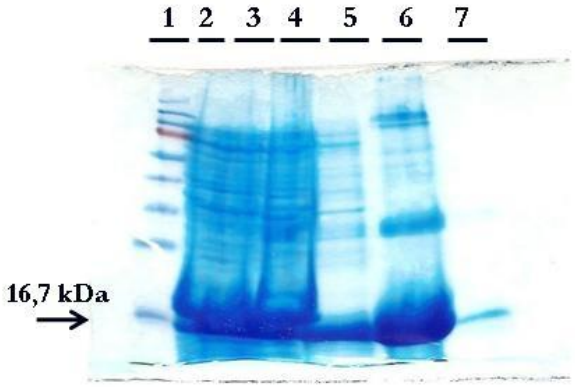


Figure 2.12 - V_LB12 protein recovered after each purification step using the protocol for purification of insoluble dAbs .
Proteins were separated by SDS-PAGE and stained with coomassie staining solution; equal volumes of sample were loaded in each well, in order to evaluate the dAb protein recovery after each step. 1) PageRuler™ Prestained Protein Ladder; 2) proteins present in the insoluble fraction after the first sonication step; 3) Proteins recovered after the second sonication step; 4) Proteins recovered after the third sonication step; 5) Proteins present in the soluble fraction after the overnight solubilization step; 6) Proteins recovered after the affinity chromatography step; 7) Proteins recovered after the protein refolding/dialysis step.

The protein recovery profile after each purification step is depicted in Figure 2.12 for the V_LB12 protein, using the protocol for purification of insoluble dAbs. It is interesting to highlight the fact that the amount of contaminants diminishes greatly on the overnight solubilization step, and that after the affinity chromatography almost no contaminants are present and the protein concentration is greatly enhanced. The factor that greatly enhanced the protein recovery in the affinity chromatography step is the presence of an N-terminal Histidine tag. In the proteins produced from the pComb3X vector there was a single C-terminal Histidine tag followed by an HA tag, and the presence of the HA tag limits the binding of the Histidines to the matrix of the affinity chromatography column and greatly reduces the amount of purified protein recovered.

Although the amount of protein produced in the pET system is much higher than in the pComb3X system, without the presence of the second Histidine tag (N-terminal) the amount of purified protein recovered was also low. As it can be perceived from Figure 2.12, the limiting step in this purification protocol is the refolding/dialysis step.

The amount of dAb recovered in the refolding/dialysis step is drastically lower, when compared to the amount of protein recovered after the affinity chromatography purification, because most of the protein does not refold properly and precipitates in the refolding/dialysis column.

Table 2.8 - Results of the dAbs purifications per liter of bacteria culture, using the insoluble dAb purification protocol.

dAb	dAb concentration	Total amount of dAb	Purity (%)[§]
V_L CD4	**75 ng/µl**	3,92 mg	95
V_L B12	**140 ng/µl**	1,96 mg	95

Although a very large amount of the purified dAb is lost in the refolding process, the total amount obtained for each dAb, per liter (Table 2.8), is almost ten times higher the the amount obtained in the soluble form (see Table 2.6).

[§] Purity was determined by densitometry using the Chemidoc software version 4.6 (Biorad, USA)

The concentrations for the dAbs, as indicated on Table 2.8, are actually the highest concentrations at which each of the dAbs is soluble in HEPES buffer. All attempts to achieve higher concentrations resulted in protein precipitation and loss of function.

2.3.6 V_LB12 and V_LCD4 binding to hCD4 – characterization and domain identification

After the purification of the V_LB12 and V_LCD4 dAbs using the protocol for purification of insoluble dAbs, further characterization of the interaction between the dAbs and the hCD4 was pursued.

The specificity of V_LB12 and V_LCD4 to the human CD4 receptor had already been established by the ELISA assays performed to evaluate the dAbs binding to soluble hCD4 (see Figure 2.6) and by the Flow cytometry assays that validated that the binding was specific to CD4 (see Figure 2.7) and that both V_LB12 and V_LCD4 had a strong affinity to the CD4 receptor (see Figure 2.8) in a cell context.

The next step was to identify the domain where V_LB12 and V_LCD4 bind to the human CD4 receptor. It has been described that the HIV-1 gp120 binds domain 1 of the CD4 receptor[36,288,305,362], so it was expected that V_LCD4 would bind the same CD4 epitope. As for V_LB12, since the grafted loop is also an HIV-1 gp120 highly conserved sequence that overlaps the gp120 CD4 binding sequence, we were also expecting binding in the domain 1 of the CD4 receptor.

V_LB12, V_LCD4 and the original V_L were submitted to ELISA assays to compare binding to soluble hCD4 and to CD4-IgG, a tetrameric fusion protein comprising human IgG2 in which the Fv portions of both heavy and light chains have been replaced by the V1 and V2 domains of human CD4 (Figure 2.13).

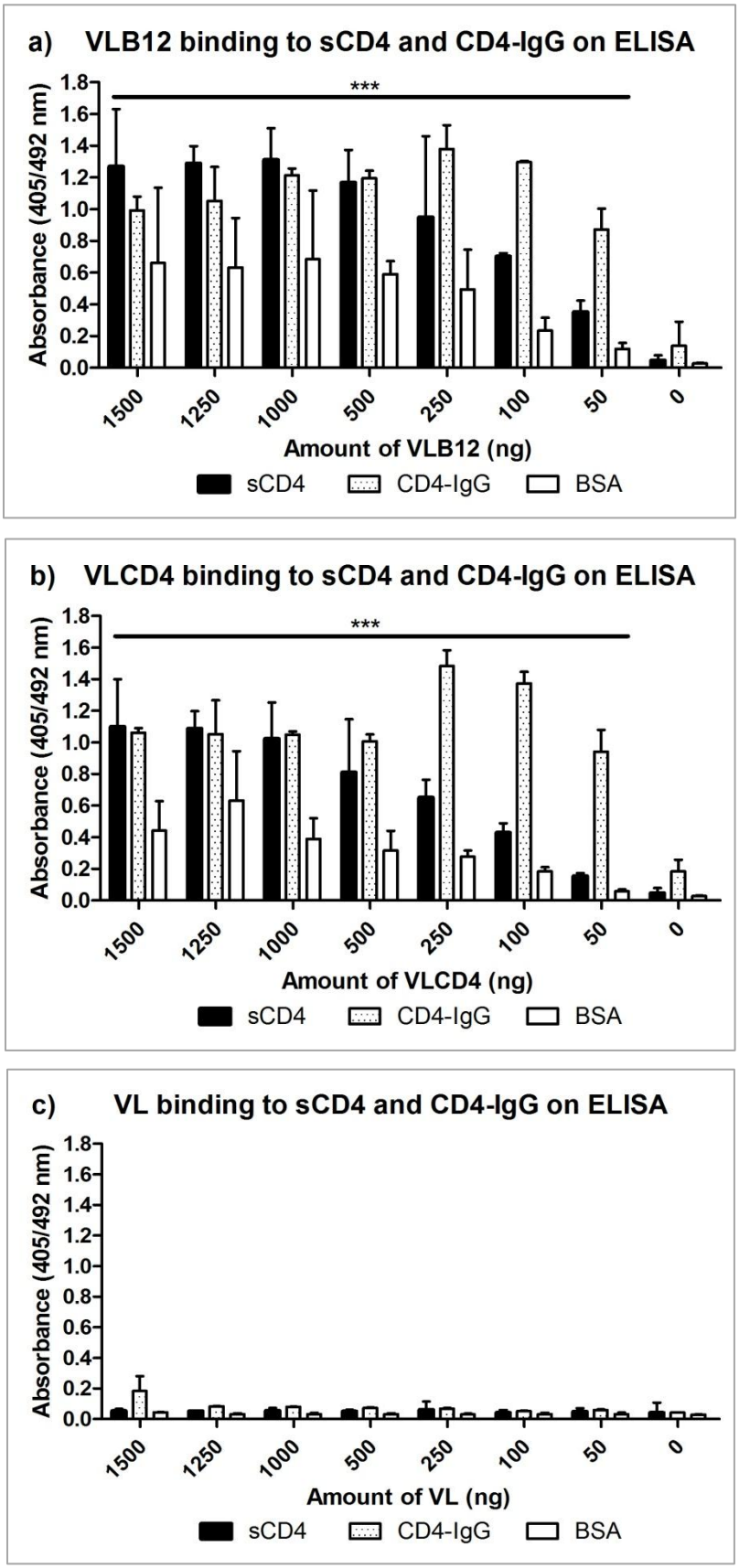


Figure 2.13 - ELISA assays were performed to evaluate V_LB12, V_LCD4 and the original V_L binding to either soluble hCD4 or a CD4-IgG.
(Continues on the next page)

a) VL B12 binding to either soluble hCD4 or a CD4-IgG on ELISA, using decreasing amounts of dAb. b) VL CD4 binding to either soluble hCD4 or a CD4-IgG on ELISA, using decreasing amounts of dAb. c) Original VL antibody binding to soluble hCD4 and to CD4-IgG on ELISA, using decreasing amounts of dAb. Assay was performed using 500 ng per well of soluble hCD4 or CD4-IgG as antigen. 3 % BSA (in PBS) was used for blocking and as negative control.
A range of decreasing amounts of dAb was used (1500 ng, 1250 ng, 1000 ng, 500 ng, 250 ng, 100 ng, 50 ng and 0 ng per well). The antibody used to detect the binding of the dAbs was α-HA-HRP (1: 1000) and the colorimetric substract used was ABTS (0,2 % H_2O_2). All incubation steps were performed at 37 ºC for 1 h. Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate. * corresponds to *P* < 0,0001.**

The ELISA assays presented on Figure 2.13, were performed to compare binding to soluble hCD4 and to CD4-IgG, using 500 ng of each antigen per well and using 3 % BSA (in PBS) as a negative control and also as a blocking agent. A range of decreasing amounts of dAb was used (1500 ng, 1250 ng, 1000 ng, 500 ng, 250 ng, 100 ng, 50 ng and 0 ng per well). From these assays it is possible to verify that both V_LB12 and V_LCD4 bind specifically to soluble hCD4 and to CD4-IgG, and that the binding to soluble hCD4 is proportional to the amount of dAb used. When analyzing the binding of both V_LB12 and V_LCD4 to CD4-IgG we see an increase in binding to CD4-IgG in the 250 ng – 50 ng range, this is probably due to the fact that each CD4-IgG molecule possesses two binding sites, duplicating the amount of epitopes available e probably these are more readily accessible in an IgG format than in the full-length extracellular domain of human CD4 and as the dAb amount decreases, there is no excess of dAb and these differences are more readily noticed.

Original VL antibody binding to soluble hCD4 and to CD4-IgG as also assessed to confirm that after the protein refolding step, V_LB12 and V_LCD4 produced using the insoluble dAb purification protocol, maintained their binding specificity. This can be confirmed when comparing either V_LB12 or V_LCD4 to the original VL antibody, where the original VL antibody presents no specific binding to either soluble hCD4 or CD4-IgG as determined by the ELISA assays presented on Figure 2.13.

These results also indicate that both V_LB12 and V_LCD4 bind to either domain 1 or domain 2 of the CD4 receptor, since all four domains are present in soluble hCD4 but only domain 1 and domain 2 are present in CD4-IgG.

2.3.7 ELISA assay to evaluate the dAbs binding to b12 mAb

The ELISA assays presented on Figure 2.14 were also performed to evaluate the dAbs binding to b12 mAb. Since V_LB12 was constructed by grafting of the gp120 b12 target sequence into the CDR1 of the original V_L framework. We wanted to evaluate if b12 target sequence is recognized by the b12 mAb antibody in the context of the V_LB12 grafting. The binding of b12 mAb to V_LCD4 was also evaluated.

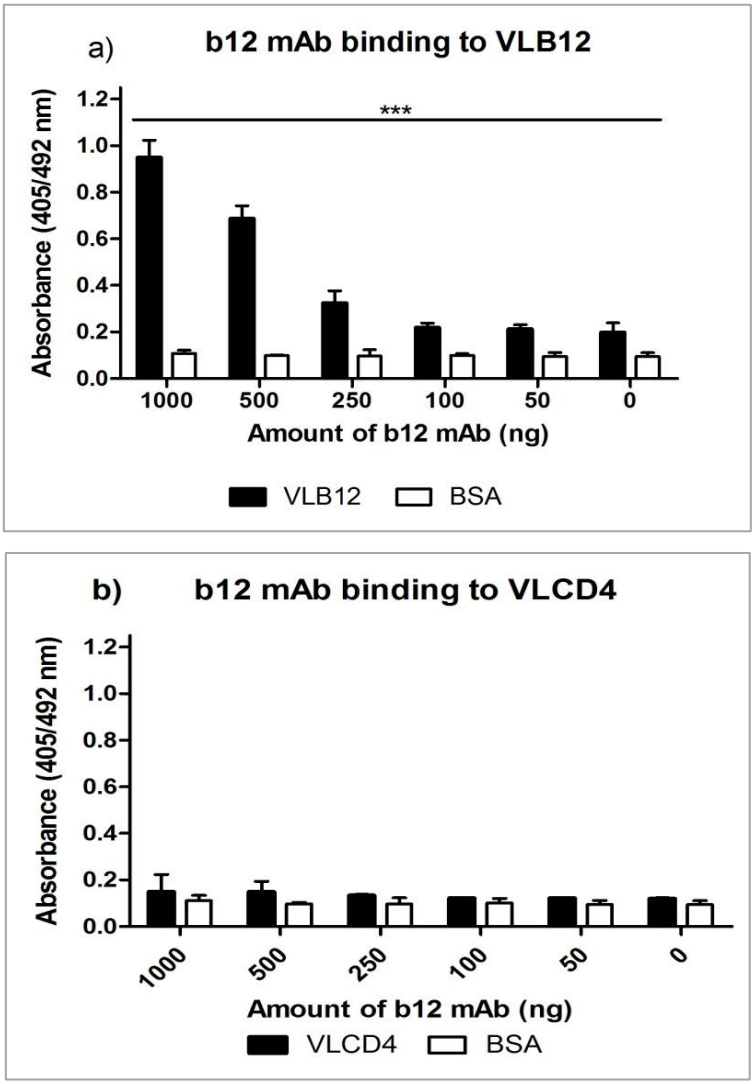


Figure 2.14 - ELISA assays were performed to evaluate b12 mAb binding to V_LB12 and V_LCD4. a) b12 mAb binding to either V_LB12 or BSA on ELISA, using decreasing amounts of b12 mAb. b) b12 mAb binding to either V_LCD4 or BSA on ELISA, using decreasing amounts of b12 mAb. Assay was performed using 500 ng per well of V_LB12 or V_LCD4 as antigen. 3 % BSA (in PBS) was used for blocking and as negative control. A range of decreasing amounts of b12 mAb was used (1000 ng, 500 ng, 250 ng, 100 ng, 50 ng and 0 ng per well). The antibody used to detect the binding of the b12 mAb was a goat α-Human IgG-HRP (1: 1000) and the colorimetric substract used was ABTS (0,2 % H_2O_2). All incubation steps were performed at 37 ºC for 1 h. Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate. * corresponds to P < 0,0001.**

From these results (see Figure 2.14) it is possible to verify that b12 mAb binds specifically to V_LB12, and this binding is proportional to the amount of b12 mAb present. Furthermore, it was also found that b12 mAb does not bind to V_LCD4. These results confirm that V_LB12 maintains the characteristics of the original gp120 epitope allowing it to be recognized by its specific antibody, b12 mAb, outside the gp120 context and that the V_LCD4, also retains the characteristics of the original gp120 epitope and is not recognized by b12 mAb.

2.3.8 V_L B12 and V_L CD4 binding to hCD4 – epitope characterization

Another important feature is epitope characterization, and in particular determining if the epitopes recognized by V_L B12 and V_L CD4 are linear or conformational.

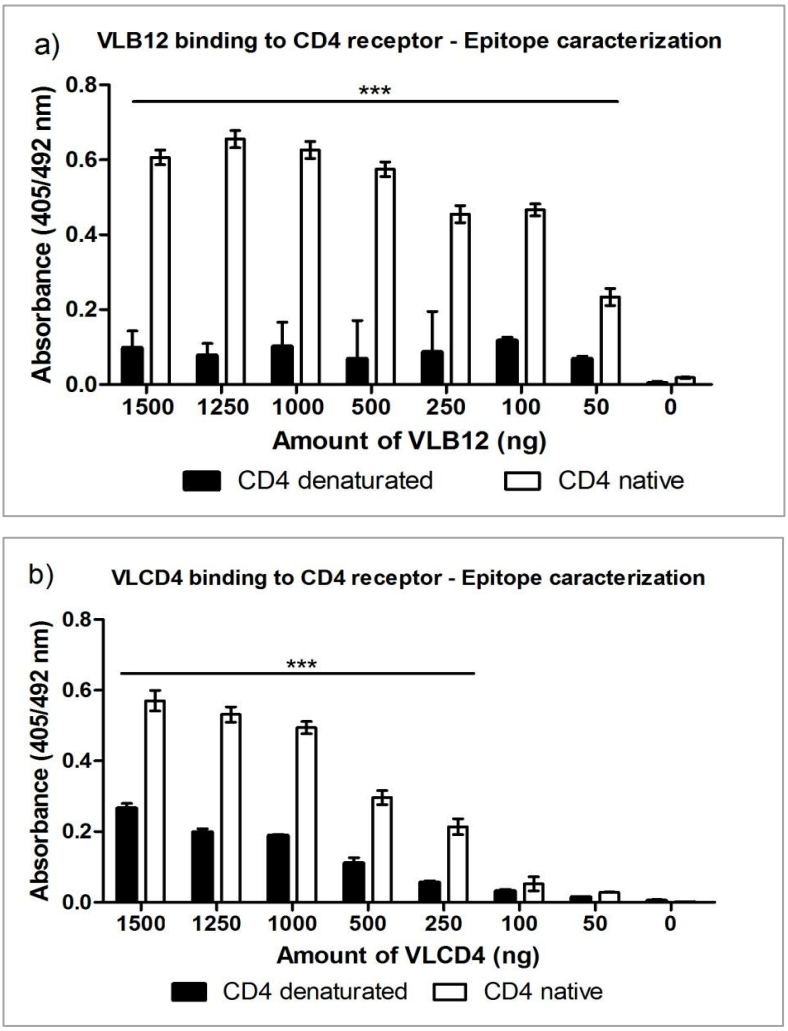


Figure 2.15 - V_LB12 and V_LCD4 binding to soluble hCD4 – Epitope characterization.
(Continues on the next page)

ELISA assays were performed using as antigen native conformation soluble hCD4 or denaturated soluble hCD4. For each assay, 500 ng of antigen (in PBS) were used per well. Denaturated soluble hCD4 was obtained by submitting the protein to heat denaturation for 10 minutes at 95 ºC. 3 % BSA (in PBS) was used for blocking and as negative control. a) A range of decreasing amounts of V_LB12 was used (1500 ng, 1250 ng, 1000 ng, 500 ng, 250 ng, 100 ng, 50 ng and 0 ng per well).
b) A range of decreasing amounts of V_LCD4 was used (1500 ng, 1250 ng, 1000 ng, 500 ng, 250 ng, 100 ng, 50 ng and 0 ng per well). For both assays the antibody used to detect the binding of the dAbs was α-HA-HRP (1: 1000) and the colorimetric substract used was ABTS (0,2 % H_2O_2). All incubation steps were performed at 37 ºC for 1 h. Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate. * corresponds to $P < 0,0001$.**

To determine if the epitopes recognized by V_LB12 and V_LCD4 are linear or conformational, ELISA assays were performed (Figure 2.15) using as antigen native conformation soluble hCD4 or denaturated soluble hCD4. For each assay, 500 ng of antigen (in PBS) were used per well. Denaturated soluble hCD4 was obtained by submitting the protein to heat denaturation for 10 minutes at 95 ºC. When comparing both V_LB12 and V_LCD4 bindings to native conformation soluble hCD4 (Figure 2.15) it was observed a strong and specific binding, in agreement with the results obtained previously (Figure 2.13). On the other hand, both V_LB12 and V_LCD4 presented very low binding to denaturated soluble hCD4, meaning that the dAbs did not recognize the denaturated (linearized) epitopes. This proved that both V_LB12 and V_LCD4 recognized conformational epitopes.

2.3.9 Flow cytometry assay to identify the hCD4 domain recognized by V_LB12 and V_LCD4

The results obtained by the ELISA assays performed to evaluate the V_LB12 and V_LCD4 binding to either soluble hCD4 or a CD4-IgG indicated that both V_LB12 and V_LCD4 bind to either domain 1 or domain 2 of the CD4 receptor. To distinguish between binding to domain 1 or domain 2, another assay was designed, where by comparison of the binding to either mouse CD4 (mCD4), human CD4 (hCD4) or to a construct where the domain 1 of the human CD4 has been replaced by a mouse domain 1(hCD4mD1) we aimed to specifically identify the exact domain recognized by V_LB12 and V_LCD4 (see Figure 2.16).

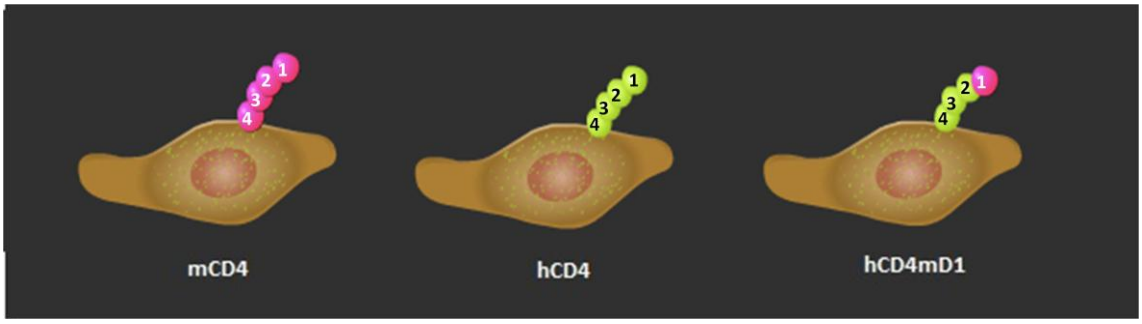
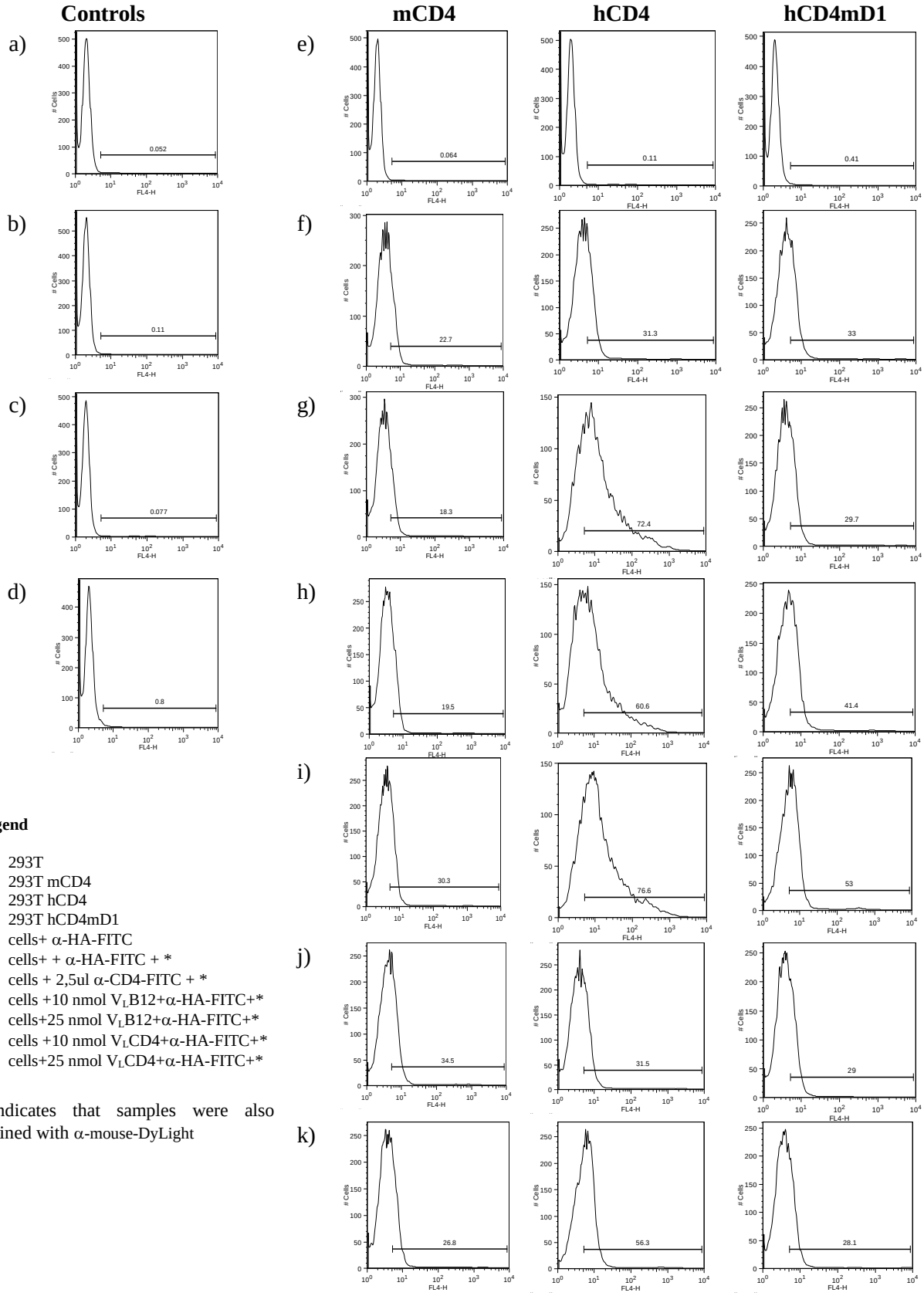


Figure 2.16 - Schematic representation of the HEK293T cells transfected with mCD4, hCD4 or hCD4mD1.
Human embryonic kidney 293T (HEK293T) (ATCC, VA, USA) cells were transfected, by calcium phosphate method, with either human CD4 (hCD4), human CD4 with mouse Domain1 (hCD4mD1) or mouse CD4 (mCD4). Briefly, 240 000 HEK293T cells were transfected with one of the plasmids in order to produce HEK293T cells expressing hCD4, hCD4mD1 or mCD4. After 48 h, the transfected cells were stained and submitted to the Flow Cytometry assay to identify the hCD4 domain recognized by VLB12 and VLCD4. This figure was designed through the Protein lounge software: Copyright www.proteinlounge.com, 2011.

Figure 2.16 presents a schematic representation of the HEK293T transfection assay with the different CD4 receptors. The dAbs, V_LB12 and V_LCD4 have already been identified as binding to either domain 1 or domain 2. If one of the dAbs does not bind to mCD4, binds to hCD4 and it does not bind to hCD4mD1, this means that the dAb is binding to the only domain that is not present in the hCD4mD1, the human domain 1. On the other hand, if one of the dAbs does not bind to mCD4, binds to hCD4 and also binds to hCD4mD1, this means that the dAb is binding to a domain that is still present in the hCD4mD1, the human domain 2.

The HEK 293T cells transfected with each of the CD4 plasmids were afterwards submitted to the Flow cytometry assays (see Figure 2.17).



Legend

a) 293T
b) 293T mCD4
c) 293T hCD4
d) 293T hCD4mD1
e) cells+ α-HA-FITC
f) cells+ + α-HA-FITC + *
g) cells + 2,5ul α-CD4-FITC + *
h) cells +10 nmol V_LB12+α-HA-FITC+*
i) cells+25 nmol V_LB12+α-HA-FITC+*
j) cells +10 nmol V_LCD4+α-HA-FITC+*
k) cells+25 nmol V_LCD4+α-HA-FITC+*

*indicates that samples were also stained with α-mouse-DyLight

Figure 2.17 - Flow cytometry assay to identify the hCD4 domain recognized by V_LB12 and VL CD4 (continues on the next page)

For each assay condition, 200 000 (293T) transfected cells were stained. 293T cells were stained 48h after Calcium phosphate transfection of mouse CD4 (mCD4), human CD4 (hCD4) or human CD4 with mouse Domain1 (hCD4mD1). For each assay condition 200 000 transfected cells were washed with PBS and incubated with 10 nmol or 25 nmol of either VLB12 or VL CD4 and also with 2,5 µl of FITC Mouse Anti-Human CD4 (α-CD4-FITC), all diluted in 1% BSA (in PBS). Afterwards, cells were washed with PBS and cells stained with the dAbs were incubated with 2,5 µl anti-HA-FITC diluted in 1% BSA (in PBS). Cells were washed with PBS. All the cells stained with either VLB12, VL CD4 or α -CD4-FITC were again washed with PBS and stained with a-mouse-DyLight 405 (1:5000). Cells were washed with PBS and fixated with 1 % p-formaldehyde (in PBS). Shown is one representative experiment out of three.

As previously mentioned, HEK293T cells were transfected with mCD4, hCD4 or hCD4mD1 (see 2.2.14) and a Flow cytometry assay was performed to identify the hCD4 domain recognized by V_LB12 and V_LCD4, as positive control it was used the FITC Mouse Anti-Human CD4 (α-CD4-FITC, clone RPA-T4) which binds to the D1 domain (CDR1 and CDR3 epitopes) of the hCD4 antigen.[164,294]

The results from the Flow cytometry assay are presented on Figure 2.17 and also on Table 2.9 (Shown is one representative experiment out of three.)

Table 2.9 - % of binding to CD4 transfected cells

	Controls	mCD4	hCD4	hCD4mD1
a)	0,052			
b)	0,11			
c)	0,077			
d)	0,8			
e)		0,064	0,11	0,41
f)		22,7	31,3	33
g)		18,3	72,4	29,7
h)		19,5	60,6	41,4
i)		30,3	76,64	53
j)		34,5	31,5	29
k)		26,8	56,3	28,1

Table 2.10 - % of binding to CD4 transfected cells.
Relative values, where the background fluorescence from unspecific α-HA-FITC + α-mouse-DyLight 405 binding (condition f) is subtracted to the % of total CD4 positive cells for each condition under evaluation.

	mCD4	hCD4	hCD4mD1
f) cells+ + α-HA-FITC + α-mouse-DyLight			
g) cells + 2,5ul α-CD4-FITC + α-mouse-DyLight	-4,4	41,1	-3,3
h) cells +10 nmol V_LB12+α-HA-FITC + α-mouse-DyLight	-3,2	29,3	8,4
i) cells +25 nmol V_LB12+α-HA-FITC + α-mouse-DyLight	7,6	45,1	20,0
j) cells +10 nmol V_LCD4+α-HA-FITC + α-mouse-DyLight	11,8	0,2	-4,0
k) cells +25 nmol V_LCD4+α-HA-FITC + α-mouse-DyLight	4,1	25,0	-4,9

From the results obtained from the Flow cytometry assays performed to identify the hCD4 domain recognized by V_LB12 and V_LCD4, we can conclude that the α-CD4-FITC (clone RPA-T4) performed according to what has been described in the literature, since the α-CD4-FITC binds to the domain 1 of the hCD4. In the Flow cytometry assay the α-CD4-FITC did not bind to the mCD4 and neither to the hCD4mD1, indicating that an antibody that specifically recognizes the domain 1 of the hCD4 will not bind to the mCD4 and neither to the hCD4mD1 since in the hCD4mD1 the domain 1 present is the same as in the mCD4.

As for V_LB12, it presented no binding to mCD4 at 10 nmol (-3,2 %), but presented a slight binding (7,6 %) at 25 nmol. When comparing the binding of V_LB12 at 25 nmol to hCD4 (45,1 %) and to hCD4mD1 (20%), the binding to mCD4 can be considered unspecific. Regarding binding to hCD4 at 10 nmol V_LB12 presented a specific binding of 29,3 % and at 25 nmol the specific binding increased to 45,1 %, similar to the specific binding of the α-CD4-FITC to the hCD4. This also correlates with the results obtained for both the α-CD4-FITC and the V_LB12 (this is the CDR1 grafting) that at 25 nmol also presented almost the same specific binding to hCD4, that was also the case n the Flow cytometry assays performed to evaluate the binding of the dAbs in Jurkat cells (see 2.3.4). V_LB12 can be considered to present no specific binding to mCD4, and specifically recognizes the hCD4. V_LB12 also presents specific binding to hCD4mD1 with a 8,1 % binding at 10 nmol and a 20,0 % binding at 25 nmol. The fact that V_LB12

presents specific binding to hCD4mD1 indicates that V_LB12 is not binding to the domain 1 of the CD4 receptor, but instead is binding to the domain 2.

V_LCD4, like V_LB12 presents some unspecific binding to mCD4, this binding can also be considered unspecific since it is higher at a lower concentration and it does not correlate to the previously determined specific binding to hCD4 (see 2.3.4 and 2.3.6) that is higher at 25 nmol (25,0 %) than at 10 nmol (0,2 nmol). Contrary to V_LB12, V_LCD4 does not bind to hCD4mD1 indicating that V_LCD4 binds specifically to the domain 1 of hCD4. The fact that V_LCD4 binds to domain 1 of the hCD4 is in agreement with what has been described in the literature regarding the interaction between gp120 and the CD4 receptor, where gp120 binds to the domain 1 of hCD4.[183,282,288] Since V_LCD4 was constructed by grafting of the gp120 CD4 binding epitope into the CDR3 of the original V_L framework, it was expected that V_LCD4 would behave in the same manner as what has been described for gp120.

2.4 Discussion

From the results obtained from the expression and purification yields of the dAbs produced in the pComb3X vector it is possible to conclude that not all CDR combinations will be supported by the V_L framework and that the sequence present in the CDRs plays an important role in antibody stability/solubility and that this is not directly correlated to the size of the CDR sequence.

Although V_LCD4 presents a higher expression and purification yields in the pComb3X system, than both V_LB12 constructs (CDR1 and CDR3), this does not translate into antigen binding ability. In fact, both V_LB12 constructs presented a higher binding to the human CD4 receptor than V_LCD4, as determined by ELISA assays and by Flow cytometry analysis.

The fact that the B12 target sequence presents a higher binding ability to the CD4 receptor than the CD4 binding epitope itself, it is probably due to the context of antibody grafting. In the V_LCD4 construct the CD4 binding epitope (10 amino acids) may not be sufficiently exposed to achieve optimal binding to the CD4 receptor,

contrary to what happens in the gp120 context. The larger B12 target sequence (23 amino acids) allows for a higher exposure of the epitope that may facilitate binding to the antigen.

When both proteins, V_LCD4 CDR3 and V_LB12 CDR1 were expressed in the pET system, due to the very high insoluble protein expression, the limiting step for the yield of purified protein was the refolding/dialysis step. Because most of the protein does not refold properly and precipitates in the refolding/dialysis column, the amount of protein recovered is drastically lower, when compared to the amount of protein recovered after the affinity chromatography purification. In fact, the maximum concentrations that we were able to obtain were 140 ng/μl for V_LB12 and 75 ng/μl for V_LCD4, because when in refolding/dialysis the recovered proteins were at higher concentrations, both proteins precipitated. As previously mentioned all attempts to achieve higher concentrations resulted in protein precipitation and loss of function.

This proves that the V_LB12 construct has a higher stability than V_LCD4 since it maintains its function at higher concentrations than V_LCD4, when subjected to refolding.

After establishing that for both constructs, V_LCD4 and V_LB12, the binding was specific to CD4 and that both V_LB12 and V_LCD4 had a strong affinity to the CD4 receptor, the next step was to identify the domain where V_LB12 and V_LCD4 bind to the human CD4 receptor.

It has been described that the HIV-1 gp120 binds domain 1 of the CD4 receptor[36,288,305,362], so it was expected that V_LCD4 would bind the same CD4 epitope. As for V_LB12, since the grafted loop is also an HIV-1 gp120 highly conserved sequence that overlaps a conformationally invariant surface that overlaps a distinct subset of the gp120 CD4 binding site, we were also expecting binding in the domain 1 of the CD4 receptor.

The ELISA assays performed to compare binding to soluble hCD4 and to CD4-IgG confirmed that both V_LB12 and V_LCD4 bind to either domain 1 or domain 2 of the CD4

receptor, since all four domains are present in soluble hCD4 but only domain 1 and domain 2 are present in CD4-IgG.

Further analysis of the CD4 domains involved in the dAbs binding to the CD4 receptor were performed in the Flow cytometry assays designed to identify the hCD4 domain recognized by each of the dAbs (Section 2.3.9) and it was possible to identify that V_LCD4 binds to domain 1, but V_LB12 on the other hand binds domain 2 of the CD4 receptor. These findings reinforce the fact that although these regions have a similar spatial distribution at the gp120 surface after binding to either the CD4 receptor (for the CD4 binding epitope, V_L CD4) or to b12 mAb (for the B12 target sequence, V_L B12) they are in fact two independent regions with different roles in the interaction between gp120 and the CD4 receptor.

Regarding epitope characterization, as previously mentioned both V_LB12 and V_LCD4 presented very low binding to denaturated soluble hCD4, meaning that the dAbs did not recognize the denaturated (linearized) epitopes. This proved that both V_LB12 and V_LCD4 recognized conformational epitopes.

In conclusion, both V_LCD4 and V_LB12 bind specifically to the hCD4 cell receptor and recognize conformational epitopes in respectively, domain 1 and domain 2 of the CD4 receptor. Both domain 1 and domain 2 of the CD4 receptor are targeted by HIV-1 neutralizing antibodies.[112,150] So, the next step was to evaluate the V_L B12 and V_L CD4 ability to inhibit HIV-1 infection.

2.5 Acknowledgments

The development of the Expression and purification protocol of single domain antibodies cloned in pET28a was performed by Catarina Santos.

Andreia Couto is supported by FCT fellowship SFRH/BD/28211/2006 and Catarina Santos was also supported by an FCT fellowship.

HIV-1 Inhibition by single domain antibodies

3 HIV-1 Inhibition by single domain antibodies

3.1 Introduction

Due to the characteristics and nature of HIV infection, including the ability to establish reservoirs in various cell lines and progressive and irreversible destruction of the immune system associated with an increased prevalence of strains resistant to drug therapy, one of the priority areas in the development of new drugs has been the development of new molecules which prevent viral entry into cells, including peptides and antibodies that prevent the entry process.

The antibody response to HIV-1 *in vivo* is directed against several viral proteins, however, essentially all neutralizing antibodies are directed toward the viral Envelope spike, in particular the surface unit glycoprotein (gp) 120.[245,356] No naturally occurring anti HIV-1 antibodies are directed against the CD4 cell receptor, although there is a nonimmunosuppressive, humanized IgG4 monoclonal antibody, derived from a mouse antibody, 5A8, which binds domain 2 of CD4. This monoclonal antibody that blocks HIV-1 entry by binding to domain 2 of human CD4 (Ibalizumab), blocks HIV-1 infection in vitro and is currently on phase III clinical trials.[40,43,87,88,148,268]

Ibalizumab is the first from a new class of entry inhibitors, the inhibitors of gp120-CD4 interaction, this paves the way for a new approach to HIV-1 inhibition. Entry inhibitors are broadening, the so far limited number of viable alternative strategies to contain the virus without recourse to antiretroviral drug cocktails.

In this chapter we wanted to evaluate the ability of our anti-CD4 antibodies, V_LB12 and V_LCD4, to inhibit HIV-1 infection, by binding to either domain 2 or domain 1 of the CD4 cell receptor, respectively. For that we performed inhibition assays against the HIV-1 NL4-3 subtype B molecular clone and two non B primary isolates, a subtype J and a subtype H primary isolates. The effect of both dAbs on cell viability was also evaluated.

3.2 Materials and Methods

3.2.1 Cell Lines and culture conditions

Human embryonic kidney 293T (HEK293T) (ATCC, VA, USA), and TZM-bl (obtained through the NIH AIDS Research and Reference Reagent Program, MD, USA, contributors Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) here on designated DMEM-10.

All cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

3.2.2 NL4-3 Viral production

HEK293T cells were transfected, by calcium phosphate method, with pHIV-1_{NL4-3} plasmids (AIDS reagent, contributor Dr. Malcolm Martin) in order to produce HIV-1_{NL4-3} virions. After 48 h, virions were collected from supernatant cultures, the amount of viral particles released in the supernatant was measured by $p24_{CA}$ ELISA (AIDS & Cancer Research Program, NCI Frederick, MD, USA) and HIV-1_{NL4-3} virions were used to infect TZM-bl cells.

3.2.3 Primary isolates viral production

Primary isolates were obtained from HIV-1-infected Angolan patients, all naïve to therapy with entry inhibitors, by cocultivation with PBMCs and stimulated with phitohemagglutinin from seronegative subjects. Cultures were maintained and expanded until the amount of viral particles released in the supernatant was measured by $p24_{CA}$ ELISA and the results obtained gave overflow. Virions were then collected from supernatant cultures, and the amount of infectious viral particles released in the supernatant was measured by performing infection assays with serial dilution in TZM-bl cells.[58]

Briefly, 10,000 cells per well were seeded in a 96-well plate in 100 µl/well of DMEM-10 and incubated overnight at 37°C. The next day, the media was removed and 200 µl of serial diluted primary isolate (subtype J or H) were prepared, in DMEM-10, and added to the cells.

The cells were incubated for 48 h at 37 °C in 5 % CO_2. Assay medium was removed from each well, cells were washed with 200 µl of PBS and 50 µl of Passive Lysis Buffer reagent (Promega, Madison, WI) were added. This was followed by a freeze-thaw step (-20 ºC – 37 ºC) afterwhich 10 µl cell lysates from each sample (in Passive Lysis Buffer) were placed in a 96 well Luminescence Plate (BD Falcon 96 Flat Bottom White Opaque Polystyrene Plate, BD Biosciences, USA). 50 µl Luciferase Assay Reagent II (LAR II) were added to each sample and luminescence was measured using an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA).

3.2.4 Virus inhibition assay using TZM-bl cells.

An inhibition assay was performed based on the methods of Wei et al. [341] with modification.[192,215] TZM-bl cells were derived from a HeLa cell line (JC.53) that stably expresses CD4 and CCR5. TZM-bl cells also express luciferase and β-galactosidase under the control of the HIV-1 promoter.

Briefly, 10,000 cells per well were seeded in a 96-well plate in 100 µl/well of DMEM-10 and incubated overnight at 37°C. The next day, the media was removed and 100 µl of serial diluted V_LB12 or V_LCD4 was added to the cells and incubated for 1 h at 37°C. Then, 100 µl of HIV-1 were prepared, in DMEM-10, for a multiplicity of infection (MOI) of 0,5 (NL4-3) or MOI of 10 (subtype J and H primary isolates) and added to the cells. **

** For the T20 assays the incubation step with the inhibitor was performed with virus and not with the cells.

The cells were incubated for 48 h at 37 °C in 5 % CO_2. Assay medium was removed from each well, cells were washed with 200 µl of PBS and 50 µl of Passive Lysis Buffer reagent (Promega, Madison, WI) were added. This was followed by a freeze-thaw step (-20 ºC – 37 ºC) afterwhich 10 µl cell lysates from each sample (in Passive Lysis Buffer) were placed in a 96 well Luminescence Plate (BD Falcon 96 Flat Bottom White Opaque Polystyrene Plate, BD Biosciences, USA). 50 µl Luciferase Assay Reagent II (LAR II) were added to each sample and luminescence was measured using an Infinite 200 Microplate Reader.

The 50 % inhibitory concentration (IC_{50}) was calculated based on the antibody dilution that caused a 50% reduction in relative luminescence units (RLU) compared to the virus control wells after subtraction of cell control RLUs.

3.2.5 Assessment of cell viability in the presence of the recombinant antibodies

Briefly, 10,000 TZM-bl cells per well were seeded in a 96-well plate in 100 µl/well of DMEM-10 and incubated overnight at 37°C. The next day, the media was removed and 100 µl of serial diluted V_LB12, V_LCD4 or HEPES buffer alone, were added to the cells and incubated for 1 h at 37°C. Then, 100 µl of DMEM-10 was added to the cells. The cells were incubated for 48 h at 37°C in 5 % CO_2. 100 µl assay medium was removed from each well and 10 µl AlamarBlue cell viability reagent (Invitrogen, UK) was added. The cells were incubated for 2 h at 37°C in 5 % CO_2. Cell viability was assessed by fluorescence emission intensity at 570 nm in an Infinite 200 Microplate Reader (Tecan i-control software, version , 1.5.14.0, Tecan, North Carolina, USA).

3.2.6 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, 2007, San Diego, California, USA, www.graphpad.com) with a level of significance of 5%. IC_{50s} were estimated by the sigmoidal dose-response (variable slope) equation - GraphPad Prism version 5.00.

3.3 Results

3.3.1 HIV-1 inhibition assays

HIV-1 inhibition assays were performed in TZM-bl cells, a HeLa cell line that was engineered to express CD4 and CCR5 and contains integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under the control of the HIV-1 LTR.

In order to evaluate the V_L B12 and V_L CD4 ability to inhibit HIV-1 infection, inhibition assays were performed with the HIV-1 NL4-3 subtype B molecular clone[2] in in TZM-bl cells in a single cycle infection assay. The HIV-1_{NL4-3} inhibition assays were performed with an MOI = 0,5 and the Luciferase activity was measured after 48 h of incubation and is directly proportional to the number of infectious virus particles that entered the cell.

The HIV-1_{NL4-3} inhibition assays performed to evaluate V_LB12 entry inhibitor capability were performed with a starting concentration of 21 μM. TZM-bl cells were incubated for 1 h with nine serial dilutions of V_LB12, afterwich HIV-1_{NL4-3} was added at the indicated MOI. As shown in Figure 3.1, V_LB12 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM.

In the case of V_LCD4, the HIV-1_{NL4-3} inhibition assays were performed with a starting concentration of 10,5 μM. TZM-bl cells were also incubated for 1 h with nine serial dilutions of V_LCD4, afterwich HIV-1_{NL4-3} was added at the indicated MOI. As shown in Figure 3.1, V_LCD4 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 6,57 μM.

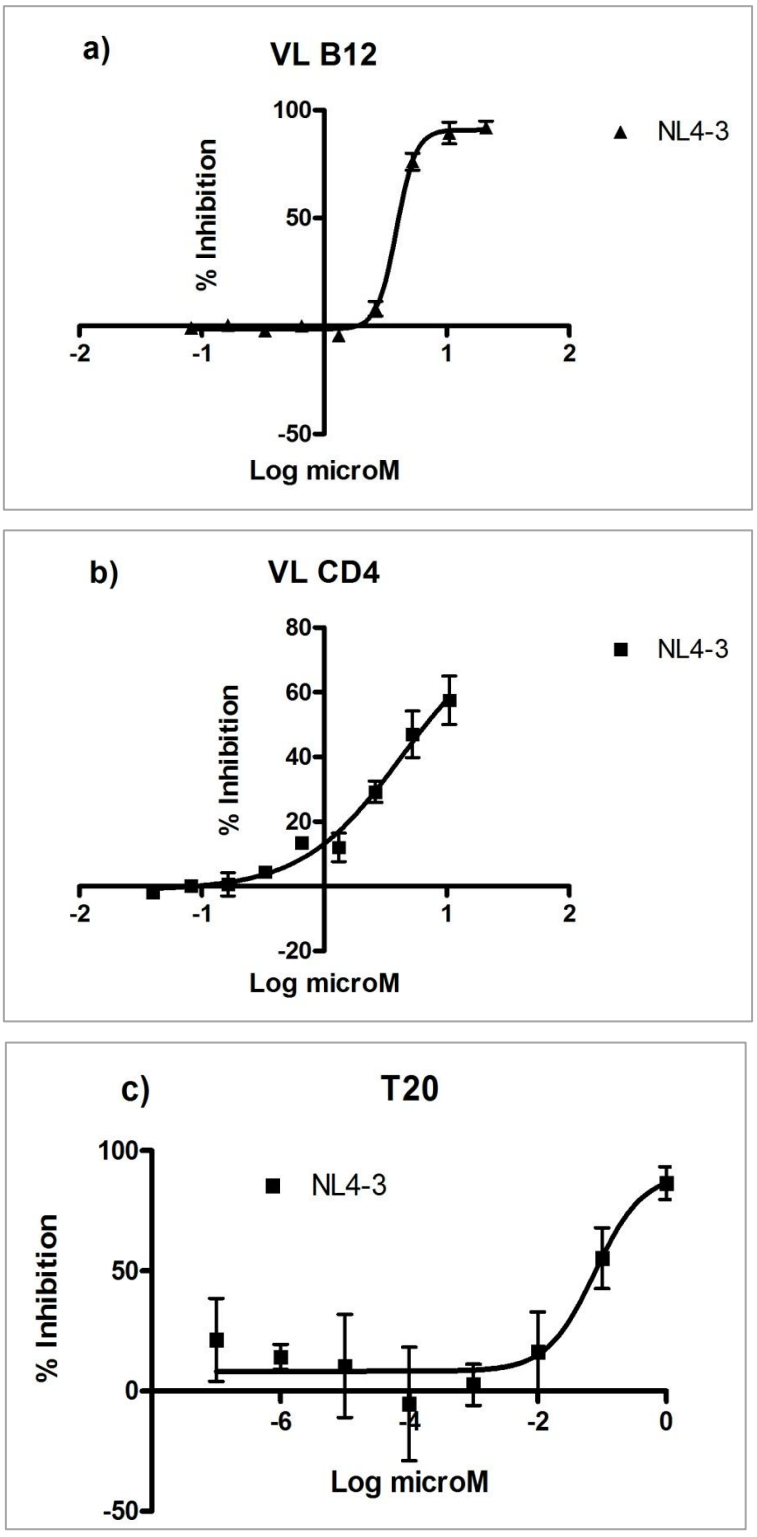


Figure 3.1 – HIV-1_{NL4-3} inhibition assays performed in TZM-bl cells.

a) HIV-1_{NL4-3} inhibition assays using V_LB12 in TZM-bl cells. TZM-bl cells were incubated for 1 h with nine serial dilutions of V_LB12, afterwich HIV-1_{NL4-3} was added at an MOI = 0,5 (200 µl final volume). Assay was performed with a V_LB12 starting concentration of 21 µM. A sigmoidal dose response curve was calculated and an IC_{50} = 3,89 µM was obtained. b) HIV-1_{NL4-3} inhibition assays using V_LCD4 in TZM-bl cells. . TZM-bl cells were incubated for 1 h with nine serial dilutions of V_LCD4, afterwich HIV-1_{NL4-3} was added at an MOI = 0,5 (200 µl final volume). (Continues on the next page)

Assay was performed with a V_LCD4 starting concentration of 10,5 μM. A sigmoidal dose response curve was calculated and an IC_{50} = 6,57 μM was obtained. c) HIV-1_{NL4-3} inhibition assays using T20 in TZM-bl cells. HIV-1_{NL4-3} was incubated for 1 h with eight serial dilutions of T20, afterwich HIV-1_{NL4-3} incubated with the T20 were added at an MOI = 0,5 (200 μl final volume) to TZM-bl cells. Assay was performed with a T20 starting concentration of 1 μM. A sigmoidal dose response curve was calculated and an IC_{50} = 0,0694 μM was obtained. Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate.

The starting concentrations for both V_LB12 and V_LCD4 were dependent on the maximum concentrations at which the purified dAbs are stable (see section 2.3.5). Since V_LCD4 is only stable at a maximum of 42 pmol/μl in HEPES buffer, and V_LB12 is stable at a maximum of 84 pmol/μl in HEPES buffer, this translated into different starting concentrations for both dAbs.

Although the obtained IC_{50} for V_LB12 was 3,89 μM and the IC_{50} obtained for V_LCD4 was 6,57 μM, the maximum percentage of inhibition achieved with V_LB12 was 92,0 % but for V_LCD4 was only 57,5 %. Moreover at 10,5 μM the percentage of inhibition achieved with V_LB12 was 89,4 % as opposed to the 57,5 % for V_LCD4. This shows that V_LB12 is a more potent entry inhibitor that V_LCD4 when tested against HIV-1_{NL4-3} (subtype B molecular clone). T20 inhibition assays of HIV-1_{NL4-3} were also performed and the IC_{50} obtained for T20 was 0,0694 μM.

While performing HIV-1 inhibition assays it was also necessary to evaluate the effect of the dAbs and also of the HEPES buffer solution in cellular viability for the TZM-bl cell line in the concentration range used for the inhibition assays. TZM-bl cell viability assay was performed in the same conditions as the HIV-1 inhibition assays, only instead of HIV-1_{NL4-3} only DMEM-10 medium was added to the cells after the 1h incubation with either HEPES buffer or the dAbs.

The effect of T20 on TZM-bl cellular viability was not evaluated since T20 is a compound already approved for clinical use and therefore does not affect cellular viability.

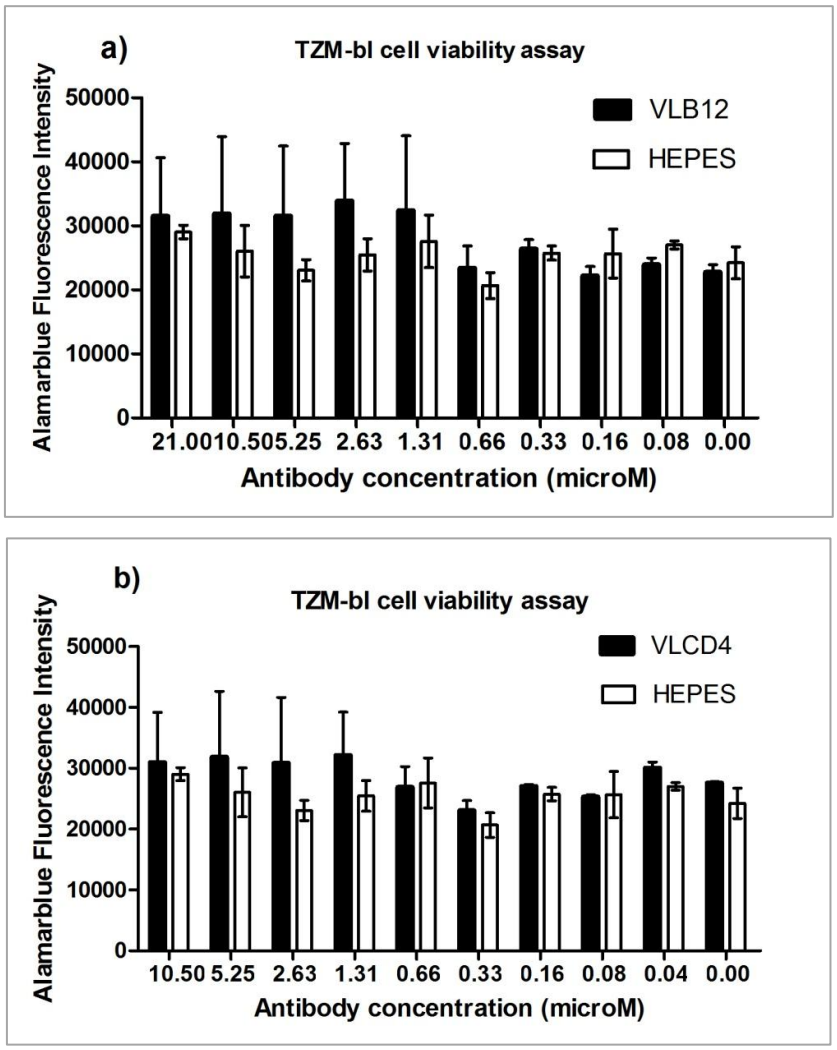


Figure 3.2 – TZM-bl cell viability was evaluated using alamarBlue.
TZM-bl cells were incubated with either HEPES buffer alone or with each dAb. Assay was peformed with a) V_L B12 and HEPES buffer or b) V_L CD4 and HEPES buffer. TZM-bl cells were incubated for 1 h with nine serial dilutions of each dAb, afterwich DMEM-10 was added to a final volume of 200 µl. Cell viability was evaluated 48 h after dAbs were added to the cells. 100 µl of cell media were removed and 10 µl of alamarBlue cell viability reagent were added to the cell medium. After 2 h of incubation at 37 ºC cell viability was assessed by fluorescence spectrophotometry. Results are expressed as mean ± SD of at least 3 independent experiments performed in triplicate.

The results shown on Figure 3.2 prove that both V_LB12 and V_LCD4 do not decrease TZM-bl cell viability, this indicates that the dAbs do not possess a cell toxicity effect as far as analysis of cell proliferation and cytotoxicity is measurable by AlamarBlue.

After performing the inhibition assays for HIV-1_{NL4-3} it was important to evaluate the inhibitory capability of both V_LB12 and V_LCD4 on HIV-1 primary isolates, and in particular for other HIV-1 subtypes.

In order to evaluate the V_LB12 and V_LCD4 ability to inhibit HIV-1 non B subtypes, inhibition assays were performed with two HIV-1 primary isolates, a subtype J primary isolate and a subtype H primary isolate. T20 inhibition assays were also performed.

HIV-1 primary isolates inhibition assays were performed using TZM-bl cells in a single cycle infection assay. Since these subtypes are not as infectious as NL4-3, infection assays were performed with an MOI = 10.

V_LB12 entry inhibitory capability for a subtype J and a subtype H primary isolates were performed with a starting concentration of 21 μM. TZM-bl cells were incubated for 1 h with nine serial dilutions of V_LB12, afterwich either subtype J or subtype H was added at the indicated MOI. As for HIV-1_{NL4-3} inhibition assays, V_LCD4 starting concentration was 10,5 μM. TZM-bl cells were also incubated for 1 h with nine serial dilutions of V_LCD4, afterwich either subtype J or subtype H was added at the indicated MOI. T20 inhibition assays for subtype J and subtype H primary isolates were performed with a starting concentration of 8,5 μM. Either HIV-1 subtype J or subtype H were incubated for 1 h with eight serial dilutions of T20, and afterwards the virus incubated with the T20 were added at the indicated MOI to TZM-bl cells.

As shown in Figure 3.3, V_LB12 was able to inhibit HIV-1 subtype H with an IC_{50} = 3,97 μM and HIV-1 subtype J with an IC_{50} = 3,86 μM. V_LCD4 was not able to inhibit either subtype J or subtype H primary isolates. As for T20, it was able to inhibit HIV-1 subtype H with an IC_{50} = 1,33 nanoM and HIV-1 subtype J with an IC_{50} = 0,42 nanoM.

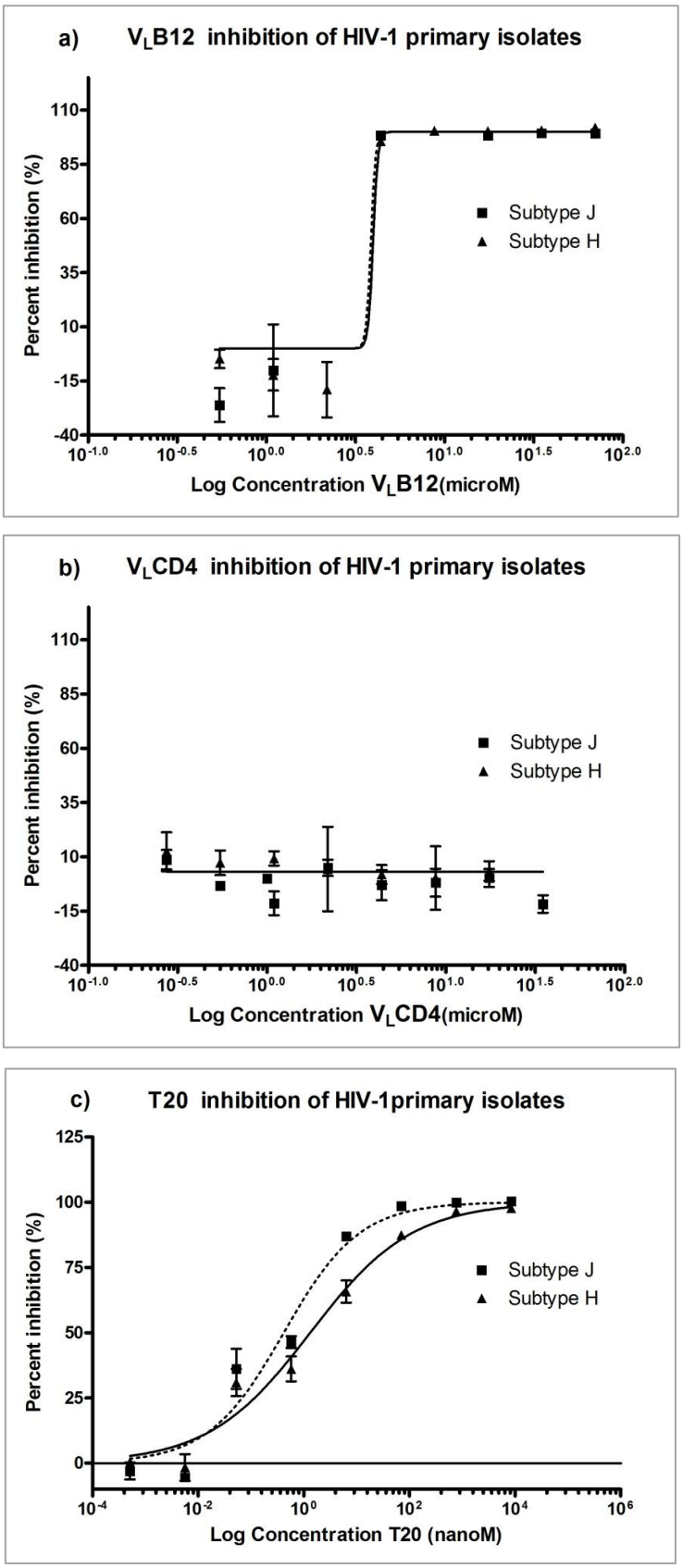


Figure 3.3 – HIV-1 primary isolates inhibition assays using TZM-bl cells.

a) HIV-1 primary isolates, subtypes H and J, neutralization assays using V_LB12 in TZM-bl cells. Assay was performed with a V_LB12 starting concentration of 21 μM. (continues on the next page)

A sigmoidal dose response curve was calculated and an IC_{50} = 3,97 μM was obtained for HIV-1 subtype H and IC_{50} = 3,86 μM was obtained for HIV-1 subtype J. b) HIV-1 primary isolates, subtypes H and J, neutralization assays using V_LCD4 in TZM-bl cells. Assay was performed with a V_LCD4 starting concentration of 10,5 μM. No sigmoidal dose response curve was calculated because no inhibition was obtained for HIV-1 subtype H or subtype J. . c) HIV-1 primary isolates, subtypes H and J, neutralization assays using T20 in TZM-bl cells. Assay was performed with a T20 starting concentration of 8,5 μM. A sigmoidal dose response curve was calculated and an IC_{50} = 1,33 nanoM was obtained for HIV-1 subtype H and IC_{50} = 0,42 nanoM was obtained for HIV-1 subtype J. Results are expressed as mean ± SD of 3 independent experiments performed in duplicate.

3.4 Discussion

The HIV-1_{NL4-3} inhibition assays performed to evaluate V_LB12 and V_LCD4 entry inhibitory capability demonstrated that V_LB12 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM and V_LCD4 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 6,57 μM.

While the maximum percentage of inhibition achieved with V_L B12 was 92,0 %, for V_LCD4 was only 57,5 %. Moreover at 10,5 μM the percentage of inhibition achieved with V_LB12 was 89,4 % as opposed to the 57,5 % for V_LCD4. This shows that V_LB12 is a more potent entry inhibitor that V_LCD4 when tested against HIV-1_{NL4-3} (subtype B molecular clone).

When compared to T20 (IC_{50} = 0,0694 μM) both dAbs present a much higher IC_{50}, indicating that the dAbs are less potent entry inhibitors than T20 against HIV-1_{NL4-3}.

With regard to the V_LB12 and V_LCD4 ability to inhibit HIV-1 non B subtypes, inhibition assays were performed with two HIV-1 primary isolates, a subtype J primary isolate and a subtype H primary isolate. V_LB12 was able to inhibit HIV-1 subtype H with an IC_{50} = 3,97 μM and HIV-1 subtype J with an IC_{50} = 3,86 μM but V_LCD4 was not able to inhibit either subtype J or subtype H primary isolates.

V_LB12 presented an IC_{50} almost identical for either HIV-1_{NL4-3}, subtype J or subtype H primary isolates that could indicate a potent broad spectrum of both laboratory-adapted and clinical HIV-1 isolates inhibitory capability.

Since V_LCD4 was unable to inhibit neither of the HIV-1 non B subtypes tested and due to its poor performance, when compared to V_LB12, it is reasonable to assume that V_LB12 is a much more promising entry inhibitor, with no cell toxicity effect as far as analysis of cell proliferation and cytotoxicity was measurable in the conditions tested.

Regarding the primary isolates inhibition assays, T20 was able to inhibit HIV-1 subtype H with an IC_{50} = 1,33 nanoM and HIV-1 subtype J with an IC_{50} = 0,42 nanoM. So, as for HIV-1_{NL4-3} inhibition assays T20 presents a much lower IC_{50} and is also a more potent entry inhibitor against the primary isolates tested, but T20 has a different target than the dAbs. T20 targets gp41 and due to the virus high frequency of mutation many strains show broad cross-resistance to antiretroviral drugs, including T20.[184,212,257]

It is also important to compare the V_LB12 with the only entry inhibitor that targets the gp120-CD4 interaction. Ibalizumab blocks CD4-dependent virus entry and inhibits a broad spectrum of both laboratory-adapted and clinical HIV-1 isolates, including CCR5-tropic and CXCR4-tropic strains from multiple subtypes, with 50% inhibitory concentrations (IC_{50}s) of 0.0004 to 0.152 μg/ml (0,0027 to 1,026 nM).[301] The IC_{50} for V_LB12 is much higher than Ibalizumab's, when comparing the IC_{50} for HIV-1_{NL4-3}, Ibalizumab has an IC_{50} of 0,73 μg/ml (4,93 nM)[360] and V_LB12 was only able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM which is almost 1000 times higher.

It has already been reported *in vitro* studies that demonstrate the synergistic activity of Ibalizumab (TNX-355) and enfuvirtide (T20) against HIV-1.[360] Implying that an entry inhibitor that targets the gp120-CD4 interaction, like V_LB12, can also prove to be useful as a therapeutic aid in improving the therapeutic response of enfuvirtide and other entry inhibitors treated patients. Although it is not as potent as Ibalizumab, V_L B12 may prove to be another non pharmacological therapeutical alternative in the treatment of HIV-1 infection, since entry inhibitors that target CD4 hopefully will be less susceptible to broad cross-resistance than antiretroviral drugs that target the virus itself.

3.5 Acknowledgments

T20 inhibition assays with HIV-1_{NL4-3} were performed by Catarina Santos. HIV-1 primary isolates inhibition assays were performed by Pedro Borrego and Cheila Rocha under the supervision of Nuno Taveira.

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V_L B12 coated nanoparticles - A new therapeutic delivery system

4 VL B12 coated nanoparticles - A new therapeutic delivery system

4.1 Introduction

4.1.1 Nanoparticles

The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action. In most of the conventional dosage forms, only a small amount of administered dose reaches the target site, while the majority of the drug distributes throughout the rest of the body in accordance with its physicochemical and biochemical properties. Therefore, developing a drug delivery system that optimizes the pharmaceutical action of a drug while reducing its toxic side effects *in vivo* is a challenging task.

One approach is the use of synthetic nonviral vectors such as cationic liposomes and cationic polymers that can provide site specific or targeted drug delivery combined with optimal drug release profiles. Liposomes present some technological limitations including poor reproducibility and stability, and low drug entrapment efficiency. Nevertheless, several low molecular weight drugs are now commercially available which employ this technology. [198,229]

Polymeric nanoparticles, which possess a better reproducibility and better stability profiles than liposomes, have been developed as alternative drug carriers that overcome many of the liposome formulation problems. These polymeric nanoparticles are composed of polysaccharides that are derived from natural sources, and therefore they are expected to be nontoxic, biocompatible, and biodegradable.[198,229]

Nanoparticles are solid colloidal particles with diameters ranging from 1-1000 nm. They consist of macromolecular materials and can be used therapeutically as drug carriers or as adjuvant in vaccines in which the active ingredient is dissolved, entrapped, encapsulated, adsorbed or chemically attached.

There are two types of nanoparticles depending on the preparation process: nanospheres and nanocapsules. Nanospheres have a monolithic-type structure (matrix) in which

drugs are dispersed or adsorbed onto their surfaces. Nanocapsules exhibit a membrane-wall structure and drugs are entrapped in the core or adsorbed onto their exterior.[8]

Nanoparticles not only have potential as drug delivery carriers as they offer non-invasive routes of administration such as oral, nasal and ocular routes, but also show to be good adjuvant for vaccines.[77,186]

Nanoparticles being compact are well suited to traverse cellular membranes to mediate drug or gene delivery. It is also expected that due to smaller size, nanoparticles will be less susceptible to reticuloendothelial system clearance and will have better penetration into tissues and cells, when used in *in vivo* therapy.[361]

4.1.2 Chitosan (CS) nanoparticles

Chitosan is a modified natural carbohydrate polymer prepared by the partial N-deacetylation of chitin, a natural biopolymer derived from crustacean shells such as crabs, shrimps and lobsters. Chitosan is also found in some microorganisms, yeast and fungi.[145,198,225]

Among water-soluble polymers available, chitosan is one of the most extensively studied. This is because chitosan possesses some ideal properties of polymeric carriers for nano- and microparticles for controlled drug release, such as biocompatible, biodegradable, nontoxic, inexpensive, and providing versatile routes of administration. Chitosan nanoparticles also avoid the use of hazardous organic solvents while fabricating particles since they are soluble in aqueous acidic solution.[225]

4.1.3 Polyethylenimine (PEI) nanoparticles

Recently, the use of non-viral vectors such nanoparticles to deliver plasmid DNA (pDNA) have been highlighted. Poly(ethylenimine) (PEI) has been revealed to be the most effective nonviral vector based on cationic polymers owing to its high pH buffering capacity that is believed to enhance the exit of vectors from the endosomal compartment for subsequent release of DNA–PEI complex.[229] Different types of polyethylenimine (PEI), branched (25 and 800 kDa) and linear (25 kDa), have been successfully used.[247]

Polycationic polymers, particularly polyethylenimine (PEI), have the ability to bind with pDNA easily, and can be applied to a wide variety of cells. PEI condenses DNA into positively charged particles, which bind to anionic cell surface residues and these condensed particles can enter the cell via endocytosis.[153,157,229,361]

These unique characteristics make PEI nanoparticles uniquely suited for delivery of DNA to a target cell, however, a major disadvantage of using PEI nanoparticles is that a considerable toxicity associated with PEI at the cellular level is widely demonstrated.[229]

4.1.4 Nanoparticle formulations with V_L B12 for FUGW-dsRed delivery to CD4 positive cells.

Bearing in mind both the advantages and disadvantages of either CS or PEI nanoparticles, in this Chapter we wanted to evaluate both nanoparticles formulations for V_LB12 coated nanoparticles delivery to CD4 positive cells. In order to evaluate nanoparticles delivery to the cells, we encapsulated in either CS or PEI nanoparticles a pDNA, FUGW-dsRed , that is a lentiviral vector that once it successfully enters the cell expresses the DsRed red fluorescent protein. The DsRed fluorescence can be detected either by immunofluorescence or by Flow cytometry.

4.2 Materials and Methods

4.2.1 Cell Lines and culture conditions

Jurkat E6-1 T-cells obtained through the NIH AIDS Research and Reference Reagent Program (MD, USA, contributor Dr. Arthur Weiss) were cultured in Roswell Park Memorial Institute medium (RPMI)-1640, supplemented with 10 % of fetal bovine serum (FBS) (RPMI-10).

Cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

4.2.2 Determination of the optimal pDNA/nanoparticle ratio for CS/DS and PEI nanoparticles

The optimal pdna/nanoparticle ratio for nanoparticle encapsulation was determined by agarose gel electrophoresis. CS/DS and PEI nanoparticle formulations we ran in a 1 % agarose gel (in tris-borate- EDTA buffer (TBE buffer)) in order to determine if the plasmid DNA was properly encapsulated in the nanoparticles in the presence of V_LB12 or in the absence of V_LB12. The DNA ladder used was a 1 kb DNA ladder (New England Biolabs, MA, USA) and was ran as a control for DNA migration in the agarose gel.

4.2.3 Preparation of chitosan-sodium deoxycholate (CS/DS) nanoparticles

The Low molecular weight chitosan (CS, 75-85% degree of deacetylation) and sodium deoxycholate (DS) were obtained from Sigma-Aldrich. CS/DS nanoparticles were prepared using an ionic gelation technique as previously described. [230] Briefly, CS and DS were separately dissolved in ultra-pure water in order to obtain 1 mg/ml solutions. CS/DS nanoparticles were formed on a 1:1 (weight:weight, w/w) ratio, when DS solution was added to a CS solution. For the nanoencapsulation, FUGW-dsRed plasmid was added to DS solution before the addition to the CS solution, at a proportion of

weight: weight of 20% pDNA: chitosan. The plasmid adsorption to the nanoparticles surface was done at 4ºC for 24 hours.

Table 4.1 -V_LB12 coated CS/DS nanoparticle formulations with encapsulated FUGW-dsred plasmid

Formulation	Amount of V_LB12 (μg/ml)	Amount of FUGW-dsREd (μg/well)
b)	0	0
c)	105	0
d)	84	0
e)	47	0
f)	0	1
g)	105	1
h)	84	1
i)	47	1

4.2.4 Preparation of PEI based nanoparticles

Polyethyleneimine (PEI 25 kDa; branched) was obtained from Sigma. A stock PEI solution was prepared in water at a final concentration of 1 mg/mL (pH 7.0) and sterilized by filtration. For each 2.5 μg of DNA and the amount of PEI necessary to have a ratio DNA:PEI 1:5 (W/W), were added separately to 50 μL of 150 mM NaCl. Prior to transfection, the PEI/NaCl solution was added to the DNA/NaCl solution and allowed to stand at room temperature for 10 min before addition to the culture.

Table 4.2 - V_LB12 coated PEI nanoparticle formulations with encapsulated FUGW-dsred plasmid

Formulation	Amount of V_LB12 (μg/ml)	Amount of FUGW-dsREd (μg/well)
b)	0	0
c)	48	0
d)	29	0
e)	11	0
f)	0	0,5
g)	48	0,5
h)	29	0,5
i)	11	0,5

4.2.5 Transfection assays for immunofluorescence assays

For transfection assays Jurkat E6-1 T-cells were grown at 37 ºC with 5% CO_2 in a humidified atmosphere in RPMI-10 medium supplemented with 100 U penicillin, and 0.1 mg streptomycin (Invitrogen, UK) per millilitre. Cells were grown in 96-well white clear bottom plates (Greiner-Bio One, Frickenhausen, Germany) to approximately 50-60% confluence and transfected using CS/DS, containing 1 μg of FUGW-dsRed plasmid per well at optimal amount of adsorbed antibody, controls of cells without nanoparticles and with empty nanoparticles were also run. 24h after transfection, the medium was changed. The fluorescence of cells was measure directly on plate, 24 h and 72 hours after transfection. The fluorescence was measured at an excitation wavelength of 554 nm and emission wavelength of 591 nm, using an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA).

4.2.6 Immunofluorescence

The fluorescence of Jurkat cells was measure at an excitation wavelength of 554 nm and emission wavelength 591 nm. Cells were recovered and counted with trypan blue and fixed for microscope fluorescence picture. The fluorescence of the cells that were transfected was subtracted from the controls and normalized by cell number and per 24h.

For fixation cells were rinsed twice with PBS and fixed for 30 min in a 4% (v/v) *p*-formaldehyde (PFA) (Merck, Darmstadt, Germany) and 4% (w/v) sucrose (Applichem, Darmstadt, Germany) solution, in PBS (Merck, Germany). After two washes with PBS, free aldehyde groups were quenched for 15 min with 50 mM NH_4Cl (Merck, Germany) in PBS. Cell slides were mounted in fluorescent mounting medium (S3023; Dako, Glostrup, Denmark) or ProLong® Gold antifade reagent with DAPI and their fluorescence was observed and recorded on an Axiovert 40CFL fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam MRc5 (Carl Zeiss) camera. Images were processed with the software AxioVision Rel. 4.6.3 (Carl Zeiss).

4.2.7 V_LB12 coated nanoparticles targeting CD4 cell receptor for FUGW-dsred plasmid delivery to Jurkat cells

Briefly, 20,000 Jurkat cells per well were seeded in a 96-well plate in 100 µl/well of RPMI-10 and incubated at 37°C in 5 % CO_2. 24h latter, 50 µl/well of RPMI-10 were added. 48 h after cells were seeded; each nanoparticle formulation (see Table 4.1 and Table 4.2) was added to the media and incubated at 37°C in 5 % CO_2. Cell culture media (RPMI-10 supplemented with gentamicin) was replaced at 24h and 48h. Cells were recovered 72 h post nanoparticle delivery.

4.2.8 Flow cytometry analysis of nanoparticle delivery to Jurkat cells

For each assay condition, of the V_LB12 coated nanoparticles targeting CD4 cell receptor for FUGW-dsred plasmid delivery to Jurkat cells (see 4.2.7), cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS).

All flow data were acquired on a BD Calibur (BD Biosciences, Franklin Lakes, NJ USA), 10 000 gated cells were acquired for each assay condition and data analysis was performed using Flowjo software 6.4.7 (BD Biosciences, Franklin Lakes, NJ USA).

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4.3 Results

In this chapter we wanted to evaluate the ability to use V_LB12 as a vehicle for target specific drug delivery, using as carriers either CS/DS or PEI nanoparticles. To validate the ability of V_LB12 to specifically deliver CS/DS or PEI nanoparticles to CD4 positive cells we used as a reporter gene dsRed, a red fluorescent protein chromophore cloned in the FUGW lentiviral vector (Addgene plasmid repository, Cambridge, MA, USA).[197] Cells with positive nanoparticles delivery of the dsRed reporter gene, will express the dsRed red fluorescent protein. The dsRed fluorescence is detectable either by immunofluorescence or by Flow cytometry.

4.3.1 Determination of the optimal pDNA/nanoparticle ratio for CS/DS and PEI nanoparticles

The optimal pdna/nanoparticle ratio (W/W) is usually between 20 to 40 % in order for a proper nanoparticle packaging to occur. Several pdna/nanoparticle ratios were tested either for CS/DS or PEI. The optimal pdna/nanoparticle ratio for nanoparticle encapsulation was determined by agarose gel electrophoresis.

Legend

lane 1- DNA Ladder
lane 2- CS/DS nanoparticles
lane 3- CS/DS nanoparticles + V_L B12
lane 4- PEI nanoparticles
lane 5- PEI nanoparticles + V_L B12
lane 6- FUGW-dsRed plasmid

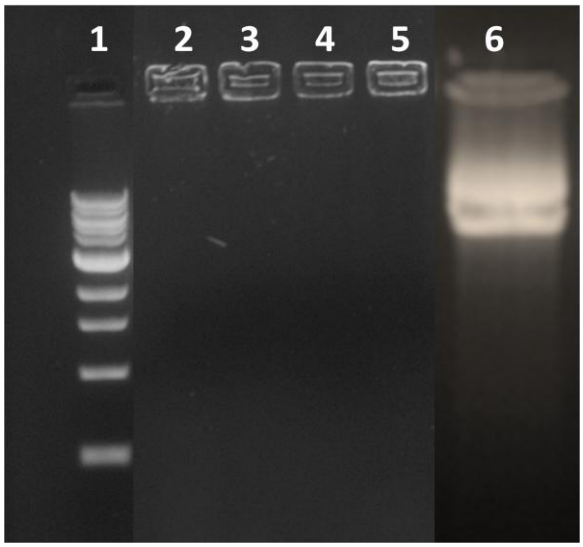


Figure 4.1 – Nanoparticle encapsulation as determined by agarose gel electrophoresis.
CS/DS and PEI nanoparticle formulations we ran in a 1 % agarose gel in order to determine if the plasmid DNA was properly encapsulated in the nanoparticles in the presence of V_LB12 (lanes 3 and 5) or in the absence of V_LB12 (lanes 2 and 4). The DNA ladder (1 kb DNA ladder, New England Biolabs, MA, USA) was ran in Lane 1 as a control for DNA migration in the agarose gel. The plasmid FUGW-dsRed was ran on lane 6 as a control for free plasmid migration profile.

The results obtained (Figure 4.1) indicate an optimal proportion of 20% (W/W) FUGW-dsRed: chitosan and a FUGW-dsRed:PEI ratio of 1:5 (W/W). There is no plasmid DNA migration in the agarose gel electrophoresis in the lanes where pDNA was encapsulated in the nanoparticles formulations (lanes 2 to 5), this is a measure of the degree of DNA packaging in the nanoparticles. The smaller the amount of free DNA the less DNA is visible migrating in the agarose gel, the more DNA is encapsulated in the nanoparticles. As a control for the migration profile of the FUGW-dsRed plasmid on agarose gel electrophoresis the plasmid alone was ran on lane 6, confirming that on lanes 2 to 5 there was no free plasmid present.

4.3.2 Immunofluorescence

To evaluate the ability to use V_LB12 as a vehicle for target specific drug delivery, using as carriers either CS/DS or PEI nanoparticles, and as a reporter gene the FUGW-dsRed, both nanoparticle systems were optimized for FUGW-dsRed packaging (see section 4.3.1) and the therapeutic delivery system was evaluated by immunofluorescence.

Based on the V_LB12 concentration obtained after the purification and followed by the protein refolding/dialysis step (see section 2.3.5) and based on the characteristics of either CS/DS or PEI nanoparticles formulation the optimal theoretical amount of V_LB12 was adsorbed to either CS/DS or PEI nanoparticles. The specificity of the V_LB12 coated nanoparticle drug delivery systems was evaluated by measuring the expression of the dsRed protein by immunofluorescence (Figure 4.2).

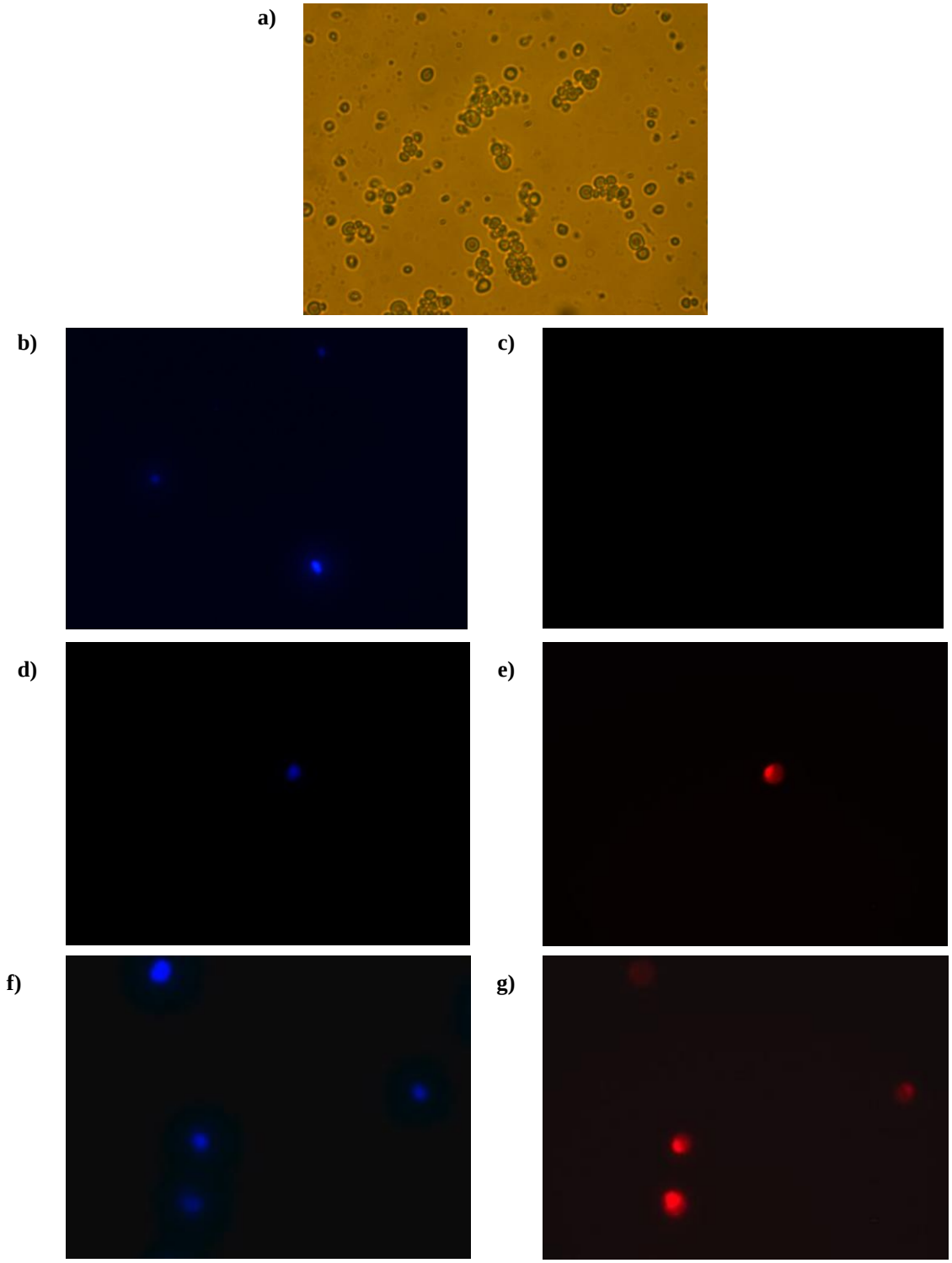


Figure 4.2 – Immunofluorescence staining of FUGW-dsRed/CS/DS nanoparticle complexes using V_LB12 for specific delivery to Jurkat cells.
a) Jurkat cells observed with a magnification of 40X (visible) for CS/DS nanoparticle delivery to Jurkat cells; b) Jurkat cells targeted by empty CS/DS nanoparticles (magnification 40X) stained with DAPI Nuclear Counterstain; **c) Jurkat cells targeted by empty CS/DS nanoparticles (magnification 40X) dsRed fluorescence; d) Jurkat cells targeted by FUGW-dsRed/CS/DS nanoparticles for delivery of the dsRed reporter gene (magnification 100X), stained with** DAPI Nuclear Counterstain; **e) Jurkat cells targeted by FUGW-dsRed/CS/DS nanoparticles for delivery of the dsRed reporter gene (magnification 100X), dsRed fluorescence; (continues on the next page)**

f) Jurkat cells targeted by V_LB12 coated CS/DS nanoparticles for delivery of the dsRed reporter gene (magnification 100X), stained with DAPI Nuclear Counterstain; g) **Jurkat cells targeted by V_LB12 coated CS/DS nanoparticles for delivery of the dsRed reporter gene (magnification 100X), dsRed fluorescence. Fluorescence of the cells was measured at an excitation wavelength 554 nm and emission wavelength of 591 nm. Fluorescence was observed and recorded on an Axiovert 40 CFL fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam MRc5 (Carl Zeiss) camera. Images were processed with the software AxioVision Rel. 4.6.3 (Carl Zeiss).**

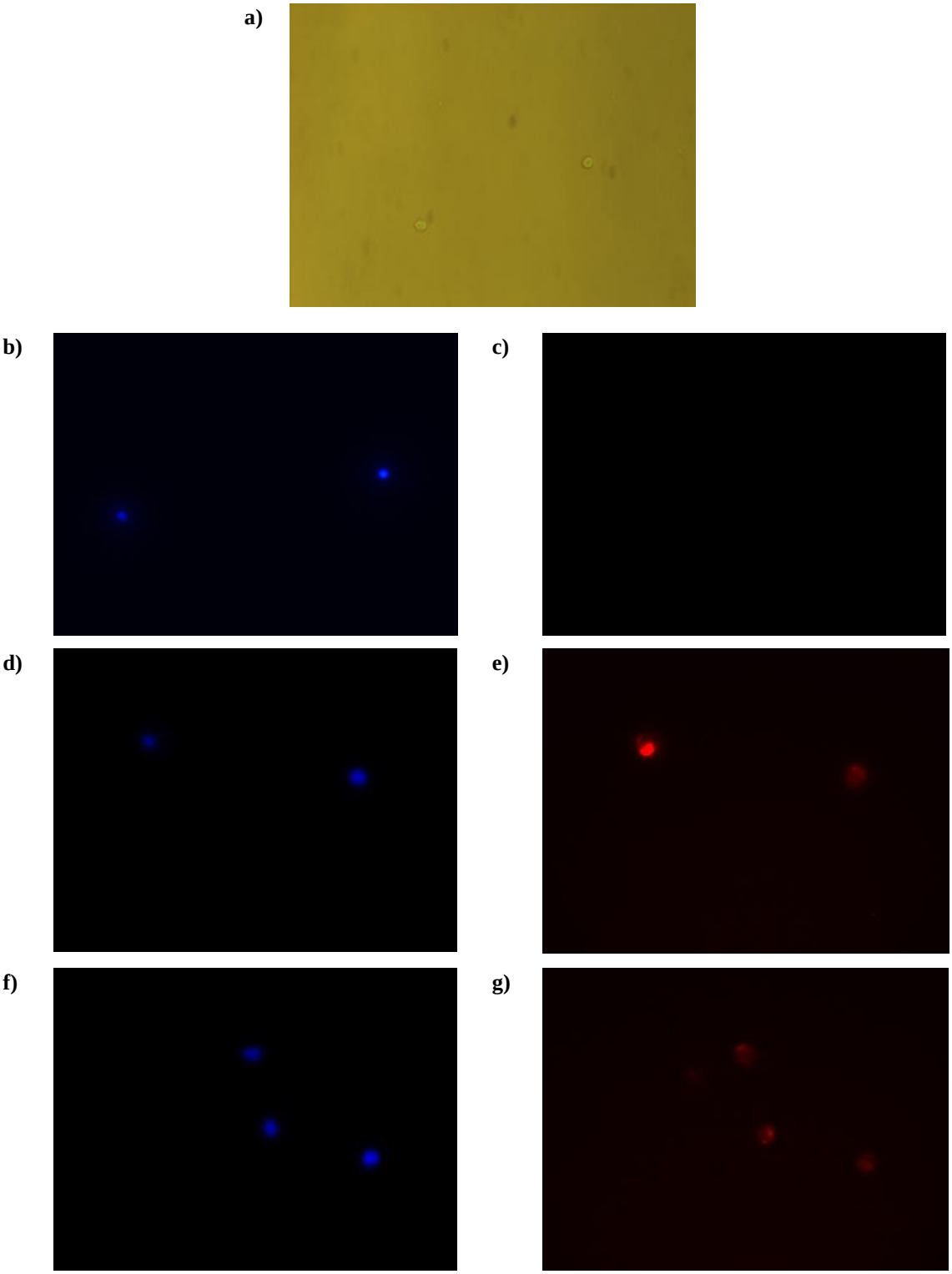


Figure 4.3 – Immunofluorescence staining of FUGW-dsRed/PEI nanoparticle complexes using V_LB12 for specific delivery to Jurkat cells.
a) Jurkat cells observed with a magnification of 40X (visible) for PEI nanoparticle delivery to Jurkat cells; b) Jurkat cells targeted by empty PEI nanoparticles (magnification 40X) stained with DAPI Nuclear Counterstain; **c) Jurkat cells targeted by empty PEI nanoparticles (magnification 40X) dsRed fluorescence; d) Jurkat cells targeted by FUGW-dsRed/ PEI nanoparticles for delivery of the dsRed reporter gene (magnification 100X), stained with** DAPI Nuclear Counterstain; **e) Jurkat cells targeted by FUGW-dsRed/ PEI nanoparticles for delivery of the dsRed reporter gene (magnification 100X), dsRed fluorescence; (continues on the next page)**

f) Jurkat cells targeted by V_L B12 coated PEI nanoparticles for delivery of the dsRed reporter gene (magnification 100X), stained with DAPI Nuclear Counterstain; g) **Jurkat cells targeted by V_LB12 coated PEI nanoparticles for delivery of the dsRed reporter gene (magnification 100X), dsRed fluorescence. Fluorescence of the cells was measured at an excitation wavelength 554 nm and emission wavelength of 591 nm. Fluorescence was observed and recorded on an Axiovert 40 CFL fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with an Axiocam MRc5 (Carl Zeiss) camera. Images were processed with the software AxioVision Rel. 4.6.3 (Carl Zeiss).**

As it is perceivable from Figure 4.2 and Figure 4.3, the presence of V_LB12 greatly increases the amount of the dsRed protein present in the cells for both CS/DS and PEI nanoparticle formulations. The normalized[††] immunofluorescence results for the V_LB12 specific targeting to the CD4 receptor vs the endocytosis of the nanoparticles are listed on Table 4.3, the same data are also represented in a graphical format in Figure 4.4.

Table 4.3 - Normalized results from the fluorescence assay, with CS/DS nanoparticles and PEI Nanoparticles. Assays are representative of n = 4

Nanoparticle formulation		**FU[‡‡]/10^5 cells 24 h post transfection**	**FU/10^5 cells 72 h post transfection**
CS/DS Nanoparticles	**DsRed**	1.530	5.353
CS/DS Nanoparticles	**DsRed+VL B12**	3.701	10.333
PEI Nanoparticles	**DsRed**	7.475	26.163
PEI Nanoparticles	**DsRed+VL B12**	105.229	293.763

The FU value/105 cells for the VLB12 specific nanoparticle/FUGW-dsRed delivery to Jurkat cells can be obtained as the difference between V_LB12 coated CS/DS nanoparticle fluorescence and the CS/DS uncoated nanoparticles, and the same applies for PEI nanoparticles. The calculations for the V_LB12 specificity for both CS/DS and PEI nanoparticles are compiled in Table 4.4.

[††] The fluorescence results were normalized against the background fluorescence from the control Jurkat cells.

[‡‡] **FU-Relative Fluorescence Units**

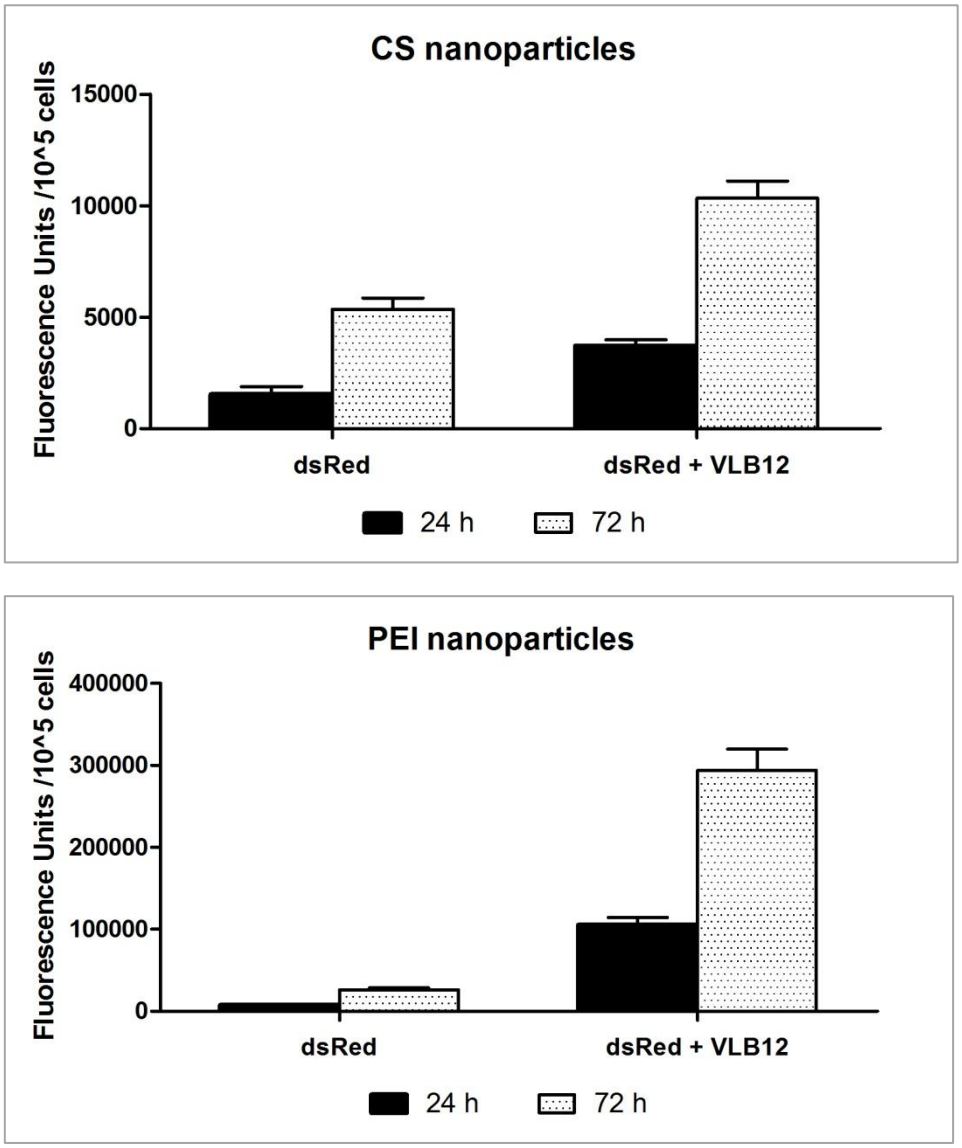


Figure 4.4 - Normalized fluorescence units/10^5 cells for CS/DS and PEI V_LB12 coated nanoparticles delivery of FUGW-dsREd.

For transfection assays Jurkat E6-1 T-cells were grown at 37 ºC with 5% CO_2 in a humidified atmosphere in RPMI-10 medium supplemented with 100 U penicillin, and 0.1 mg streptomycin (Invitrogen, UK) per millilitre. Cells were grown in 96-well white clear bottom plates (Greiner-Bio One, Frickenhausen, Germany) to approximately 50-60% confluence and transfected using CS/DS, containing 1 μg of FUGW-dsRed plasmid per well at optimal amount of adsorbed antibody, controls of cells without nanoparticles and with empty nanoparticles were also run. 24h after transfection, the medium was changed. The fluorescence of cells was measure directly on plate, 24 h and 72 hours after transfection. The fluorescence was measured at an excitation wavelength of 554 nm and emission wavelength of 591 nm, using an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA). Assays are representative of n = 4.

Table 4.4 - Calculations from the fluorescence assay, with CS/DS nanoparticles and PEI Nanoparticles

Nanoparticle formulation	**FU[§§]/10^5 cells 24 h post transfection**	**FU/10^5 cells 72 h post transfection**
CS/DS Nanoparticles	2 171 (58,7 %)	4 980 (48,2 %)
PEI Nanoparticles	97 754 (92,9 %)	267 600 (91,1 %)

For the CS/DS nanoparticles the increase in dsRed fluorescence due to V_LB12 specific CD4 targeting increases by 58,7 % at 24 h post transfection and still presents a 48,2 % increase in fluorescence at 72 h post transfection. In the case of the PEI nanoparticles the V_LB12 specific CD4 targeting increases dsRed fluorescence by 92,9 % at 24 h post transfection and maintains a 91,1 % of increase in fluorescence at 72 h post transfection.

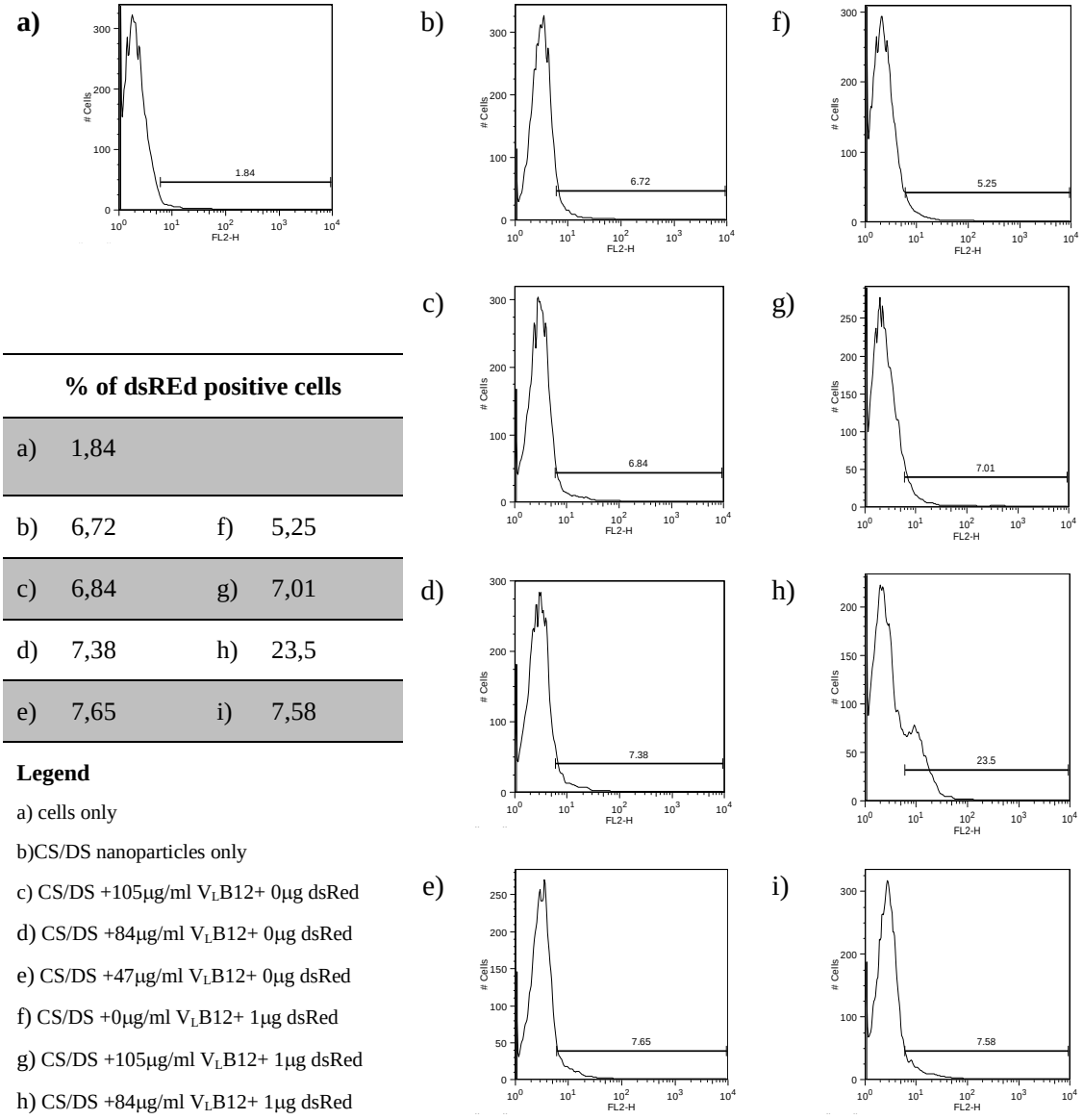
When comparing both nanoparticle systems in terms of FU/10^5 cells only, PEI nanoparticles present a much more effective pDNA delivery system than the CS/DS nanoparticles.

CS/DS nanoparticles show a 10 % decrease in V_LB12 specific dsRed fluorescence from the 24 h time point to the 72 h time point although the normalized FU increased over time. Since the goal is to evaluate not only the increase in dsRed fluorescence due to V_LB12 specific CD4 targeting but also the correlation with maintained cell viability over time, other methods should be used to complement the immunofluoresce assays. In order to obtain more detailed information on specific dsRed fluorescence/cell viability these nanoparticle CD4 targeted delivery systems were also evaluated by Flow cytometry analysis.

[§§] **FU-Relative Fluorescence Units**

4.3.3 V_LB12 coated nanoparticles for delivery of FUGW-dsREd plasmid to CD4 positive Jurkat cells.

Controls **Chitosan nanoparticles**



% of dsREd positive cells			
a)	1,84		
b)	6,72	f)	5,25
c)	6,84	g)	7,01
d)	7,38	h)	23,5
e)	7,65	i)	7,58

Legend

a) cells only

b)CS/DS nanoparticles only

c) CS/DS +105µg/ml V_LB12+ 0µg dsRed

d) CS/DS +84µg/ml V_LB12+ 0µg dsRed

e) CS/DS +47µg/ml V_LB12+ 0µg dsRed

f) CS/DS +0µg/ml V_LB12+ 1µg dsRed

g) CS/DS +105µg/ml V_LB12+ 1µg dsRed

h) CS/DS +84µg/ml V_LB12+ 1µg dsRed

i) CS/DS +47µg/ml V_LB12+ 1µg dsRed

Figure 4.5 – Flow cytometry analysis of chitosan nanoparticles coated with V_LB12 for delivery of FUGW-dsREd plasmid to CD4 positive Jurkat cells.

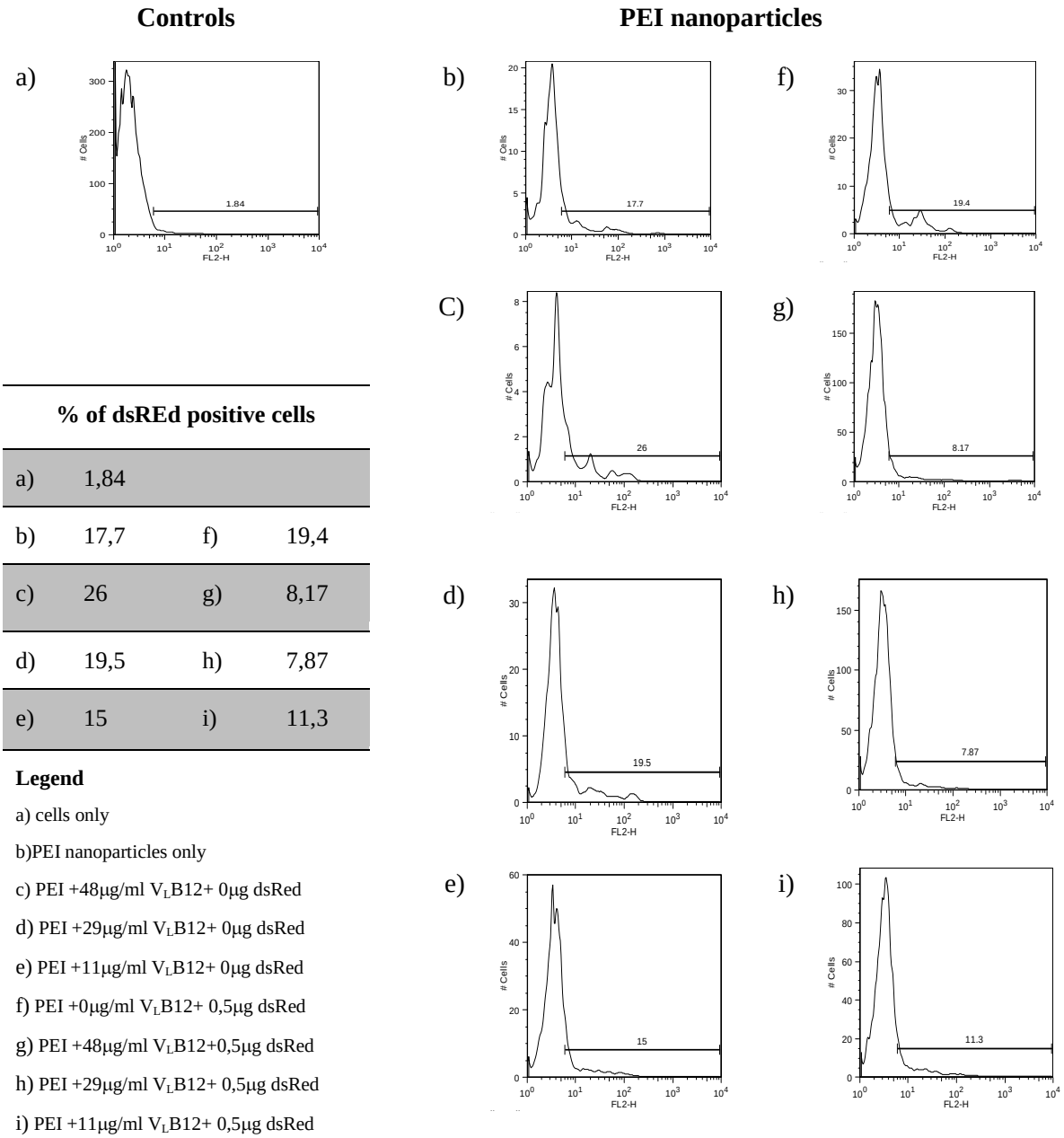
80 000 Jurkat cells were incubated with each nanoparticle formulation. Cell culture media (RPMI-10 supplemented with gentamicin) was replaced at 24h and 48h. Cells were recovered 72 h post nanoparticle delivery. Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS). Condition a) displays the cells only control. Conditions b to i) correspond to the V_LB12/CS/DS nanoparticles/FUGW-dsREd combinations listed on Table 4.1. Shown is one representative experiment out of three.

After the CS/DS formulation for specific delivery of the FUGW-dsRed plasmid to Jurkat cells was optimized and evaluated by immunofluorescence (see 4.2.6), optimal adsorbed amount of V_LB12 was also evaluated by FACS analysis. From the results displayed on

Figure 4.5 it is possible to conclude that background fluorescence is the same for empty particles (condition b), as for particles with FUGW-dsREd plasmid only (condition f).

CS/DS nanoparticles coated with either 105 or 47 μg/ml (conditions c and e) without FUGW-dsREd encapsulated, present approximately the same percentage of dsRed positive cells as CS/DS nanoparticles coated with either 105 or 47 μg/ml (conditions g and i) with encapsulated FUGW-dsREd meaning that there was no FUGW-dsREd delivery to the cells.

Only the formulation with 84 μg/ml of V_LB12 (condition h) is expressing the dsRed gene. Therefore this is the only combination of V_LB12/CS/DS nanoparticles/ FUGW-dsREd where there is a successful delivery of the plasmid expressing the red fluorescent protein. The percentage of effective dsRed positive cells is 16,1 % (23,5 - 7,38) which represents a good performance value for this type of nanoparticles.



% of dsREd positive cells			
a)	1,84		
b)	17,7	f)	19,4
c)	26	g)	8,17
d)	19,5	h)	7,87
e)	15	i)	11,3

Legend

a) cells only

b)PEI nanoparticles only

c) PEI +48µg/ml V_LB12+ 0µg dsRed

d) PEI +29µg/ml V_LB12+ 0µg dsRed

e) PEI +11µg/ml V_LB12+ 0µg dsRed

f) PEI +0µg/ml V_LB12+ 0,5µg dsRed

g) PEI +48µg/ml V_LB12+0,5µg dsRed

h) PEI +29µg/ml V_LB12+ 0,5µg dsRed

i) PEI +11µg/ml V_LB12+ 0,5µg dsRed

Figure 4.6 – Flow cytometry analysis of PEI nanoparticles coated with V_LB12 for delivery of FUGW-dsREd plasmid to CD4 positive Jurkat cells.
80 000 Jurkat cells were incubated with each nanoparticle formulation. Cell culture media (RPMI-10 supplemented with gentamicin) was replaced at 24h and 48h. Cells were recovered 72 h post nanoparticle delivery. Cells were washed with PBS and fixated with 1 % *p*-formaldehyde (in PBS). Condition a) displays the cells only control. Conditions b to i) correspond to the V_L B12/PEI nanoparticles/FUGW-dsREd combinations listed on Table 4.2. Shown is one representative experiment out of three.

The results depicted on Figure 4.6 represent a maximum of approximately 900 gated cells acquired. In the presence of the PEI nanoparticles, most of the conditions depicted present a percentage of dsRed positive cells higher in the controls without the FUGW-dsREd plasmid than in the corresponding V_LB12/PEI nanoparticles/FUGW-dsREd conditions, this is the result of a very high autofluorescence due to the very low

numbers of live cells as opposed to very high amounts of dead cells present in all the samples containing PEI nanoparticles. From approximately 6,4 x 10^5 cells per well expected to be present at the end of the assay (all the cells from each well were collected for Flow cytometry analysis) less than 1 x 10^3 live cells per well could be acquired. This represents that less than 0,15 % of the expected number of cells were alive, on average, at the end of the assay.

4.4 Discussion

The V_LB12 has proved to be an efficient method for specific delivery of nanoparticle encapsulated drugs to CD4 positive cells. When comparing the CS/DS nanoparticles to the PEI nanoparticles we reach the conclusion that PEI nanoparticles although present a good dsRed delivery as obtained by immunofluorescence are very toxic and cause very high levels of cell death.

The results obtained by immunofluorescence indicated that PEI was a much more effective system for drug delivery, and both CS/DS and PEI nanoparticle systems allowed us to validate V_LB12 as an efficient method for specific delivery of nanoparticle encapsulated drugs to CD4 positive cells.

When V_LB12 amounts different from the amount previously optimized by immunofluorescence assays, were used in the Flow cytometry assays no specific targeting could be achieved, this could be due to the fact that process used for coating the nanoparticles with V_LB12 was a not controlled adsorption method, that may not work in all formulations and also the dilution factor associated with this process also interferes in the particle's superficial charge. These may be the reasons why only one of the V_LB12 amounts worked for specific CS/DS nanoparticle formulation and subsequent cell targeting.

In this Chapter we wanted to evaluate the V_LB12 potential for specific delivery of nanoparticle encapsulated drugs to CD4 positive cells and its subsequent use for gene therapy. Consequently, although CS/DS nanoparticles present a much more modest pDNA delivery to the target cells than PEI nanoparticles, PEI's high toxicity limits its application in gene therapy.[229,361]

So, in conclusion the V_LB12/CS/DS nanoparticles is a promising system for specific delivery of nanoparticle encapsulated drugs to CD4 positive cells.

4.5 Acknowledgments

Nanoparticles formulations were performed by Lídia Gonçalves and António J. Almeida. The cloning of the dsREd gene in the FUGW plasmid was performed by Lídia Fonseca.

Andreia Couto was supported by FCT fellowship SFRH/BD/28211/2006 and Lídia Fonseca was also supported by a PhD fellowship from FCT.

V_L B12 as a vaccine antigen

5 V_L B12 as a vaccine antigen

5.1 Introduction

In order to use V_LB12 as a vaccine antigen some considerations must be taken into account, like the type of formulation and the route of administration. Regarding the type of formulation several methods can be addressed, as for the route of administration these range from the classical parenteral routes to the mucosal membranes delivery as well as different delivery systems.[10,77]

5.1.1 Vaccine formulations and vaccine administration

The rout for vaccine administration is of great importance in the development of protective immunity and the choice is most of the times a matter of pharmacokinetics. The pharmacokinetics is of course related with the location of the target effect of active substances. In the case of HIV-1 vaccines, vaccine administration is clearly associated with parenteral routes or with mucosal membranes administration.

Among the non-parenteral routes of administration, mucosal membranes, which possess associated lymphoid tissue with all of the immunocompetent cells required for the induction of antigen specific immune responses, are emerging as attractive routes for vaccination.[161,227]

There are two main administration routes for mucosal vaccine delivery, oral and nasal. The main targeted for oral delivery vaccine are Peyer's patches. By incorporating vaccine into nanoparticles systems, the vaccine is protected against enzymatic degradation on its way to the mucosal tissue and efficiently taken up by M-cells. In contrast to oral administration, nasal administered vaccines have to be transported over a very small distance, remain only about 15 minutes in the nasal cavity, and are not exposed to low pH values and degradative enzymes. Thus, nasally delivery vaccines may not necessary be formulated as nanoparticles.[146,324-326]

Since the nasal cavity has been investigated as a promising site for nanoparticle vaccine administration, different delivery systems have been explored by the nasal route, including CS-based nanoparticles incorporating soluble antigens, and plasmid DNA, which have shown to be efficient for intranasal vaccination, inducing important and long-lasting immune responses.[329,330]

5.1.2 Nanoparticles for delivery of vaccines

Nanoparticles often exhibit significant adjuvant effects in parenteral vaccine delivery since they may be readily taken up by antigent presenting cells.[173] Moreover, oral and nasal delivery of nanoparticles are thought to have the potential to provide mucosal protective immune responses, one of the most desired goals of modern vaccinology. The submicron size of nanoparticles allows them to be taken up by M-cells, in mucosa associated lymphoid tissue (MALT) i.e. gut-associated, nasal-associated and bronchus-associated lymphoid tissue,[146,324-326] initiating sites of vigorous immunological responses. Immunoglobulin A (IgA), a major immunoglobulin at mucosal surface, and the generation of B-cell expressing IgA occur primarily inMALT. The B-cell then leaves the MALT and reaches systemic circulation where they clonally expand and mature into IgA plasma cells. Therefore, providing not only protective IgA at the pathogen entered sites, but also systemic immunity.

5.1.3 Antiviral activities of antibodies *in vivo* – their role in vaccine development

In principle, antibodies can act against both free virus and infected cells. Probably the most marked antiviral activity of antibody and the activity that is most important for antibody-mediated protection *in vivo* is the neutralization of free virus particles.[44]

Animal models provide direct evidence that mechanisms other than neutralization can be important for protection by neutralizing antibodies. In some cases, protection is found to be as effective with $F(ab)_2$ fragments as with the corresponding whole IgG

molecule. However, in other cases, $F(ab)_2$ fragments that are as effective as whole IgG molecules at neutralization *in vitro* are ineffective at protection *in vivo*. It has been shown that neutralizing mouse IgG1 antibodies (which are poor activators of effector functions) are ineffective at protection, whereas IgG2a molecules (which are good activators) of the same specificity are effective.[293] In many examples in mouse models, protection requires the Fc part of IgG, but is independent of complement. This implies that, in these cases, protection by neutralizing antibodies probably requires activity against infected cells and involves ADCC or phagocytosis.[44]

Also, for viruses whose primary route of entry are also mucosal surfaces, antibodies that are present in the mucosal compartments at the time of exposure may protect against viral challenge.[155,232] Both mucosal secretory IgA (sIgA) and systemic IgG have been shown to be effective. And may play an important role in the development of an efficient protective AIDS vaccine.[44]

In summary, a vaccine may generate antibodies that are are ineffective in *in vitro* neutralization assays, but may simply require the activation of the effector functions, involving ADCC or phagocytosis and may also trigger mucosal secretory IgA that may protect against viral challenge. In order to properly evaluate the potential of a particular antigen as a vaccine candidate, several different markers should be evaluated, like IgA and IgG titers, and also IgG1 versus IgG2a titers.

5.2 Materials and Methods

5.2.1 Cell Lines and culture conditions

Human embryonic kidney 293T (HEK293T) (ATCC, VA, USA), and TZM-bl (obtained through the NIH AIDS Research and Reference Reagent Program, MD, USA, contributors Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc.) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) here on designated DMEM-10.

All cell cultures were maintained at 37 °C in 5 % CO_2. All cell culture media and reagents, otherwise indicated, were from Lonza (Basel, Switzerland).

5.2.2 Viral production

HEK293T cells were transfected, by calcium phosphate method, with pHIV-1_{NL4-3} plasmid (AIDS reagent, contributor Dr. Malcolm Martin) in order to produce HIV-1_{NL4-3} virions. After 48 h, virions were collected from supernatant cultures; the amount of viral particles released in the supernatant was measured by $p24_{CA}$ ELISA (AIDS & Cancer Research Program, NCI Frederick, MD, USA) and HIV-1_{NL4-3} virions were used to infect TZM-bl cells.

5.2.3 Virus neutralization assay using TZM-bl cells.

A neutralization assay was performed based on the methods of Wei et al. (28)[341] with modification.[192,215] TZM-bl cells were derived from a HeLa cell line (JC.53) that stably expresses CD4 and CCR5. TZM-bl cells also express luciferase and β-galactosidase under the control of the HIV-1 promoter.

Briefly, 10,000 cells per well were seeded in a 96-well plate in 100 µl/well of DMEM-10 and incubated overnight at 37°C. The next day, 200 µl of HIV-1_{NL4-3} were prepared, in DMEM-10, for a multiplicity of infection (MOI) of 0,1 and serial diluted

serum from each mice group (1/40 – 1/81920 dilution) was added to the virus solution and incubated for 1 h at 37°C. The media was removed and the virus solution added to the cells.

The cells were incubated for 48 h at 37°C in 5 % CO_2. Assay medium was removed from each well, cells were washed with 200 µl of PBS and 50 µl of Passive Lysis Buffer reagent (Promega, Madison, WI) were added. This was followed by a freeze-thaw step (-20 ºC – 37 ºC) afterwhich 10 µl cell lysates from each sample (in Passive Lysis Buffer) were placed in a 96 well Luminescence Plate (BD Falcon 96 Flat Bottom White Opaque Polystyrene Plate, BD Biosciences, USA). 50 µl Luciferase Assay Reagent II (LAR II) were added to each sample and luminescence was measured using an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA).

5.2.4 Preparation of CS/DS

The Chitosan/Deoxycholate (CS/DS) nanoparticles were prepared using an ionic gelation technique as described.[50] Briefly, chitosan (Chitosan low molecular weight (LMW) with degree of deacetylation (DD) 75-85%, Sigma-Aldrich, UK) and sodium deoxycholate (Sigma-Aldrich, UK) were separately dissolved in ultra-pure water in order to obtain 1 mg/ml solutions. Empty nanoparticles were formed when 1mL DS solution was added to a 1mL CS solution. For protein V_LB12 encapsulation, protein was first liofilized in order to concentrate and ressuspended in DS solution before the addition to CS solution. CS/DS nanoparticles were prepared using an ionic gelation technique as previously described. [51]

The encapsulation efficiency of nanoparticle formulations was determined as described,[50] after centrifugation of the samples at 29,000 × *g* for 30 min. Supernatants were analyzed by the BCA protein assay (Pierce, USA) to determine the free protein concentration. Calibration curves were made with corresponding solutions of blank

nanoparticles, and all samples were measured in triplicate. The encapsulation efficiency was determined by difference as follows:

$$Encapsulation\ efficiency\ (\%) = \frac{[Total\ Protein] - [Free\ Protein]}{[\ Total\ protein]} \times 100$$

The percentage of protein encapsulation efficiency was 68 ± 10%.

5.2.5 Nanoparticles Physicochemical Characterization

Mean particle size and polydispersion index (PI) were determined by photon correlation spectroscopy (Zetasizer Nano-S, Malvern Instruments, UK). Samples were diluted with 0.22 μm filtered ultra-pure water. The zeta potential was measured through electrophoresis mobility (Zetasizer 2000; Malvern Instruments, UK) using samples diluted with ultra-pure water. In all cases, mean values were obtained from the analysis of three different batches, each of them measured three times. Results were expressed as mean ± standard deviation (SD).

5.2.6 Mice immunisation schedule

Female BALB/c mice obtained from Charles River (Spain) (n=5/group), 6–8weeks old provided with food and drink *ad libitum*, were used in the *in vivo* studies, which were performed in strict accordance with Directive of 24 November (nº 86/609 EEC), the Portuguese laws D.R. nº 31/92, D.R. 153 I-A 67/92, and all following legislations.

Seven groups (25 g; n 5/group) were immunised by intranasal (i.n.) and subcutaneous (s.c.) routes on day 1 and boosted on day 21, for i.n. route a micropipette tip was used to administer 20 µl of sample (10 µl in each nostril) containing 50 µg of V_LB12 protein. The formulations were delivered slowly onto nares, so that the mice could inhale it i.n.

Controls consisted of free antigen or empty nanoparticles. All formulations were freshly and aseptically prepared by particles dispersion in PBS pH 7.4 in a biological safety cabinet, immediately prior dosing. A group was vaccinated by s.c. route with protein adsorbed to gel of aluminium hydroxide (Sigma-Aldrich, UK): the protein was mixed with a solution of gel of aluminium hydroxide in a proportion in order that the dose of the vaccine did not exceed the 0.2 mg of aluminium containing 50 µg of protein.

Blood samples were collected from the tail vein after 2, 4, 6, 8, 10, 12, 14 and 16 weeks of immunization and sera was separated by centrifugation (18000 x g, 5min at 4 ºC, Allegra 64R, Beckman, USA) and stored at −20 ºC until tested by antigen specific enzyme-linked immunosorbent assay (ELISA) for IgG, IgG subclass 1 (IgG1) and IgG subclass 2 (IgG2a).

5.2.7 Quantification of antigen-specific IgG and subtypes by ELISA

The antibody responses (IgG, IgG1 and IgG2a) to V_LB12 cell extracted proteins were determined based on a previously reported method.[13]

Plates (Microlon®, High binding flat bottom plates, Greiner, Germany) were coated overnight with 5.0 µg/ml V_LB12 protein in 100mM sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 5% (w/v) skimmed milk powder (Merck KGaA, Germany) dissolved in 10mM PBS at pH 7.4 containing 0.05% (v/v) of Tween® 20 (PBST; Sigma Aldrich, Co., Germany). Plates were again washed and sera were tested by serial two-fold dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG, (Sigma, Pool Dorset, UK), IgG1 and IgG2a (Serotec, UK) (diluted 1:1000) were applied as secondary antibody. Finally, the substrate OPD (SigmaFAST™ OPD Kit, Sigma Aldrich, Germany) was used to develop the plates, the color reaction was stopped after 15 min, by adding 2.5N H_2SO_4 to the wells, and absorbance was read on an Infinite 200 Microplate Reader (Tecan i-control software, version , 1.5.14.0, Tecan, North Carolina, USA) at 490 nm. The titters reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading.

5.2.8 ELISA assay for mice serum IgG specificity to gp120

For the mice serum IgG specificity to gp120, ELISA assays were performed using as antigen, recombinant HIV-1_{BaL} gp120 (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1BaL gp120 from DAIDS, NIAID) was used at 500 ng per well (in phosphate-buffered saline, PBS). Recombinant HIV-1_{BaL} gp120 was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, NY, USA). The plates were then blocked with 3 % bovine serum albumin (BSA) in PBS. 3 % BSA (in PBS) was also used and as negative control. The serums from the immunized mice groups were diluted with 1% BSA in PBS. The mice serums were used at a 1:100, 1:1000 or 1:10000 dilutions. The b12-IgG (obtained through the NIH AIDS Research and Reference Reagent Program, contributor: Dr. Dennis Burton and Dr. Carlos Barbas III)[18,45,47,276] was used as a positive control. b12-IgG was used at 1000 ng, 100 ng or 10 ng per well. The G1 to G7 serums and b12-IgG were added to the wells, and incubated for 1 h. The plates were washed with Tween 20 (0.05 % in PBS) and incubated with either horseradish peroxidase (HRP)-conjugated goat anti-Human IgG (Santa Cruz Biotechnology, Santa Cruz, USA) or HRP-conjugated goat anti-Mouse IgG (Bio-Rad Laboratories, CA, USA), the α-Human-HRP and the α-Mouse-HRP antibodies were used at a 1:1000 dilution in 1% BSA (in PBS). All incubations were performed for 1 h at 37 ºC. The plates were then washed with PBS and developed with an HRP substrate, ABTS solution (citric acid (pH 4.0) with 0,2 % H_2O_2 and read on an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA) at an optical density (OD) of 405/492 nm.

5.2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, 2007, San Diego, California, USA, www.graphpad.com) with a level of significance of 5%.

5.3 Results

BALB/c mice were immunized by intranasal (i.n.) and subcutaneous (s.c.) routes on day 1 and boosted on day 21 according to the immunization conditions described on section 5.2.6. Mice were divided in seven groups as described on Table 5.1.

Table 5.1 – Mice groups used for the immunization assay of the V_L B12 antigen.

Group	
I	CONTROL
II	i.n. protein encapsulated in CS/DS nanoparticles
III	i.n. empty CS/DS nanoparticles
IV	s.c. protein encapsulated in CS/DS nanoparticles
V	s.c. protein + gel of aluminium hydroxide
VI	i.n. free antigen
VII	s.c. free protein

To evaluate the vaccine antigen properties of V_LB12 serum was collected from each group at 2, 4, 6, 8, 10 and 12 weeks (Bleed 1, 2, 3, 4, 5 and 6 respectively) and antigen specific enzyme-linked immunosorbent assay (ELISA) was determined for total IgG, IgG subclass 1 (IgG1) and IgG subclass 2 (IgG2a). The results for antigen specific ELISA determined for total IgG are summarized in Figure 5.1.

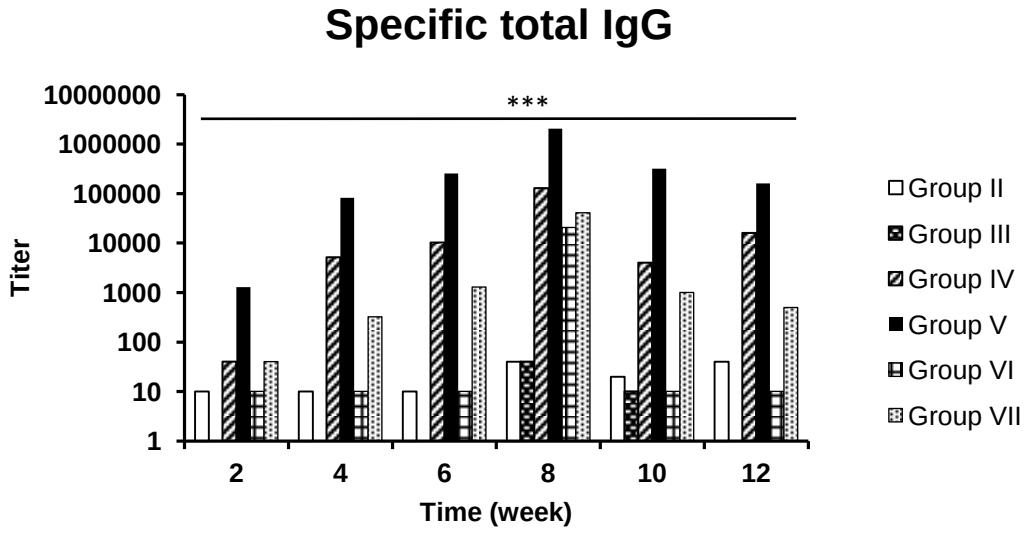


Figure 5.1 – ELISA assay performed to determine antigen specific total IgG titers.
(Continues on the next page).

Plates (Microlon®, High binding flat bottom plates, Greiner, Germany) were coated overnight with 5.0 µg/ml VLB12 protein in 100mM sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 5% (w/v) skimmed milk powder (Merck KGaA, Germany) dissolved in 10mM PBS at pH 7.4 containing 0.05% (v/v) of Tween® 20 (PBS-T; Sigma Aldrich, Co., Germany). Plates were again washed and sera were tested by serial two-fold dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG (Sigma, Pool Dorset, UK) (diluted 1:1000) were applied as secondary antibody. Finally, the substrate OPD (SigmaFAST™ OPD Kit, Sigma Aldrich, Germany) was used to develop the plates, the color reaction was stopped after 15 min, by adding 2.5N H_2SO_4 to the wells, and absorbance was read at 490 nm. The titters reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading. Results are expressed as mean of a pool of five mice. * corresponds to $P < 0,0001$.**

Group II doesn't appear to have a significant increase in total specific IgG overtime, but the total specific IgG titer rose on week 8 which might indicate that the immune response is starting to develop. Group III is only starting to develop an increase in total specific IgG at week 8, but since group III are intranasaly delivered empty CS/DS nanoparticles it is not expected a significant increase in total IgG since CS/DS nanoparticles are not immunogenic.

Groups IV, V and VII were the firsts to present a significant rise in total specific IgG titters with is related to the rout of administration since subcutaneous delivery usually triggers a much faster immune response that an intranasal administration.

Group VI is also only starting to present high IgG titer at week 8, which also correlates with intranasal administration.

From week 8 onword the total specific IgG titters present a slight drop but titer proportions between groups remain constant.

The results for antigen specific ELISA determined for IgG1 and IgG2a are summarized in Figure 5.2. This subclass titers were only determined from week 8 onword. Regarding specific IgG1 only Groups IV, V, VI and VII present elevated titers of IgG1, in particular Group V presents the highest specific IgG1 titer which correlates with the extremely high total specific IgG titer. This is particularly significant at week 10

Regarding specific IgG2a only Groups IV and V present IgG2a, indicating that these are starting to develop a Th2 response that could significantly improve the vaccine's protective immunity.

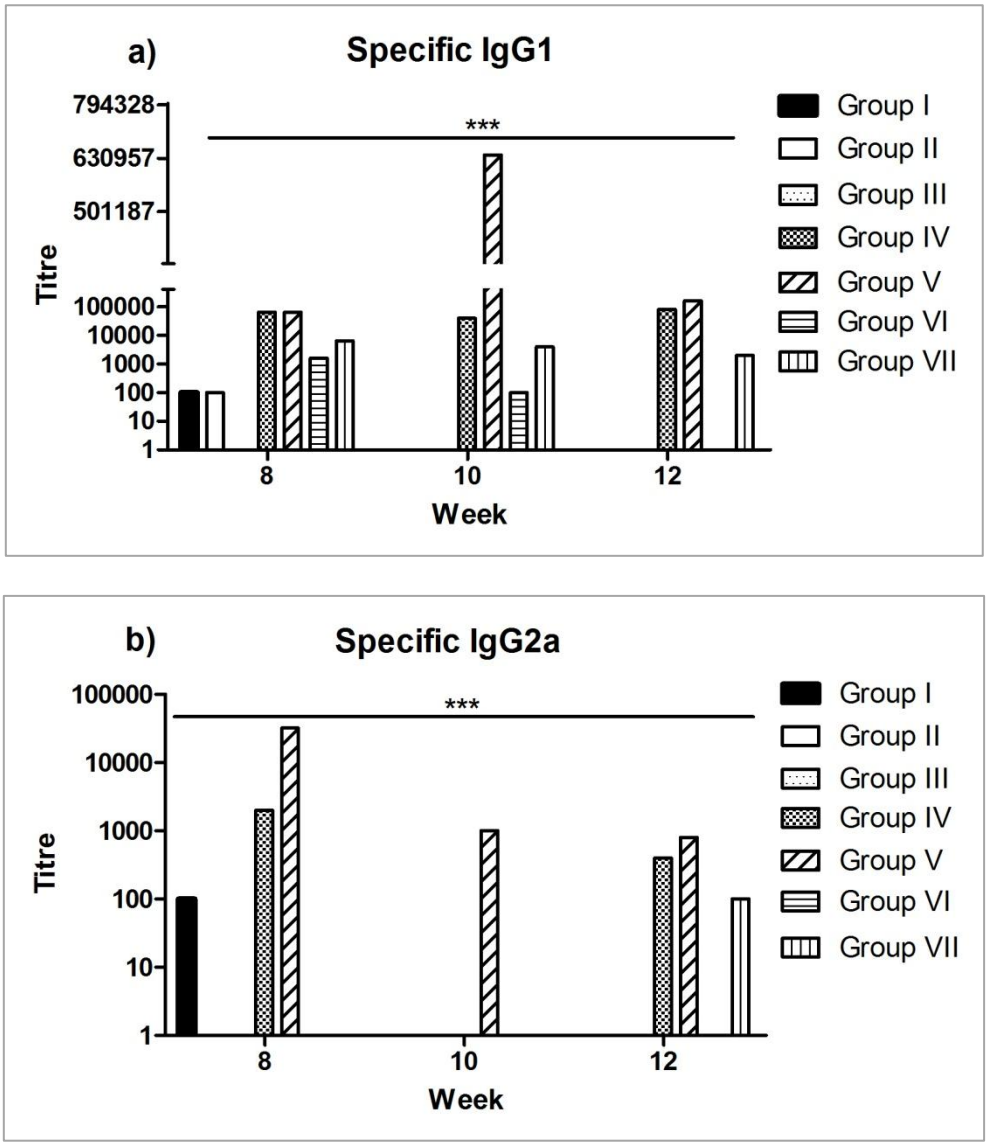


Figure 5.2 – ELISA assay performed to determine antigen specific IgG1 and IgG2a titres.
Plates (Microlon®, High binding flat bottom plates, Greiner, Germany) were coated overnight with 5.0 µg/ml VLB12 protein in 100mM sodium carbonate buffer (pH 9.6), washed and afterwards blocked with a 5% (w/v) skimmed milk powder (Merck KGaA, Germany) dissolved in 10mM PBS at pH 7.4 containing 0.05% (v/v) of Tween® 20 (PBS-T; Sigma Aldrich, Co., Germany). Plates were again washed and sera were tested by serial two-fold dilutions. Sera obtained from naive mice were used as a control. Horseradish peroxide conjugate goat anti-mouse IgG1 and IgG2a (Serotec, UK) (diluted 1:1000) were applied as secondary antibody. Finally, the substrate OPD (SigmaFAST™ OPD Kit, Sigma Aldrich, Germany) was used to develop the plates, the color reaction was stopped after 15 min, by adding 2.5N H_2SO_4 to the wells, and absorbance was read at 490 nm. The titters reported are the reciprocal of serum dilutions that gave an optical density 5% higher than the strongest negative control reading. Results are expressed as mean of a pool of five mice. * corresponds to $P < 0,0001$.**

From week 8 to week 10 there is a decrease in the titter of IgG2a and this lower values of the titter of IgG2a were maintained at week 12.

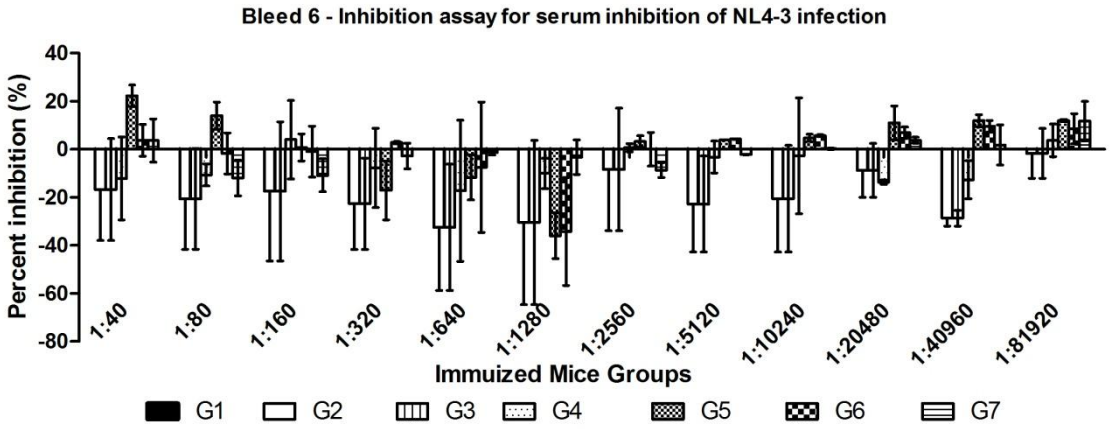


Figure 5.3 - Inhibition assay performed with serial dilutions of G1 to G7 serum from Bleed 6 for HIV-1_{NL4-3} inhibition.
10,000 TZM-bl cells per well were seeded in a 96-well plate in 100 µl/well of DMEM-10 and incubated overnight at 37°C. The next day, 200 µl of HIV-1NL4-3 were prepared, in DMEM-10, for a multiplicity of infection (MOI) of 0,5 and serial diluted serum from each mice group (1/40 – 1/81912 dilution) was added to the virus solution and incubated for 1 h at 37°C. The media was removed and the virus solution added to the cells. The cells were incubated for 48 h at 37°C in 5 % CO2. Assay medium was removed from each well, cells were washed with 200 µl of PBS and 50 µl of Passive Lysis Buffer reagent (Promega, Madison, WI) were added. This was followed by a freeze-thaw step (-20 ºC – 37 ºC) afterwhich 10 µl cell lysates from each sample (in Passive Lysis Buffer) were placed in a 96 well Luminescence Plate (BD Falcon 96 Flat Bottom White Opaque Polystyrene Plate, BD Biosciences, USA). 50 µl Luciferase Assay Reagent II (LAR II) were added to each sample and luminescence was measured using an Infinite 200 Microplate Reader (Tecan i-control software, version 1.5.14.0, Tecan, North Carolina, USA). Results are expressed as mean ± SD of two independent experiments performed in duplicate.

Surprisingly although the specific IgG titers were very high, particularly for Group V (subcutaneous protein with aluminium adjuvant), none of the serums from the different immunization groups presented a specific inhibitory effect on HIV-1_{NL4-3} (see Figure 5.3) .

Groups II and IV seem to increase HIV-1_{NL4-3}, while groups V, VI and VII presented a fluctuating inhibition pattern, that decreases as the serial dilution increases, only to increase again at lower concentrations. The overall conclusion is that no group presents an inhibitory effect on HIV-1_{NL4-3}. These results are in agreement with the results obtained for bleed 5 (data not shown).

Since there was no inhibitory effect detected for the antibodies generated by each of the different mice groups used in the V_LB12 immunization assay, the need to arrive at an explanation for these facts prompted us to perform an ELISA assay in order to confirm

if the high levels of specific total IgG were recognizing the gp120 glycoprotein (see Figure 5.4).

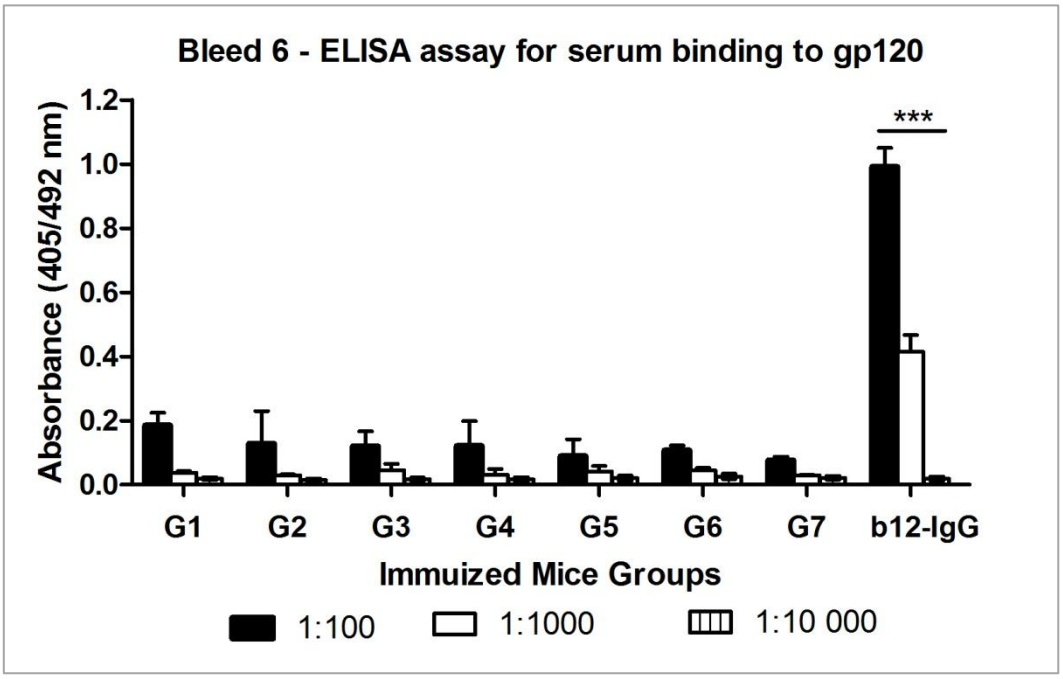


Figure 5.4 - ELISA assay for serum binding to gp120.
ELISA assays were performed using as antigen, recombinant HIV-1_{BaL} gp120 (obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HIV-1BaL gp120 from DAIDS, NIAID) was used at 500 ng per well (in phosphate-buffered saline, PBS). Recombinant HIV-1_{BaL} gp120 was adsorbed onto 96 well flat bottom, high binding non-sterile, polystyrene ELISA plates (Corning, NY, USA). The plates were then blocked with 3 % bovine serum albumin (BSA) in PBS. 3 % BSA (in PBS) was also used and as negative control. The serums from the immunized mice groups were diluted with 1% BSA in PBS. The mice serums were used at a 1:100, 1:1000 or 1:10000 dilutions. The b12-IgG was used as a positive control. b12-IgG was used at 1000 ng, 100 ng or 10 ng per well. The G1 to G7 serums and b12-IgG were added to the wells, and incubated for 1 h. The plates were washed with tween 20 (0.05 % in PBS) and incubated with either α-Human-HRP or α-Mouse-HRP, the antibodies were used at a 1:1000 dilution in 1% BSA (in PBS). All incubations were performed for 1 h at 37 ºC. The plates were then washed with PBS and developed with an HRP substrate, ABTS solution (citric acid (pH 4.0) with 0,2 % H_2O_2 and read on an Infinite 200 Microplate Reader at an optical density (OD) of 405/492 nm. Results are expressed as mean ± SD of three independent experiments performed in triplicate. * corresponds to $P < 0,0001$.**

The ELISA assay for serum binding to gp120 determined that the mice serum antibodies were not recognizing gp120, and that this was true for all the groups in the immunization assay. Even more so since the G1 is the one that at a 1:100 dilution presents some binding to gp120, but this group is the control were the mice received no treatment. This confirms that the high total specific IgG titers obtained are not targeting the b12 epitope, but must be targeting the remaining regions of the V_L framework. The

usage of b12-IgG as a positive control validates the assay confirming that the recombinant HIV-1_{BaL} gp120 protein used as antigen is being recognized by ELISA assay by antibodies targeting gp120, and the b12 epitope in particular. In conclusion, the V_L framework, which is of rabbit origin,[121] is apparently highly immunogenic and since the b12 epitope is only 23 amino acids long this is not being sufficient for the development of high titers of b12 specific antibodies.

5.4 Discussion

To evaluate the vaccine antigen properties, of V_LB12 the mucosal immunity was tested by using encapsulated V_LB12 in nanoparticles of chitosan in comparison with subcutaneous routes with aluminium adjuvant and without adjuvant. Th1 or Th2 responses were evaluated by the ratio of antigen-specific IgG2a-IgG1 in the serum samples.

All groups presented an increase in total specific IgG over time until week 8. From week 8 onword the total specific IgG titters present a slight drop but titer proportions between groups remain constant.

The groups where the antigen was encapsulated in CS nanoparticles and particularly where the antigen was delivered intra nasally present lower titers of total specific IgG as was expected since mucosal response develops more slowly than when the antigen is delivered subcutaneously.

Th2 response was also present in the groups that presented the highest specific IgG titers indicating a possibility of development of a cellular immunity component. And since the immune response elicited by a successful vaccine likely will require both antibodies and T cells that recognize, neutralize and/or inactivate diverse strains of HIV and that reach the site of infection before the infection becomes irreversibly established this may reveal a global effect different than the one obtained from the HIV-1 neutralization assay alone.[158]

Surprisingly although the specific IgG titers were very high, particularly for Group V (subcutaneous protein with aluminium adjuvant), none of the serums from the different immunization groups presented inhibitory effect on HIV-1_{NL4-3}. This may be caused by the fact that the V_L framework is highly immunogenic and the majority of the antibodies are being generated against the V_L framework and not our sequence of interest that is present in the CDR1 of the antigen. This was confirmed by the ELISA assay for serum binding to gp120, where none of the immunized mice groups serum could bind to the gp120, confirming the fact that the antibodies are only being generated against the framework and not to the b12 epitope present in the CDR1. This would explain the fact that the groups

where the antigen is encapsulated in CS nanoparticles that reduces the exposure of the framework to the mice immune system, are developing lower specific IgG titers.

The effect caused by the presence of the rabbit framework may be minimized by introducing key amino acid substitutions in the framework in order to make the framework less immunogenic to the mouse immune system, a process similar to "antibody humanization".[144,196,259,260] By using a framework less immunogenic to the mouse immune system it may be possible to have a higher prevalence of antibodies generated that target our sequence of interest and therefore this V_LB12 construct may yet be a useful vaccine antigen.

The occurrence of an epitope that is targeted by a broadly neutralizing mAb (2F5) and when engrafted onto a different protein scaffold to serve as an immunogen, while possessing high structural fidelity, but eliciting antibodies that lack HIV-1 neutralizing activity has also been reported.[159] This indicates that an antigen-driven B cell selection of HIV-1 neutralizing antibodies may require a complementary immunization strategy. Interestingly both the b12 mAb and 2F5 possess long CDRH3. For instances sequences of human CDRH3 vary from 2 to 28 amino acids residues, but mouse antibodies only show CDRH3 loops ranging from 1–19 residues and the b12 mAb CDRH3 is 18 amino acids long [368] that may prove to be difficult to achieve in mice and therefore b12 like mAbs may not be reproducible in the mouse system.

In conclusion, the eliciting of b12 like mAbs may not be reproducible in the mouse system although the introduction of key amino acid substitutions in the framework to reduce immunogenicity to the mouse immune system should still be evaluated. If this strategy fails, another animal model that does not possess the same CDR length constraints as the mouse should be used to evaluate V_LB12 as a vaccine antigen.

General Discussion

6 General Discussion

The work presented on this Thesis is divided in four parts; the first part was the construction of single domain antibodies that target the hCD4 cell receptor, through grafting of the b12 target sequence or CD4 binding epitope into either CDR1 or CDR3 of a highly stable rabbit single domain V_L antibody and the selection of the antibodies with the highest binding ability to the CD4 receptor.

In the second part of the Thesis we wanted to evaluate the V_LB12 and V_LCD4 ability to inhibit HIV-1 infection, and for that inhibition assays were performed with the HIV-1_{NL4-3} subtype B molecular clone and with HIV-1 subtypes J and H primary isolates.

In the third part of the Thesis we wanted to take advantage of V_LB12's high binding affinity to the CD4 receptor and to evaluate the potential of V_LB12 coated nanoparticles as a new therapeutic delivery system. For that, two different types of nanoparticle formulations were tested, Chitosan and PEI nanoparticles, for the delivery of FUGW-dsRed plasmid DNA to Jurkat cells.

And finally, in the fourth part of the Thesis we wanted to evaluate the V_LB12 in another perspective. Taking into account the fact that the B12 target sequence is a gp120 highly conserved and constitutively exposed region we wanted to evaluate its potential as a vaccine antigen.

From the results obtained in the first part of the work (Chapter 2) it was possible to obtain a large amount of information. From the results obtained from the expression and purification yields of the dAbs produced in the pComb3X vector it is possible to conclude that not all CDR combinations will be supported by the V_L framework and that the sequence present in the CDRs plays an important role in antibody stability/solubility and that this is not directly correlated to the size of the CDR sequence.

The fact that the B12 target sequence presents a higher binding ability to the CD4 receptor than the CD4 binding epitope itself, it is probably due to the context of

antibody grafting. In the V_LCD4 construct the CD4 binding epitope (10 amino acids) may not be sufficiently exposed to achieve optimal binding to the CD4 receptor, contrary to what happens in the gp120 context. The larger B12 target sequence (23 amino acids) allows for a higher exposure of the epitope that may facilitate binding to the antigen.

After establishing that for both constructs, V_LCD4 and V_LB12, the binding was specific to CD4 and that both V_LB12 and V_LCD4 had a strong affinity to the CD4 receptor, the next step was to identify the domain where V_LB12 and V_LCD4 bind to the human CD4 receptor and it was possible to determine that V_LCD4 is binding to domain 1 and V_LB12 is binding to the CD4 domain 2. We were also able to evaluate that, both V_LB12 and V_LCD4 bind specifically to the hCD4 cell receptor and recognize conformational epitopes of the CD4 receptor. Since domain 1, but also domain 2, of the CD4 receptor are targeted by HIV-1 neutralizing antibodies, the next step was to evaluate the V_LB12 and V_LCD4 ability to inhibit HIV-1 infection.

For that, in the second part of the Thesis (Chapter 3) we evaluated the V_LB12 and V_LCD4 ability to inhibit HIV-1 infection, inhibition assays were performed with the HIV-1 NL4-3 subtype B molecular clone and with HIV-1 subtypes J and H primary isolates, in TZM-bl cells in a single cycle infection assay. V_L B12 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM and V_LCD4 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 6,57 μM. We also performed inhibition assays with T20 and obtained an IC_{50} = 0,0694 μM for HIV-1_{NL4-3} . As for the primary isolates, V_LB12 was able to inhibit HIV-1 subtype H with an IC_{50} = 3,97 μM and HIV-1 subtype J with an IC_{50} = 3,86 μM but V_LCD4 was not able to inhibit either subtype J or subtype H primary isolates. T20 was able to inhibit HIV-1 subtype H with an IC_{50} = 1,33 nanoM and HIV-1 subtype J with an IC_{50} = 0,42 nanoM.

These results indicate that V_LB12 presents a potential broad inhibition spectrum as opposed to V_LCD4 that was not able to inhibit either of the non subtype B primary isolates. The fact that V_LCD4 was able to inhibit HIV-1_{NL4-3} with an IC_{50} = 6,57 μM but was unable to inhibit either of the primary isolates may be related to the fact that NL4-3 is a laboratory adapted molecular clone that may be more susceptible to blockage of viral attachment to CD4 that the subtype J or subtype H primary isolates tested.

Regarding T20, it presents a much lower IC_{50} than the dAbs and is also a more potent entry inhibitor against both HIV-1_{NL4-3} and the primary isolates tested, but T20 has a different target than the dAbs. T20 targets gp41 and due to the virus high frequency of mutation many strains show broad cross-resistance to antiretroviral drugs, including T20.

So, it is important to compare the V_LB12 with the only entry inhibitor that targets the gp120-CD4 interaction, Ibalizumab.[301] It is also important to highlight the fact that both V_LB12 and Ibalizumab bind to domain 2 of the CD4 receptor and therefore may have similar mechanisms of action and one of the main advantages common to both antibodies is that since they both bind to domain 2 they will not interfere with the receptor's effector functions.

The IC_{50} for V_LB12 is much higher than Ibalizumab's, when comparing the IC_{50} for HIV-1_{NL4-3}, Ibalizumab has an IC_{50} of 0,73 μg/ml (4,93 nM)[360] and V_LB12 was only able to inhibit HIV-1_{NL4-3} with an IC_{50} = 3,89 μM which is almost 1000 times higher. Despite of the fact that V_LB12 is not an entry inhibitor as potent as Ibalizumab, there have already been reported *in vitro* studies that demonstrate the synergistic activity of Ibalizumab (TNX-355) and enfuvirtide (T20) against HIV-1.[360] Implying that an entry inhibitor that targets the gp120-CD4 interaction, like V_LB12, can also prove to be useful as a therapeutic aid in improving the therapeutic response of enfuvirtide and other entry inhibitors treated patients. Therefore, V_LB12 may prove to be another non pharmacological therapeutical alternative in the treatment of HIV-1 infection.

One of the interesting characteristics of the V_LB12 construct is its versatility, since it can be used not only to inhibit HIV-1 entry into the cells, but due to the ability to bind CD4 can also be used as a drug delivery system when attached to nanoparticles.

This was the purpose of the third part of this Thesis (Chapter 4), to explore the potential of V_LB12 coated nanoparticles as a new therapeutic delivery system for gene therapy.

For that, two different types of nanoparticle formulations were tested, Chitosan and PEI nanoparticles, for the delivery of FUGW-dsRed plasmid DNA to Jurkat cells. Both nanoparticle formulations were successful in delivery of specific CD4 targeted FUGW-dsRed plasmid DNA as determined by immunofluorescence. The results obtained by

immunofluorescence indicated that PEI was a much more effective system for drug delivery, and both CS/DS and PEI nanoparticle systems allowed us to validate V_LB12 as an efficient method for specific delivery of nanoparticle encapsulated drugs to CD4 positive cells.

Chitosan nanoparticle formulation was proved to be more effective due to the lower cell toxicity, as determined by Flow cytometry. The optimal amount of V_LB12 adsorbed to the nanoparticles gave a 16 % positive dsREd fluorescent population with a specific V_LB12 targeting to CD4.

So the goal of using V_LB12 coated nanoparticles as a new therapeutic delivery system that had the potential to be used for gene theraphy was achieved and the V_L B12/CS/DS nanoparticle system applications will be further explored in the future.

As previously mentioned, in the fourth part of the Thesis (Chapter 5) we wanted to evaluate the V_LB12 in another perspective. Taking into account the fact that the B12 target sequence is a gp120 highly conserved and constitutively exposed region we wanted to evaluate its potential as a vaccine antigen. For that we performed a mice immunization assay to evaluate V_LB12 as a vaccine antigen were we also incorporated the knowledge obtained from Chapter 4 regarding V_LB12 and CS/DS nanoparticle formulations.

To evaluate the vaccine antigen properties, of V_LB12 the mucosal immunity was tested by using encapsulated V_LB12 in nanoparticles of chitosan in comparison with subcutaneous routes with aluminium adjuvant and without adjuvant. Th1 or Th2 responses were evaluated by the ratio of antigen-specific IgG2a-IgG1 in the serum samples. HIV-1 neutralization assays using serum from different groups to evaluate the V_LB12 as potential vaccine antigen were also performed.

Surprisingly although the specific IgG titers were very high, particularly for Group V (subcutaneous protein with aluminium adjuvant), none of the serums from the different immunization groups presented inhibitory effect on HIV-1_{NL4-3}. This may be caused by the fact that the V_L framework is highly immunogenic and the majority of the antibodies are being generated against the V_L framework and not our sequence of interest that is present in the CDR1 of the antigen.

This was confirmed by the ELISA assay for serum binding to gp120, where none of the immunized mice groups serum could bind to the gp120, confirming the fact that the antibodies are only being generated against the framework and not to the b12 epitope present in the CDR1. This would explain the fact that the groups where the antigen is encapsulated in CS/DS nanoparticles that reduces the exposure of the framework to the mice immune system, are developing lower specific IgG titers.

The effect caused by the presence of the rabbit framework may be minimized by introducing key amino acid substitutions in the framework in order to make the framework less immunogenic to the mouse immune system, a process similar to "antibody humanization". By using a framework less immunogenic to the mouse immune system it may be possible to have a higher prevalence of antibodies generated that target our sequence of interest and therefore this V_LB12 construct may yet be a useful vaccine antigen.

The occurrence of an epitope that is targeted by a broadly neutralizing mAb (2F5) and when engrafted onto a different protein scaffold to serve as an immunogen, while possessing high structural fidelity, but eliciting antibodies that lack HIV-1 neutralizing activity has also been reported.[159] This indicates that an antigen-driven B cell selection of HIV-1 neutralizing antibodies may require a complementary immunization strategy. Interestingly both the b12 mAb and 2F5 possess long CDRH3. For instances sequences of human CDRH3 vary from 2 to 28 amino acids residues, but mouse antibodies only show CDRH3 loops ranging from 1–19 residues and the b12 mAb CDRH3 is 18 amino acids long [368] that may prove to be difficult to achieve in mice and therefore b12 like mAbs may not be reproducible in the mouse system. This indicates that the eliciting of b12 like mAbs may not be reproducible in the mouse system although the introduction of key amino acid substitutions in the framework to reduce immunogenicity to the mouse immune system should still be evaluated. If this strategy fails, another animal model that does not possess the same CDR length constraints as the mouse should be used to evaluate V_LB12 as a vaccine antigen.

In conclusion, first and foremost this work has proved that both gp120 epitopes can be stably expressed in a recombinant antibody grafting and both V_LCD4 and V_LB12 recognize the CD4 cell receptor outside the gp120 context indicating that these loops are in fact independent and both must play a part in the gp120 binding to CD4.

V_LCD4 is a synthetic antibody that targets the domain 1 of the CD4 receptor of human cells but presents poor inhibitory effect on HIV-1 infection.

V_LB12 on the other hand is also a synthetic antibody that targets the domain 2 of the CD4 receptor of human cells but inhibits HIV-1 infection in laboratory and primary HIV-1 strains. This molecule can also be used for gene therapy as a carrier for DNA biomolecules encapsulated in CS/DS nanoparticles. Its usefulness as a vaccine candidate needs further evaluating and a "humanized" or modified version of V_LB12 may yet be a good candidate as an HIV-1 vaccine antigen.

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7 Bibliography

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