UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

Development of specific recombinant single-domain antibodies against gp120 HIV-1 glycoprotein and their selection by Phage Display

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Doutora Ana Isabel Simões Pereira Duarte

Doutor Frederico Nuno Castanheira Aires da Silva

ORIENTADOR:

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2018

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Às “meninas” da equipa “FAS”: Ana, Joana e Sandra, muito obrigada por me terem recebido tão bem. Constituem uma equipa espetacular. O vosso apoio foi constante e perfeito. O vosso espírito de equipa é incrível e tenho muito orgulho em ter testemunhado o vosso trabalho. São excelentes profissionais que contribuem em muito para a evolução da medicina humana e veterinária em Portugal. Ana foste, sem dúvida, o meu braço direito no desenvolvimento prático deste trabalho e sem ti, sem a tua calma e partilha de experiência teria sido muito mais difícil e frustrante conseguir chegar ao fim com sucesso.

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ABSTRACT

Development of specific recombinant single-domain antibodies against gp120 HIV-1 glycoprotein and their selection by Phage Display

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS), a condition in which progressive failure of the immune system allows life-threatening opportunistic infections to succeed. One of the most important factors in the worldwide spread of HIV is its enormous genetic variability and rapid evolution. The revealing of all stages of HIV replication cycle led to the identification of potential therapeutic targets in order to decrease the replicative process. However, the acquisition and transmission of HIV drug resistance still poses a major risk to the success of antiretroviral therapy. Thus, new targets and more promising strategies must emerge quickly to improve treatment options for patients who are infected with viruses already resistant to currently available antiretrovirals. The inhibition of HIV entry into the host cell is an extremely promising strategy, and glycoprotein gp120 plays a central role in this process. In this context, the aim of this project consisted in the development and selection, by Phage Display, of specific single-domain antibodies (sdAbs) against the surface HIV gp120 glycoprotein. To achieve this goal, rabbit antibody immunized libraries were generated and a pool of VH and VL sdAbs were selected by Phage Display. This approach allowed the construction of highly diverse sdAbs libraries and a posterior specific selection of VH and VL sdAbs with high binding activity to gp120. With the results obtained in this project, the potential of rabbit derived sdAbs as therapeutic molecules were once again demonstrated. At this stage the results are promising and further studies will be implemented in the future, to characterize the selected antibodies and to obtain more data regarding the binding activity to the target molecule and their potential to neutralize HIV infection.

Keywords: Human Immunodeficiency Virus, gp120, rabbit immunization, recombinant single domain antibodies, Phage Display

RESUMO

Desenvolvimento de anticorpos recombinantes de pequeno domínio contra a glicoproteína gp120 do VIH-1 e sua seleção por *Phage Display*

O vírus da imunodeficiência humana (VIH) é o agente causador da síndrome da imunodeficiência adquirida, uma condição na qual a falha progressiva do sistema imunitário permite que infeções oportunistas, potencialmente letais, se instalem. Um dos fatores mais importantes na disseminação mundial do VIH é a sua enorme variabilidade genética e rápida evolução. O conhecimento de todas as etapas do ciclo replicativo do VIH levou à identificação de possíveis alvos terapêuticos a fim de diminuir o processo replicativo. No entanto, a aquisição e transmissão de resistência aos fármacos contra o VIH ainda representa um grande risco para o sucesso da terapia antirretroviral. Assim, novos alvos e estratégias mais promissoras devem emergir rapidamente para melhorar as opções de tratamento dos pacientes infetados com vírus resistentes aos antirretrovirais atualmente disponíveis. A inibição do processo de entrada do VIH é uma estratégia extremamente promissora, e a glicoproteína gp120 desempenha um papel central neste processo. Neste contexto, o objetivo deste projeto consistiu no desenvolvimento e seleção, por *Phage Display*, de anticorpos específicos contra a gp120. Para alcançar este objetivo, foram criadas bibliotecas imunizadas de anticorpos de coelho e foi selecionado um conjunto de anticorpos de domínio pequeno (anticorpos no formato VH e VL) por *Phage Display*. Esta abordagem permitiu a construção de bibliotecas altamente diversificadas e uma posterior seleção específica de anticorpos com elevada afinidade de ligação à gp120. Com os resultados obtidos neste projeto, o potencial, como moléculas terapêuticas, dos anticorpos de pequeno domínio derivados do coelho foi novamente demonstrado. Nesta fase, os resultados são promissores e mais estudos serão implementados no futuro de modo a caracterizar os anticorpos selecionados e obter mais dados sobre a afinidade de ligação à molécula alvo e o seu potencial de neutralização da infeção pelo VIH.

Palavras-chave: Vírus da Imunodeficiência Humana, gp120, imunização de coelhos, anticorpos recombinantes de pequeno domínio, *Phage Display*

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LIST OF ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
ART	Antiretroviral treatment
BM	Bone Marrow
bnAbs	Broadly neutralizing antibodies
C	Conserved regions
CA	Capsid protein
cDNA	complementary DNA
CDR	Complementary Determining Region
CD4i	CD4 induced site
CH	Constant domain of heavy-chain
CHR	C-terminal heptad repeat
CL	Constant domain of light-chain
CRFs	Circulating Recombinant Forms
DNA	Deoxyribonucleic Acid
E. coli	*Escherichia coli*
ELISA	Enzyme- Linked Immunosorbent Assay
ENV	Envelope
ER	Endoplasmic Reticulum
Fab	Fragment antigen binding
Fc	Constant Fragment
FDA	Food and Drug Administration
FIV	Feline Immunodeficiency Virus
FRs	Framework residues
FR1	First framework region
Fv	Variable fragment
Gag	Group Specific Antigen
gp	Glycoprotein
H	Heavy
HAART	Highly active antiretroviral therapy
HCDR3	heavy chain CDR3
HIV	Human Immunodeficiency Virus
Ig	Immunoglobulin
iMed.ULisboa	Center for Molecular Pathogenesis and Research Institute of Medicine
IN	Integrase
L	Light
LB+amp	LB-agar medium with Ampicillin
LTR	Long Terminal Repeat

M	Major
MA	Matrix protein
mAbs	Monoclonal antibodies
MHC	Major histocompatibility complex
M-tropic	Macrophage-tropic
N	Nonmajor and Nonoutlier
NC	Nucleocapsid protein
Nef	Negative factor
NHR	N-terminal heptad repeat
NNTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside/nucleotide reverse transcriptase inhibitors
O	Outlier
ORFs	Open Reading Frames
PBS	Phosphate Buffered Saline XI
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI	Protease inhibitors
PIC	Pre-Integration Complex
Pol	Polymerase
PR	protease
Pr 55Gag	Gag precursor
P-TEFb	Positive Transcription Elongation Factor b
Rev	Regulator of virion expression
RNA	Ribonucleic Acid
RNA Pol II	RNA polymerase II
rpm	Rotations per minute
RRE	Rev Response Element
RT	Reverse transcriptase
R5 tropism	M-tropic strains
R6	Rabbit 6
R8	Rabbit 8
R5X4 tropism	Dual-tropic strains
scFv	Single-chain variable fragment
sdAbs	Single Domain antibodies
SIVs	Simian Immunodeficiency Viruses
Sp	Spleen
SU	Surface glycoproteins
Tat	Transcriptional transactivator
TCL-tropic	T cell line–tropic
TM	Transmembrane protein
T0-T5	Blood collection time points

U	Units
URFs	Unique Recombinant Forms
UV	Ultra violet
V	Variable regions
VH	Variable domain of heavy-chain
Vif	Virion Infectivity Factor
VIH	Vírus da imunodeficiência humana
VL	Variable domain of light chain
Vpr	Viral Protein Regulatory
Vpu	Viral protein U
Vpx	Viral protein X
VV	Vaccinia virus
X4 tropism	TLC-tropic strains
WHO	World Health Organization

1. INTERNSHIP REPORT

The curricular internship to obtain a Master's Degree in Veterinary Medicine was carried out at the Laboratory of Immunology and Virology at Faculty of Veterinary Medicine of Lisbon University, between September 11, 2017 and January 15, 2018, with a total of 620 hours.

The internship was in the area of laboratory research where the goal was to construct a rabbit derived single domain antibody immune library and whose results contributed to the development of the final dissertation.

During the internship, I was able to learn and execute several laboratory techniques, such as:

- Titration of antibodies through enzyme-linked immunosorbent assay (ELISA);
- Synthesis of cDNA by reverse transcription of RNA;
- Amplification of DNA through Polymerase Chain Reaction (PCR);
- Quantification of RNA and DNA;
- Electrophoresis in agarose gel;
- DNA precipitation;
- DNA purification;
- Digestion of DNA using restriction enzymes;
- Ligation reactions between target DNA and a pComb3x vector;
- Bacteria transformation by electroporation;
- DNA extraction from bacteria;
- Culture of bacteria in liquid and solid mediums;
- Preparation of culture mediums;
- Maintenance of cell cultures;
- Phage culture;
- Phase precipitation;
- Phage titration;
- Infection of bacteria with phage;
- Phage Display technology for antibody selections
- DNA sequencing;
- Alignment of DNA sequences.

2. INTRODUCTION

2.1 Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) is a lentivirus that causes HIV infection and over time can cause AIDS, a condition in which progressive failure of the immune system allows life-threatening opportunistic infections to thrive (World Health Organization (WHO), 2017).

HIV infection *per se* affects in complex ways every organ system, at all ages from the neonate to the elderly (Lucas & Nelson, 2015). Like many viruses, HIV has the ability to mutate and change over time - within the main types of HIV there are many genetically distinct subgroups (Taylor, Sobieszczyk, McCutchan & Hammer, 2008). There are two major types of HIV: HIV-1 (the most common) and HIV-2 (relatively uncommon and less infectious). Both virus are associated with the same mode of transmission and cause AIDS and similar opportunistic infections, although, HIV-2 is prevalent in West Africa and some countries in Western Europe, while HIV-1 is prevalent worldwide and is responsible for the global HIV pandemic (Clavel et al., 1987; Clavel et al., 1986).

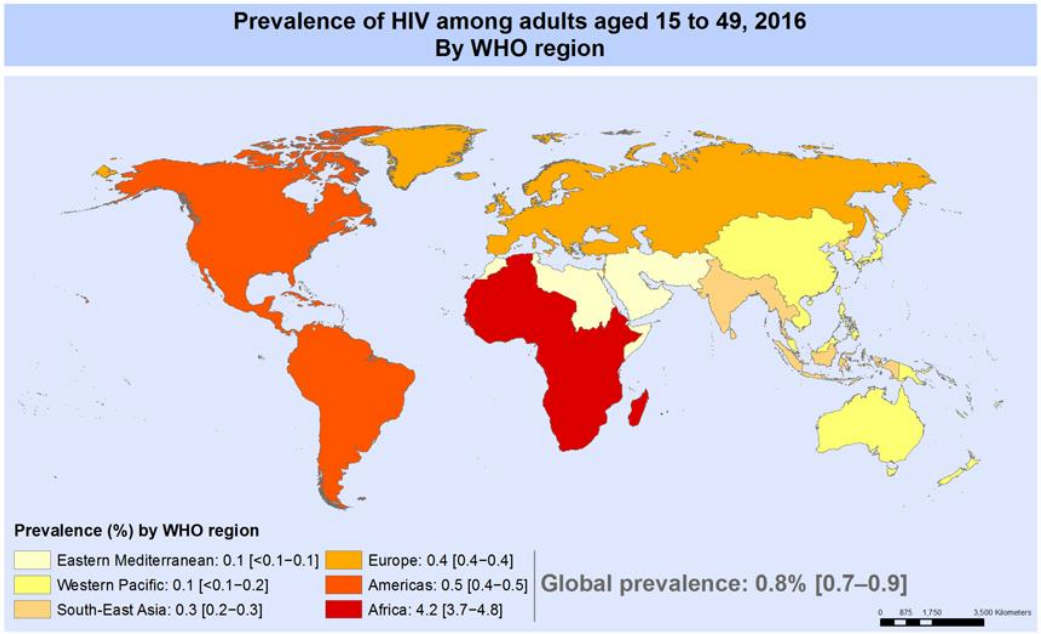
The earliest direct evidence of HIV infection in humans was found retrospectively in a serum sample and a lymph node biopsy specimen stored in 1959 and 1960, respectively, in Kinshasa, Democratic Republic of the Congo (Worobey et al., 1998; Zhu et al., 1998).

HIV-1 in humans resulted from at least four cross-species transmissions of simian immunodeficiency viruses (SIVs) from chimpanzees and gorillas in West Central Africa, while HIV-2 viruses resulted from at least eight independent transmissions of SIVs infecting sooty mangabeys in West Africa only (Peeters, Jung & Ayouba, 2013). Cross-species transmission probably occurred through bloodborne transmission in the process of hunting and butchering of primates for bushmeat and the capture, trade and keeping of monkeys as pets (Hahn, Shaw, De Cock & Sharp, 2000).

Enormous progress against AIDS for the past 15 years has inspired a global commitment to end the epidemic by 2030. In 2016 there were: 36,7 million people living with HIV, 19,5 million people living with HIV on antiretroviral therapy, 1,8 million people newly infected with HIV and 1 million people died from AIDS-related illnesses worldwide, compared to 1.9 million in 2005 and 1.5 million in 2010 (Figure 1) (UNAIDS, 2017).

With the development of effective antiretroviral treatment (ART), the life expectancy for people with HIV is now approaching the one seen in the general population. As ART is given to growing proportions of individuals with HIV infection, and is life-extending but not curative, interest has been directed on to the outcomes of the interactions of HIV, anti-retroviral therapy and the ageing process, and also on how to manage the growing number of persons with life-long HIV infection (Smith et al., 2014).

Figure 1. HIV prevalence.



Total number of existing cases of adults and adolescents, aged 15-49, infected with HIV by 2016. Adapted from WHO, 2017.

2.1.1 HIV Taxonomy

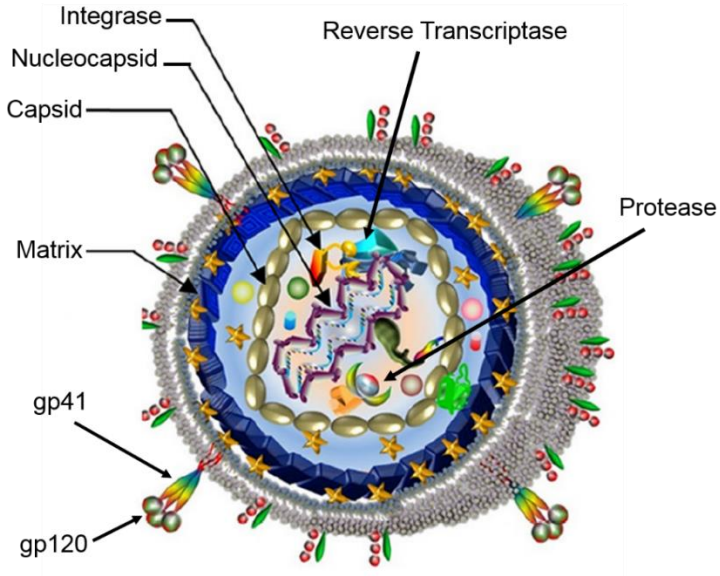
HIV is a member of the genus Lentivirus, which belongs to the family Retroviridae (Turner & Summers, 1999). The family Retroviridae is classified currently into two subfamilies (Orthoretrovirinae and Spumaretrovirinae) and seven genera. The family includes many viruses of importance in veterinary and human medicine, and to biomedical science in general. The prefix “Retro” refers to reverse and is due to the reverse transcriptase (RT), a particular enzyme which characterizes this family (Maclachlan & Dubovi, 2011). Like all retroviruses, HIV's genome is encoded by ribonucleic acid (RNA), which is reverse-transcribed to viral deoxyribonucleic acid (DNA) by the viral RT upon entering a new host cell. Other examples of lentiviruses include the simian immunodeficiency virus, visna virus, equine infectious anemia virus (Turner & Summers, 1999) and feline immunodeficiency virus (FIV) (Sparger et al., 1989). Since the 1980s, retroviruses are demonstrated to cause a number of important human diseases, including lymphomas, leukemias, and AIDS, which has further catalyzed intensive investigation of both human and animal retroviruses (Maclachlan & Dubovi, 2011).

2.1.2 HIV Structure and Genome

Lentivirus genus includes retroviruses that possess complex genomes and exhibit cone-shaped capsid core particles (Figure 2). The HIV virus is enveloped by a lipid bilayer that is derived from the membrane of the host cell (Turner & Summers, 1999) and contains several

cellular membrane proteins, including major histocompatibility antigens, actin and ubiquitin also derived from the host cell (Arthur et al., 1992). The virus particle contains an inner core containing the viral genome as well as enzymes that are required for early replication events. The inner core is protected by a protein capsid which, in turn, is surrounded by the lipid bilayer membrane. Approximately 2000 copies of virus matrix protein (MA, p17) is inserted into the inner surface of the membrane forming the matrix shell. A protein membrane, also called the glycoprotein envelope (Env), protrudes through the membrane and forms the outer surface of the virus particle (Haseltine, 1991). A conical capsid core particle comprising about 2000 copies of the capsid protein (CA, p24) is located in the center of the virus. The capsid encapsidates two copies of the unspliced viral genome, which is stabilized as a ribonucleoprotein complex with approximately 2000 copies of the nucleocapsid protein (NC, p7), and also contains three essential virally encoded enzymes: integrase (IN), RT and protease (PR) (Turner & Summers, 1999).

Figure 2. Schematic view of the HIV-1 particle.



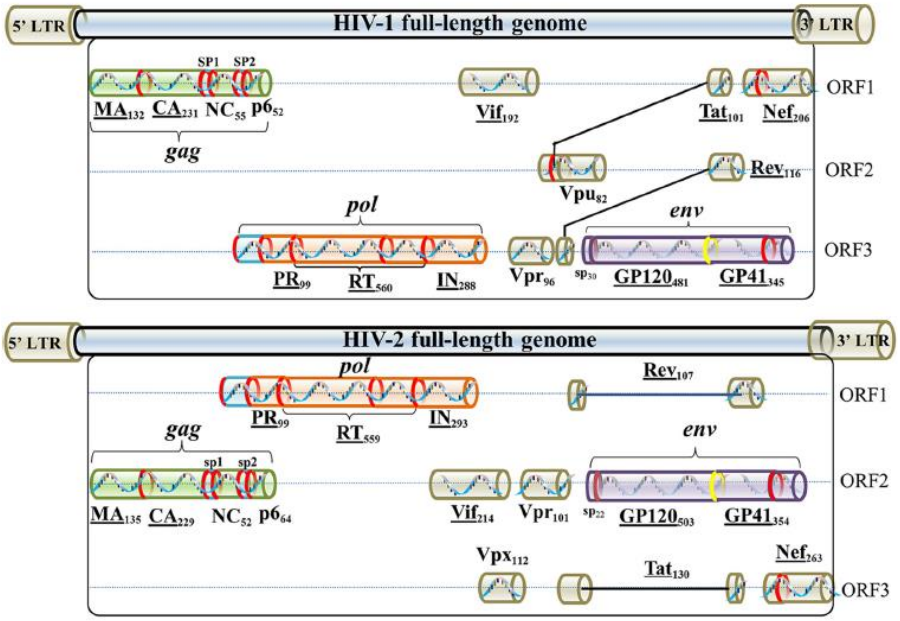
The Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum (ER) using the spliced env mRNA gene as the message. The protein appears to oligomerize to a trimeric structure in the ER, and is heavily glycosylated. Env is posttranslationally modified in the ER and Golgi apparatus and is cleaved to produce the non-covalently associated TM-SU trimeric glycoprotein complex. Adapted from Li et al., 2015.

The virus genome is diploid, consisting of a homodimer of two single-stranded RNA approximately 9 kb in length and contains 9 open reading frames (ORFs). The ends of each HIV RNA strand contain an RNA sequence called the long terminal repeat (LTR). Regions in the LTR act as “switches” to control production of new viruses and can be triggered by proteins from HIV or from the host cell. The largest three reading frames contains the three

major genes, which encode several proteins that have essential roles during the HIV life cycle (Freed, 2015; Turner & Summers, 1999; Frankel & Young, 1998) (FIGURE 3):

- *Gag* (group specific antigen), which encodes for structural proteins (Ma, CA, NC and p6);
- *Pol* (polymerase), which encodes for viral enzymes such as PR, RT and IN. The most important enzyme is RT, which has different functions depending on the part of the protein envolved in the biological process, being an RNA-dependent DNA polymerase, a DNA-dependent DNA polymerase and an RNase;
- *Env* (envelope) which encodes for specific surface protein (SU) gp120 and transmembrane protein (TM) gp41, determining cell tropism and contributing for pathogenicity. The superficial cone-shaped capsid core particles, also called envelope spikes, comprise trimers of non-covalently linked heterodimers consisting of the SU gp120 and the TM gp41. When triggered, these spikes initiate a cascade of conformational changes that culminates in fusion between the viral and host cell membranes and release of the viral core into the cytoplasm (Engelman & Cherepanov, 2012; Turner & Summers, 1999).

Figure 3. Schematic model of the HIV-1 and HIV-2 full-length genome. Adapted from Li et al., 2015.



The remaining genes code for regulatory proteins (Tat - *Transcriptional transactivator* and Rev - *Regulator of virion expression*) that control the ability of HIV to infect cells, produce new copies of virus, or cause disease (Frankel & Young, 1998); and accessory proteins (Vif - *Virion Infectivity Factor*; Vpu - *Viral protein U*, Vpx - *Viral protein X*, Vpr- *Viral Protein*

Regulatory; and Nef - *Negative factor*) (Malim & Emerman, 2008). Vpu is found exclusively in HIV-1, whereas Vpx is carried by HIV-2 (Li et al., 2012). Virus particles package the accessory proteins, Nef, Vif and Vpr however, the three additional accessory proteins that function in the host cell, Rev, Tat and Vpu, do not appear to be packaged (Turner & Summers, 1999).

Vpu promotes degradation of CD4 when there is the formation of complexes of Env glycoproteins with newly synthesized CD4 molecules in the ER, thus allowing Env transport to the cell surface for assembly into viral particles (Frankel & Young, 1998). Nef, like Vpu, reduces the levels of cellular CD4, serving as a direct bridge between CD4 and the cellular endocytic machinery. By downregulating CD4, Nef may enhance Env incorporation into virions, promote particle release, and possibly affect CD4 T-cell signaling pathways. It can also downregulate expression of Major Histocompatibility Complex (MHC) class I molecules, which may help protect infected cells from cytotoxic T cells (Schaeffer, Geleziunas & Greene, 2001). Vif has an important role in the production of highly infectious mature virions by blocking the innate antiviral activity of a cytidine deaminase APOBEC3G (CEM15) that induces guanine to adenine hypermutation in the viral genome (Mariani et al,. 2003; Frankel & Young, 1998). Vpr intervenes in the transport of the viral genome from the cytoplasm to the nucleus (Frankel & Young, 1998). Vpx enhances HIV-2 replication by neutralizing the host factor SAMHD1 activity. SAMHD1 restricts HIV-2 replication by depleting the cytoplasmic pool of deoxynucleoside triphosphates needed for viral DNA production (Lahouassa et al., 2012). Tat and Rev have a regulatory function and are essential for HIV replication by activating the transcriptional process and regulating the expression of viral proteins, respectively (Frankel & Young, 1998).

Although HIV genome code for only 16 viral proteins (Li et al., 2012), a great number of physical interactions between pairs of HIV proteins, so-called HIV pairwise protein interactions, as well as interactions between HIV and human proteins provide essential mechanisms for HIV to achieve efficient viral replication at different stages of the HIV life cycle (Li, 2014).

2.1.3 Genetic diversity in HIV

One of the most important factors in the worldwide spread of HIV is its enormous genetic variability and rapid evolution (Hamelaar, 2012). This genetic variability of HIV results from the high mutation and recombination rates of the RT enzyme, together with high rates of virus replication (Roberts, Bebenek & Kunke, 1988; Ho et al., 1995). The RT of HIV lacks proofreading activity, which is the ability to confirm that the DNA transcript it makes is an accurate copy of the RNA code, and confers a mutation rate of approximately 3.4×10^{-5} mutations per base pair, per cycle of replication (Mansky & Temin, 1995). Recombination is also one of the reasons for high variability of HIV, which involves shuttling of mutations

between viral genomes and leads to major antigenic shifts or alterations in virulence (Burke, 1997). Recombination occurs when there is dual infection. Dual infection is detected in 10–20% of HIV-infected individuals in regions in Africa where different variants cocirculate (Powell, Urbanski, Burda, Kinge & Nyambi, 2009). Dual infection can be the result of either the simultaneous infection with two heterologous strains that are multiplying in the same cell (co-infection) or sequential infection, in which a second infection with a heterologous strain occurs after seroconversion to the initial infection (superinfection) (Gottlieb et al., 2004; Grobler et al., 2004).

Phylogenetic analysis of HIV-1 from nonhuman primates suggests that, early in the 20th century, three independent transmission events gave rise to three HIV-1 groups: major (M) outlier (O), and nonmajor and nonoutlier (N). Strains related to the M and N groups have been found in chimpanzees, however, there is evidence that suggests that group O may have originated in gorillas (Keele et al., 2006; Van Heuverswyn et al., 2006). Group M is the predominant circulating HIV-1 group (90% of reported HIV/AIDS cases) and viral envelopes have diversified so greatly that this group has been subclassified into nine clades including A–D, F–H, J and K, which in turn are divided by sub-subtypes denoted with numerals (Taylor et al., 2008). Intra-subtype genetic variation can be 15 to 20%, whereas inter-subtypes variation is usually 25 to 35% (Korber et al., 2001).

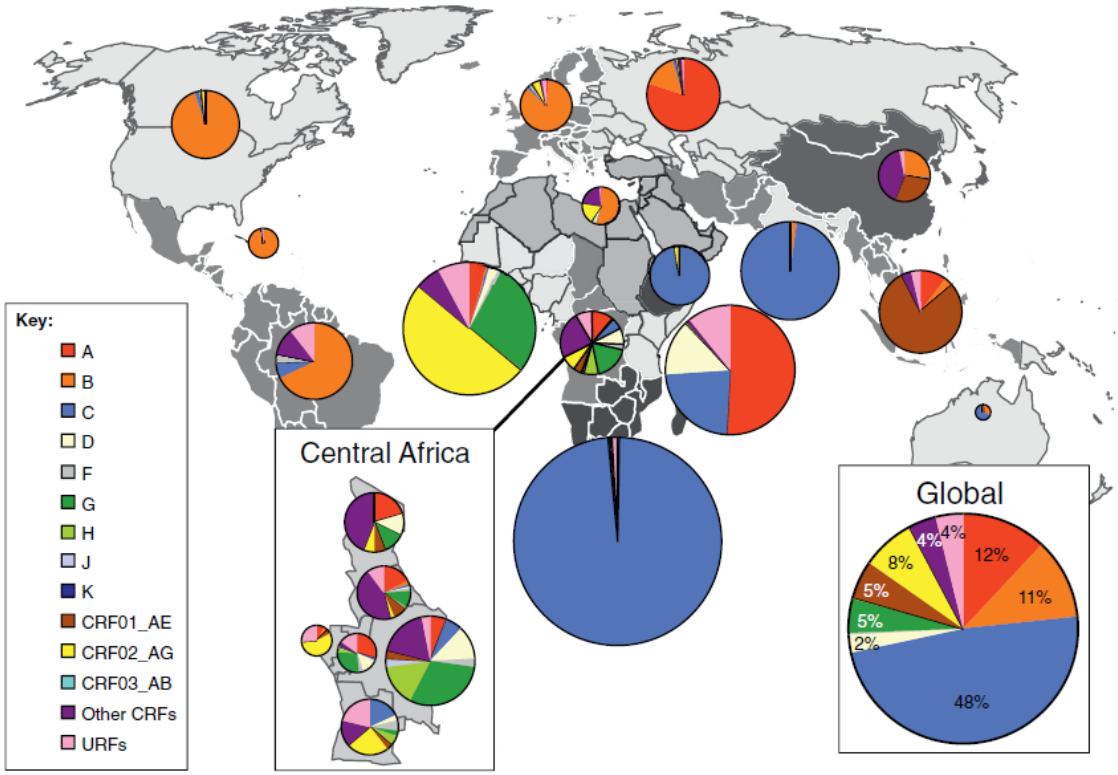
Recombinants between different HIV-1 group M subtypes are designated as either circulating recombinant forms (CRFs) or as unique recombinant forms (URFs). These are the result of recombination between subtypes within a dually infected person, from whom the recombinant forms are then passed to other people. If fully sequenced and found in three or more epidemiologically unlinked individuals, recombinants are classified as CRFs; if not meeting these criteria, recombinants are classified as URFs (Robertson et al., 2000). Over 20 different CRFs have been defined within group M alone, where the subtypes A and C account for the majority of HIV cases in the pandemic (Klimas, Koneru & Fletcher, 2008).

Differential characteristics of viral subtypes and their interactions with the human host may influence HIV transmission and disease progression. For example, HIV strains capable of using the chemokine coreceptor CCR5 are more frequently transmitted than strains that use the CXCR4 coreceptor; and the latter emerge later in infected patients and are associated with more rapid disease progression (Berger et al., 1998).

The global distribution of subtypes and recombinant forms (Figure 4) reflects the molecular epidemiological complexity of HIV (Taylor et al., 2008). In 2004–2007, subtype C accounted for 48% of all infections worldwide, followed by subtypes A (12%) and B (11%), the CRFs CRF02_AG (8%) and CRF01_AE (5%), subtype G (5%) and D(2%). The Subtypes F, H, J and K together cause fewer than 1% of global infections. All recombinant forms taken together were responsible for 20% of infections worldwide. (Hamelaar, Gouws, Ghys & Osmanov, 2011).

The extensive genetic variation and rapid evolution that has been observed in the HIV genome, makes HIV one of the fastest evolving organisms. Therefore, The HIV diversity has a major impact on HIV diagnosis, pathogenesis, transmission, clinical management and vaccine development (Hamelaar, 2012; Freed, 2001).

Figure 4. Global distribution of HIV-1 subtypes and recombinant forms.



Pie charts representing the distribution of HIV-1 subtypes and recombinants from 2004 to 2007 in each region are superimposed on the regions. Adapted from Hamelaar, 2012.

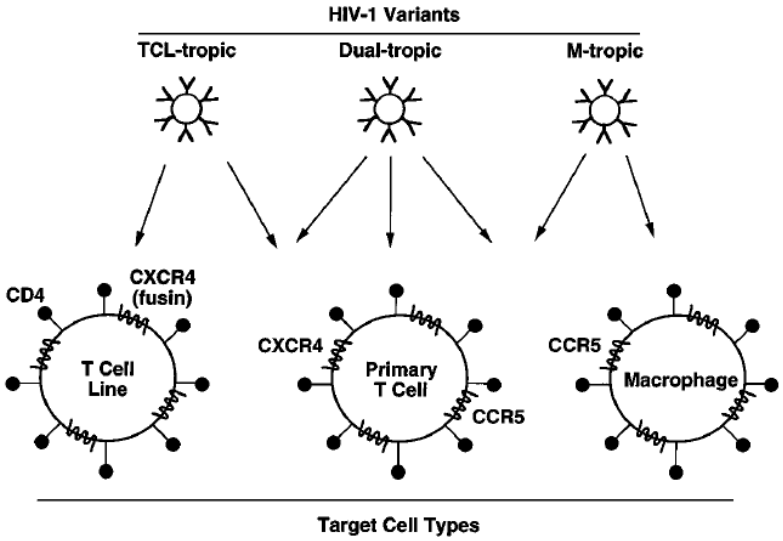
2.1.4 The host-cells and HIV interaction

CD4+ T cells and monocytes/macrophages are the two major cell types infected by HIV-1 *in vivo* (Rice, 2015). The state of differentiation of host cells has an important effect on the replication level of the virus. In contrast to activated CD4+ T cells and differentiated macrophages, resting CD4+ T cells and monocytes are non-permissive for HIV-1 replication. CD4+ T cells must be activated to become highly permissive for viral replication, whereas monocytes must undergo the macrophage differentiation process (Chiang & Rice, 2012).

HIV-1 particles bind specifically to cells bearing CD4, a protein that normally functions in immune recognition. Binding occurs via interactions between the gp120 and the amino-terminal immunoglobulin domain of CD4. However this interaction is sufficient for binding but not for fusion of the viral and the cellular membranes and consecutive virus entry and host

infection (Turner & Summers, 1999). Therefore, in addition to CD4, HIV requires a coreceptor for entry into target cells. The chemokine receptors CXCR4 and CCR5 are members of the G protein-coupled receptor superfamily and have been identified as the principal coreceptors for HIV fusion process. The abilities of the corresponding Env to use CXCR4 and/or CCR5, and the expression patterns of these coreceptors on different CD4 target cells are the two considerations that can explain the tropism of different HIV-1 strains (Figure 5). HIV-1 isolates that show efficient infectivity for continuous CD4 T cell lines, but poor infectivity for primary macrophages are designated T cell line–tropic (TCL-tropic) and they induce the formation of syncytia in the infected cells. On the other hand, HIV-1 strains that show the opposite preference, infecting primary macrophages much more efficiently than continuous T cell lines are designated macrophage-tropic (M-tropic) or nonsyncytium-inducing. There are also dual-tropic isolates that replicate efficiently in both target cell types. Continuous T cell lines abundantly express CXCR4, primary macrophages express CCR5, and primary T cells express both coreceptors. Moreover, M-tropic strains prefer CCR5 (R5 tropism), TCL-tropic strains preferentially use CXCR4 (X4 tropism) and dual-tropic strains can use both coreceptors (R5X4 tropism) (Berger, Murphy &Farber, 1999).

Figure 5. HIV-1 tropism.



HIV strains can be broadly divided into three categories: T cell line–tropic (TCL-tropic), macrophage-tropic (M-tropic) and dual-tropic. TCL-tropic strains are specific for CXCR4 coreceptor and can infect continuous CD4 T cell lines and primary CD4 T cells. M-tropic strains are specific for CCR5 coreceptor and can infect macrophages and primary CD4 T cells. Dual-tropic strains can use both CXCR4 and CCR5 coreceptors, and can infect continuous CD4 T cell lines, macrophages, and primary T cells. Adapted from Berger et al., 1999.

Early in the course of HIV infection, the M-tropic strain viruses predominate. However, as the infection progresses to AIDS, TCL-tropic isolates can be isolated from many patients. These

strains typically display higher cytopathic effects *in vitro*, suggesting that they may have a particularly important role in the decline of CD4 T cells *in vivo*, which is the hallmark of AIDS (Berger et al., 1999).

2.1.5. HIV Transmission

The mainly known routes for HIV-1 transmission are: sexual contact across mucosal surfaces, maternal-infant exposure and percutaneous exposure (Shaw & Hunter, 2012).

During sexual intercourse, HIV can cross the mucosal barrier of the vagina, vulva, penis, and rectum by first coming into contact with immune cells (dendritic cells) that then carry the virus across the mucosa (Lekkerkerke, Kooyk & Geijtenbeek, 2006). Factors such as concurrent sexually transmitted diseases, rough sex, or a partner with a very high viral load are known to increase the risk of transmission (Atkins, Carlin, Emergy, Griffiths & Boag, 1996). Generally women are at a higher risk of acquiring HIV during heterosexual intercourse due to female physiological characteristics, such as the large amount of mucosal surface area that is exposed to seminal fluid (Klimas et al., 2008).

Maternal-infant exposure can lead to infection of the fetus or newborn during pregnancy, delivery or by breastfeeding (Klimas et al., 2008). The mother-to-child transmission of HIV decreased in the last 20 years due to great prophylactic strategies. In places where one could implement all the prophylactic measures, the transmission rate reduced from 25-42% without any interventions to 1% or less. Prophylactic measures include the use of antiretroviral protocols during pregnancy, labor and delivery, and postnatally to the infant; and elective cesarean delivery before amniotic membrane rupture if HIV load is still detectable in late pregnancy (Panel on Treatment of HIV-Infected Pregnant Women and Prevention of Perinatal Transmission, 2018; WHO, 2013).

HIV transmission can also occur by contact with infected blood, where the most important way is through the practice of reusing and sharing syringes and needles for drug administration (Chitwood et al., 1990).

The transmission of HIV in healthcare workers is a reality, accidental needlestick and mucosal splash with contaminated blood are the mainly routes (Klimas et al., 2008).

2.1.6 HIV Replication Cycle

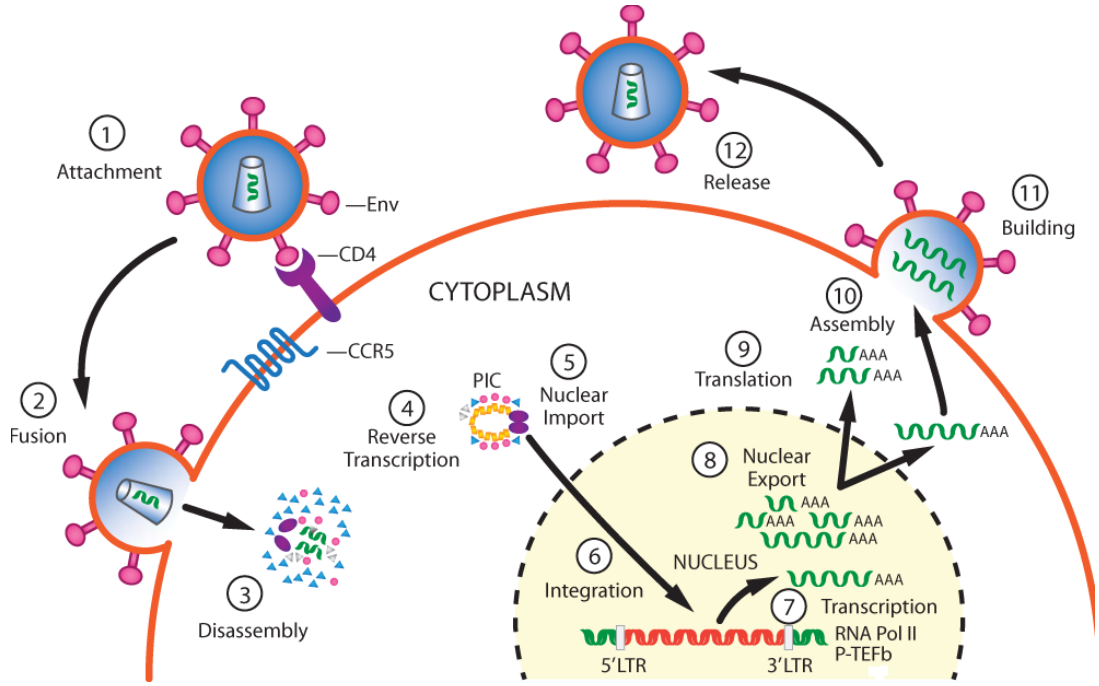
The HIV replication cycle (Figure 6), closely resembles that of other retroviruses and can be divided into an early and a late phase. The early phase is initiated with recognition of target cells by the mature virion and encompasses the entire process leading to and including integration of the genomic DNA into the host cell genome (Turner & Summers, 1999). This includes virus binding to cell surface receptors, cell entry, reverse transcription of the viral RNA to DNA, uncoating of the viral capsid, nuclear import of viral DNA, and DNA integration (Freed, 2015). The HIV-1 replication cycle begins when the virion Env binds to the CD4

receptor and CCR5 or CXCR4 co-receptor on the surface of CD4+ T cells or monocytes/macrophages (Rice, 2015). Binding with the CCR5 coreceptor typically predominates in initial infection. However, as infection progresses, mutations in the viral envelope allows the virus to use the CXCR4 coreceptor instead of or in addition to CCR5. The CCR5:CXCR4 ratio on CD4+ cells is 1:9; thus the switch from CCR5 to CXCR4 as a coreceptor allows infection of a much greater number of CD4+ cells and is associated with accelerated HIV disease progression (Doms, 2004). The gp120 subunit binds to the CD4 cell receptor, witch induces conformational changes in the glycoprotein envelope, exposing a previously inaccessible highly conserved domain that binds to the co-receptor. Co-receptor binding induces, in turn, conformational changes at the gp41 subunit level, resulting in the fusion of viral and host cellular membranes (Doms, 2004).The membrane fusion reaction enables the viral core to gain access to the cytoplasm and is thus central to the infection process. Into the cytoplasm, the viral core begins a regulated process of disassembly. In this process, the viral capsid is disrupted and the viral RT enzyme is fully activated converting the two RNA molecules in the virion to a linear double-stranded DNA, which is then transported to the nucleus as part of a pre-integration complex (PIC). This complex appears to include the IN, MA, RT, and Vpr proteins. After active transport to the nucleus, the viral DNA is covalently integrated into the host genome by the catalytic activity of IN (Freed, 2001; Turner & Summers, 1999). HIV integration is strongly favored in active transcription units, which may promote efficient viral gene expression after integration (Craigie & Bushman, 2012). The integrated viral genome is termed provirus. From the integrated provirus it is possible to synthesize mRNAs that encode structural, regulatory and accessory proteins necessary for viral replication (Freed, 2001).

Integration marks the transition from the early to late phase of HIV-1 replication. The late phase of the virus life cycle begins with the regulated expression of the integrated proviral genome and involves all processes up to and including virus budding and maturation (Tuner et al., 1999). As a result of the natural metabolism of the host cell, if the cell is activated, the DNA, containing the viral genome, is transcribed. This process is catalyzed by the cellular enzyme RNA polymerase II. If host cells are not activated, it is possible for the virus to persist in a latent stage for many years (Potter et al., 2007). Transcription requires the viral Tat, witch recruits the general transcriptional elongation factor P-TEFb to the Pol II complex, for efficient elongation. The transcription process gives rise to viral mRNAs of a variety of alternatively spliced species that can be divided into three major classes: unspliced RNAs (mRNAs for the Gag and Gag-Pol polyprotein precursors), partially spliced mRNAs (encoding the Env, Vif, Vpu, and Vpr proteins) and small multiple spliced mRNAs (enconding Rev, Tat, and Nef) (Tazi et al., 2010, Freed, 2001). After HIV mRNA is processed in the cell's nucleus it is transported to the cytoplasm. The smaller messages are exported readily from the nucleus, whereas the unspliced and singly spliced mRNAs require the action of Rev

protein. Rev binds to the Rev Response Element (RRE), located within the env mRNA coding region, resulting in the formation of a complex capable of interacting with the cellular nuclear export machinery (Daugherty, Liu & Frankel, 2010).

Figure 6. Schematic overview of the HIV-1 replication cycle.



The infection begins when Env glycoprotein spikes engage the receptor CD4 and the membrane co-receptor CCR5, leading to fusion of the viral and cellular membranes and entry of the viral particle into the host cell. In the host cell, reverse transcription yields the pre-integration complex (PIC). After import into the cell nucleus, PIC-associated integrase carries out the formation of the integrated provirus. Proviral transcription is mediated by host RNA polymerase II (RNA Pol II) and positive transcription elongation factor b (P-TEFb), and yields viral mRNAs of different sizes, which are then exported to the cytoplasm. mRNAs serve as templates for protein production and RNA genome is incorporated into viral particles with protein components. Viral-particle budding and release from the host cell is accompanied or soon followed by protease-mediated maturation to create an infectious viral particle. Steps in the viral replication cycle are numbered. LTR - long terminal repeat. Adapted from Rice, 2015

Once the proteins necessary for viral replication are synthesized, the process of formation of new virions begins. Thus, to assemble a virus, the structural proteins must be produced first. For HIV- 1, the genomic RNA is also the mRNA that codes for the Gag precursor (Pr 55Gag) and the Gag-Pol fusion protein (PR 160Gag-Pol). Pr 55Gag is as polyprotein precursor that can be processed into MA (p17), CA (p24), NC (p7) and p6. Pr 55Gag plays a key role in the new virions formation process since it 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.

The genes encoding the viral enzymes PR, RT and IN are translated as part of the fusion Gag-Pol polyprotein. The Env gp160 is synthesized in the ER and is modified, after translation, in the ER and Golgi apparatus and then cleaved by a host protease to produce the two envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the Env complex in the host membrane and associates non-covalently with gp120. The Gag and Gag-Pol polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. As the particle buds and is released from the cell surface coated with gp120 and gp41, the virion undergoes a morphologic change known as maturation, involving proteolytic processing of the Gag and Gag-Pol polyproteins by viral protease. The mature virion is then ready to infect the next cell and start a new infection cycle (Freed, 2001).

HIV disseminates rapidly in the absence of preexisting immune pressures, leading to a burst of viremia manifested in a substantial proportion of patients by an acute HIV syndrome (Moir, Chun & Fauci, 2011). In the initial infection, there is massive systemic lympho-reticular infection by HIV with loss of CD4+ T cells, most severe in the gut mucosa, which never recovers its pre-infection lymphoid population (Lucas & Nelson, 2015). With migration of infected T lymphocytes or virions into the bloodstream, secondary amplification in the gastrointestinal tract, spleen, and bone marrow results in massive infection of susceptible cells. Early after infection, a pronounced depletion of activated as well as memory CD4+ T cells, located in the gut associated lymphoid tissues, has been seen in individuals (Simon, Ho & Karim, 2006). The mechanisms of CD4+ cell depletion are complex and they include apoptosis of productively infected and activated cells via activation of caspase 3 and pyroptosis triggered by abortive viral infection, via activation of caspase 1, which may account for the great majority of T cells lost (≥ 95%). Therefore, this death pathways links CD4 T-cell depletion and chronic inflammation, which are the two signature events in HIV infection, and creates a vicious pathogenic cycle where dying CD4 T-cells release inflammatory signals that attract more cells to die. (Doitsh et al, 2014). Since HIV-infected T helper cells are lysed and their production and maturation are simultaneously inhibited by Nef and Tat viral proteins, a gradual decline of these cells is observed and part of the newly produced T helper lymphocytes do not develop normal functions. After a long-lasting HIV infection the continuous loss of T helper lymphocytes results in immunodeficiency, since they play a central role in the immune response, signaling other cells such as the cytotoxic T cell and the B cells to perform their functions (Zeng et al., 2012). Depletion of susceptible CD4+ T cell targets, together with the evolution of an incompletely effective HIV-specific immune response, leads to the establishment of a plasma viral set point and to qualitative changes within each immune cell population that ultimately affect overall immunologic competence (Moir et al., 2011).

2.2. Current and emerging therapies for HIV

The revealing of all stages of HIV replicative cycle led to the identification of potential therapeutic targets in order to decrease the replicative process. This resulted in tremendous scientific progress in the drug discovery and development process (Pau & George, 2014). Approximately 30 different antiretroviral drugs, targeting four different steps in the replicative cycle of the virus, have been developed and approved for use in the treatment of HIV. There are six classes of ART available that interrupt viral replication (Table 1): nucleoside/nucleotide reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI), which target the reverse transcription step that converts the viral genomic RNA into linear double-stranded DNA; protease inhibitors (PI), which inhibit the protease activity that is critical for the maturation of viral particles which bud from infected cells; CCR5 antagonists and fusion inhibitors, which block entry of the virus into new target cells by thwarting either the interaction between gp120 and the co-receptor CCR5 or the formation of the six-helix bundle of gp41, thus blocking fusion between the viral and cellular membranes; and finally, integrase inhibitors, which blocks the strand transfer activity of integrase required for insertion of viral DNA into the host cell chromosome (Englemen et al., 2012; Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011).

Zidovudine, a NRTI, was the first approved antiretroviral agent for the treatment of HIV in 1987. With the administration of NTRIs, in a monotherapy regimen, the patients showed a reduction in viral load, delay in disease progression and prolonged survival, however, the use of a single agent did not provide sustained viral suppression (Pau & George, 2014). With the development of drugs with different spectra of activity, the possibility of making therapeutic combinations emerged, which dramatically changed the course of the HIV epidemic. Current treatment consists of highly active antiretroviral therapy (HAART), that is, at least three drugs belonging to two classes of antiretroviral agents. Normally, these classes are two NRTIs plus either a PI or a NNRTI or an integrase inhibitor (Menendez-Arias, 2013). HAART is effective in >70% of patients in bringing down blood viral loads to undetectable levels (<50 virus particles/ml blood) and enabling the blood CD4+ T cell count to rise to nearer normal levels (>500 cells/mm3 blood) (Lucas & Nelson, 2015).

Drug adherence, tolerability and long-term toxicity constituted major limitations to the clinical use of HAART. However, improvements in potency, safety and dosage simplification have alleviated those problems. Nevertheless, the acquisition and transmission of HIV drug resistance still poses a major risk to the success of ARTs (Menendez-Arias, 2013). The combinatorial approach to drug treatment significantly suppresses the probability of selection for, and resulting outgrowth of, resistant HIV-1 strains that quickly arise during monotherapy (Englemen et al., 2012). HIV resistance to ART can be divided into two categories: primary resistance, which reflects acquisition of a drug-resistant strain by a newly infected person;

and secondary or acquired resistance, which develops after a period of HIV treatment and which results from drug selection pressure accelerating specific mutations in the virus (Taylor et al., 2008). It should be considered that HIV-1 group O, HIV-2 and some HIV-1 group M subtypes already have natural resistance against NNRTIs and PI (Charpentier et al., 2015; Menendez-Arias, 2013). Therefore, a genotypic resistance test should be done prior to treatment in order to avoid a reduced effectiveness of the chosen therapy (Menendez-Arias, 2013).

Table 1. Antiretroviral therapies available. Adapted and modified from Lucas & Nelson, 2015.

Class	Action point on HIV life cycle	Examples	Specific toxicities
Nucleoside/Nucleotide reverse transcriptase inhibitors	Inhibits reverse transcriptase activity, preventing the conversion of HIV RNA into dsDNA	Zidovudine Lamivudine Abacavir Didanosine Tenofovir	Mitochondrial toxicity Muscle and nerve damage Lactic acidosis Liver steatosis Hepatitis Hypersensitivity reaction Kidney tubular damage
Non-nucleoside reverse transcriptase inhibitors	Inhibits reverse transcriptase activity, preventing the conversion of HIV RNA into dsDNA	Efavirenz Nevirapine	Liver necrosis Stevens–Johnson syndrome Toxic epidermal necrolysis
Protease inhibitors	Inhibits the assembly line of new HIV viruses	Ritonavir Darunavir Atazanavir	Dyslipidaemias Lipodystrophy Liver damage
Integrase inhibitors	Blocks HIV integrase, preventing integration of its genes into the host cell DNA	Raltegravir	Uncommon
Fusion inhibitors	Interfere with HIV's ability to fuse to gp120 receptors on the outer surface of the cell, and blocking entry	Enfuvirtide	Uncommon
Entry inhibitors	Interfere binding of HIV with chemokine receptors, blocking entry	Maraviroc Ibalizumab	Uncommon

The limitations of HAART and the difficulty encountered to develop an effective vaccine against HIV-1 infection have require a high demand for the development of new therapeutic approaches for AIDS treatment. Thus, the HIV research community continue to explore novel therapeutic strategies, including those that target steps in the viral replication cycle that are not disrupted by currently available drugs (Freed, 2015). More promising targets need to emerge quickly, with the aim of blocking further steps in the virus replication cycle and countering the more resilient forms of HIV (Menendez-Arias, 2013). Increased knowledge

about the virus entry process, as well as its participants, has helped to discover and design new molecules capable of inhibiting the infection process. Entry into host cells is an essential step in HIV replication cycle and inhibition of this stage would complement approaches targeting other aspects of the viral replication cycle, such as inhibition of the viral RT, PR and IN enzymes (Acharya et al., 2015; Doms, 2004). Thus, the entry process is quite promising to develop new therapeutic molecules and the entry inhibitors represent a novel approach in prevention and antiretroviral therapy. New classes of drugs such as biologic small-molecule entry inhibitors (antibodies) will improve treatment options and clinical prospects particularly for patients who are infected with viruses already resistant to currently available antiretrovirals (Adamson & Freed, 2008). The development of this type of therapeutic molecules, targeting the virus entry process, represents the focus of this work.

2.2.1. HIV entry inhibitors

The unfolding elucidation of viral entry into cells has paved the way for the development of entry inhibitors. Since there are several protein molecules involved in the entry process, both at the virus and at the host cell level, there are multiple potential therapeutic targets. Unlike most currently used drugs that act only after infection has occurred, entry inhibitors stop the virus before it infects the host cell. Therefore, they may be useful as prophylactic agents, creating a barrier to the initial infection event, and all the knowledge gained in developing these molecules can provide useful leads for effective HIV-1 vaccines (Eckert & Kim, 2001). Viral entry inhibition additionally has the advantage of blocking the progress of the infection at the early steps, preserving an uninfected population of target cells (Flores & Quesada, 2013). Since each step of the viral entry pathway is a potential target for antiviral agents, depending on which step entry inhibitor block, they can be divided into three major classes: CD4-gp120-binding inhibitors; CCR5- or CXCR4-based inhibitors; and fusion inhibitors (Adamson & Freed, 2008).

CD4-gp120-binding inhibitors act on the binding between the gp120 viral glycoprotein and the CD4 cell receptor, which means that they prevent the first step of the virus entry process in the host cell (O'Hara & Olson, 2002). A variety of different candidate molecules with different mechanisms of action have been studied. These include PRO-542, BMS- 378806, BMS-488043, BMS-663068 and Ibalizumab (Adamson & Freed, 2008). Early attempts to develop specific inhibitors of CD4-gp120-binding focused on the design and testing of recombinant soluble CD4 molecules that lack the transmembrane and cytoplasmic domains, but retain the ability to bind gp120, thus functioning as molecular decoys. These molecules showed good *in vitro* activity against tissue culture-adapted strains of HIV-1, but activity in early phase clinical trials was disappointing (Henrich & Kuritzkes, 2013). The conjugation of soluble CD4 molecules to *Pseudomonas aerugniosa* exotoxin PE40 to create an immunotoxin (sCD4-PE40) led to similarly disappointing results (Davey et al., 1994).

Preliminary studies of PRO 542 generated more promising data. PRO 542 is a fusion protein in which the gp120 binding site of the tetrameric structure of the CD4 receptor is bound to the constant region of an immunoglobulin, with the aim to mimic the CD4 receptor. In patients with advanced HIV disease it was observed a modest reduction in plasma HIV-1 RNA levels in a phase 1-2 trial of PRO 542 (Jacobson et al., 2004). However, no additional studies of PRO 542 were developed (U. S. National Library of Medicine, 2018).

Small molecule inhibitors, such as BMS-378806 and BMS-488043, that bind to a specific region within the CD4 binding pocket of gp120 and block the gp120-CD4 interaction showed greater promising. BMS-378806 showed a potent activity *in vitro* against HIV-1 subtype B, but was less active against other subtypes and inactive against HIV-2 (Lin et al., 2003). Although there has been a reduction in plasma HIV-1 RNA in treatment- naïve subjects with BMS-488043, additional development of this molecule was discontinued due to suboptimal pharmacokinetics (Henrich & Kuritzkes, 2013). However this limitation has been overcome with the development of BMS-663068 (a prodrug of the attachment inhibitor BMS-626529), which demonstrated improved pharmacokinetics and increased potency against a greater range of HIV-1 subtypes (Nowicka-Sans et al., 2012). BMS-663068 has not been approved by FDA and there are no ongoing clinical trials (U. S. National Library of Medicine, 2018).

Ibalizumab (formerly TNX-355) is an anti-CD4 humanized monoclonal antibody of murine origin that has displayed promising antiviral activity and safety in early clinical trials. Ibalizumab binds between domain 1 and domain 2 of the extracellular region of CD4. In contrast to attachment inhibitors, ibalizumab does not prevent gp120 binding to CD4, but is thought to decrease the flexibility of CD4, thereby hindering access of CD4-bound gp120 to CCR5 or CXCR4. Its mechanism of action allows antiviral activity regardless of chemokine receptor tropism and suggests that cross-resistance with other classes of antiretrovirals is unlikely. Since ibalizumab binding site is located away from the binding site for major histocompatibility complex class II molecules, it does not appear to interfere with immunological functions that involve antigen presentation (Henrich & Kuritzkes, 2013; Bruno & Jacobson, 2010; Song et al., 2010; Burkly et al., 1992). On March 2018, the U.S. Food and Drug Administration (FDA) approved ibalizumab for adult patients infected with HIV who were previously treated with multiple HIV drugs and whose HIV infections are resistant to currently available therapies (U.S. Department of Health and Human Services, 2018).

Another strategy to inhibit HIV-1 entry is aimed at blocking the interaction between gp120 and the CCR5 or CXCR4 coreceptors. These molecules target the cellular rather than the viral component of the interaction (Adamson & Freed, 2008). Chemokine receptors are attractive targets because they are static targets not prone to mutation, in contrast to viral targets (Eckert & Kim, 2001). The small molecule CCR5 antagonists have been given generic names with the suffix “-viroc”, an abbreviation for viral receptor occupancy (Kuritzkes et al., 2009). Four of these compounds—aplaviroc, maraviroc, vicriviroc and INCB009471

have been developed, but only maraviroc is currently approved for clinical use (FDA, 2018; Henrich & Kuritzkes, 2013). CCR5 is an especially attractive target, since the natural genetic absence of surface-expressed CCR5 in Δ32 protects homozygous genotype individuals from infection with R5 strains of HIV-1 and heterozygous individuals from rapid disease progression, with little apparent impact on their immune status or general health. This epidemiological evidence together with the fact that CCR5 is the coreceptor for the most commonly transmitted HIV-1 strains, which predominate during the early stages of infection and remain the dominant form in more than 50% of late stage HIV-1-infected patients, encouraged researchers towards developing CCR5 ligands as a means to inhibit viral entry (Adamson & Freed, 2008; Dorr et al., 2005). However, the finding that CXCR4 knockout mice suffer from severe disorders suggests that drugs targeting CXCR4 coreceptor may be less well tolerated, and as a result, the development of CXCR4 inhibitors has been more challenging (Henrich & Kuritzkes, 2013; Adamson & Freed, 2008).

Given the clinical efficacy of maraviroc, its relatively low toxicity profile, and its ability to antagonize viral entry, there has been much interest in using the drug for antiretroviral treatment intensification (Henrich & Kuritzkes, 2013). By targeting exclusively CCR5 coreceptor, maraviroc does not inhibit X4 or dual tropic viral isolates (Dorr et al., 2005). Moreover, the possibility that treatment with CCR5 antagonists would promote detrimental selection for viruses with altering tropism and accelerating disease progression (such as X4 viruses), is a significant concern (Henrich & Kuritzkes, 2013). Virologic failure to maraviroc was associated with emergence of CXCR4 tropism in 57% of subjects in whom a repeat tropism test was obtained at the failure time-point (Fatkenheuer et al., 2008). Hence, FDA approval of maraviroc stipulates that it is used as therapy for treatment of adult patients in which only R5 tropic HIV-1 is detectable by baseline coreceptor screening (Adamson & Freed, 2008).

PRO 140 is a humanized anti-CCR5 monoclonal antibody that potently inhibits HIV-1 entry at concentrations that do not affect CCR5's chemokine receptor activity (Trkola et al., 2001). Unlike maroviroc which is a small molecule CCR5 inhibitor with agonist activity, PRO 140 binding to CCR5 does not result in agonist activity but instead in competitive activity (allosteric inhibitor). PRO 140 has completed 7 human clinical trials to determine its therapeutic impact on HIV infection and is currently in two US FDA approved phase IIb/III clinical trials in HIV infected patients (Burger, Parker, Guinta & Lindner, 2018).

Fusion inhibitors act on the gp41 viral protein, in particular on the intermediary states that originate from the conformational changes resulting from the entry process. By binding to gp41, they prevent the conformational changes that are necessary to the success of the fusion process between the cell membranes of the virus and the host cell (O'Hara & Olson, 2002). There are three classes of fusion inhibitors: Class 1 fusion inhibitors bind to the trimeric N-terminal heptad repeat (NHR); class 2 fusion inhibitors bind to the C-terminal

heptad repeat (CHR); and class 3 fusion inhibitors promote the disruption of the trimeric structure of the NHR region (Hrin et al., 2008). To date, the only FDA-approved fusion inhibitor is enfuvirtide (T-20), which is a class 1 fusion inhibitor and was the first entry inhibitor approved for clinical use (Henrich & Kuritzkes, 2013). Enfuvirtide is a 36-amino-acid synthetic peptide, whose sequence corresponds to that of the HR-2 region of the HIV-1 envelope gp41 subunit. Enfuvirtide is thought to block the fusion process by binding to HR1, thereby competitively inhibiting the HR1-HR2 interaction and blocking the six-helix bundle formation (Henrich & Kuritzkes, 2013; Adamson & Freed, 2008). Despite enfuvirtide being the only membrane fusion inhibitor available for treatment of viral infection, its use entails some drawbacks: it requires high doses of administration and has a low genetic barrier for resistance (Ding et al., 2017). Other limitations associated with T20 treatment include the high cost of the peptide manufacturing process and its mode of delivery, which entails twice a day subcutaneous injection (Adamson & Freed, 2008). Searching for fusion inhibitors with higher efficacy and less limitations was a logical next step. Thus, a synthetic peptide with 36 amino acid residues, called Sifuvirtide, was designed based on the crystal structure of gp41 (Wang et al., 2009). Sifuvirtide covers the deep pocket of gp41 so that it has higher binding activity to gp41 and thus, comparing to efuvirtide, is more efficient in inhibiting HIV fusion. It shows potent activity against both primary and laboratory-adapted HIV-1 isolates, and is also highly effective against enfuvirtide resistant HIV-1 variants (Miyamoto & Kodama, 2012). Sifuvirtide can be injected once daily and has entered early phase human clinical studies in Asia (Henrich & Kuritzkes, 2013).

F63 is a recently engineered VL sdAb with elongated CDRs which was selected by Phage Display technology from a synthetic library. The antibody showed to broadly and potently inhibit HIV-1 and HIV- 2 primary isolates infection by targeting a different sequence within the highly conserved hydrophobic pocket of HR1 (Cunha-Santos et al., 2016).

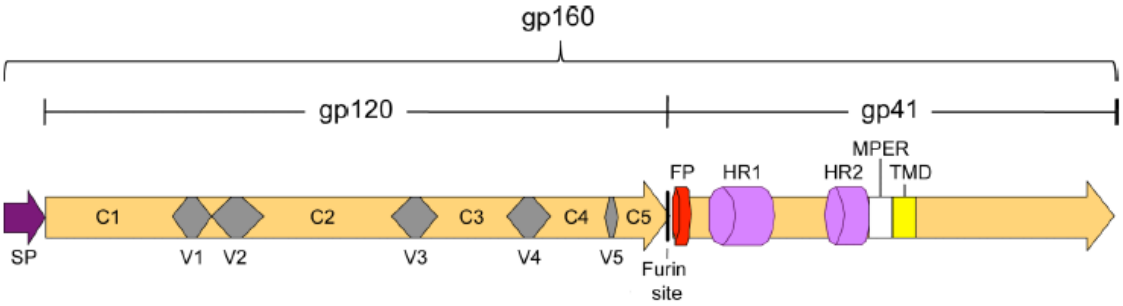
Although several entry inhibitors have already been developed, only three have been approved by FDA. Enfuvirtide was the first drug to be used in clinical practice, followed by maraviroc and ibalizumab. However, none of these agents offers a complete solution to the inhibition of HIV entry (Nowicka-Sans et al., 2012). The discovery and development of novel agents directed towards other targets of the entry process of the replicative cycle of the virus, such as gp120, emerges nowadays as an imperative need (Flores & Quesada, 2013).

2.3. gp120 HIV-1 glycoprotein

2.3.1 Structure and Function of gp120 HIV-1 glycoprotein

The Env is the only virally encoded antigen on the surface of HIV-1 and is responsible for viral entry into host cells (Julien et al., 2013). The *env* gene encodes the envelope spike proteins as a gp160 polyprotein precursor that is post-translationally cleaved by proteases of the furin family to the mature SU gp120, which is responsible for receptor and coreceptor attachment, and the TM gp41 subunit, that contains the fusion machinery and that anchors the envelope spikes into the viral membrane (Figure 7). gp120 and gp41 are named after their approximate molecular weights of 120,000 and 41,000 Da, respectively (Weiss, 2013). These proteins form trimers of noncovalently linked dimers before the native complex reaches the surface of infected cells and is then packaged onto virions (Checkley, Luttge & Freed, 2011**;** Decroly et al., 1994). Each HIV-1 particle shows only 7-14 Env spikes on their surface (Zhu et al, 2006).

Figure 7. Schematic representation of the constituent domains of HIV-1 Env.

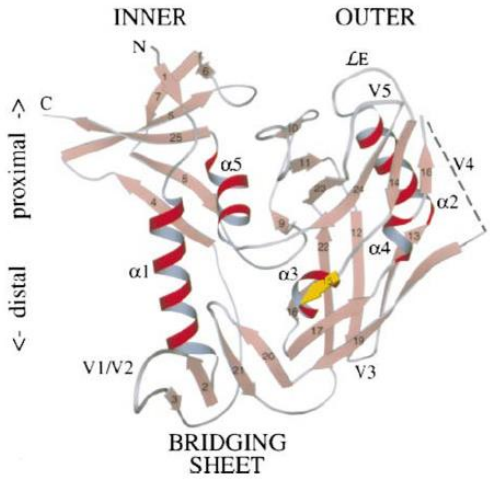


gp160 precursor is cleaved into the SU subunit (gp120) and TM subunit (gp41) in the Golgi complex at the furin site indicated. gp120 contains five variable domains (V1-V5) and five constant domains (C1-C5). Sp- signal peptide, which is cleaved during translation. Adapted from Checkley et al., 2011.

The overall conformation of gp120 is somewhat flexible, what proved to be a major obstacle in solving its structure in the late 1990s. High-resolution structure of the HIV-1 SU core was obtained in 1998 by Kwong and his colleagues, were they complex gp120 with a two-domain fragment of CD4 (comprising the two N-terminal immunoglobulin domains) and an antigen-binding fragment of a neutralizing antibody (Fab 17b) that blocks chemokine-receptor binding. In this way they stabilized the gp120 core to allow crystallization and solution of a core gp120 structure (Figure 8). The gp120 core structure consists of an inner and outer domain, which refer to portions of the SU that are respectively closer to the inner and outer regions of the trimeric spike. These domains are joined by a small “bridging” domain. The

inner domain is appreciably more conserved than the outer domain (Turner & Summers, 1999; Kwong et al., 1998).

Figure 8. Structure of the HIV-1 SU gp 120 core. Adapted from Kwong et al., 1998.



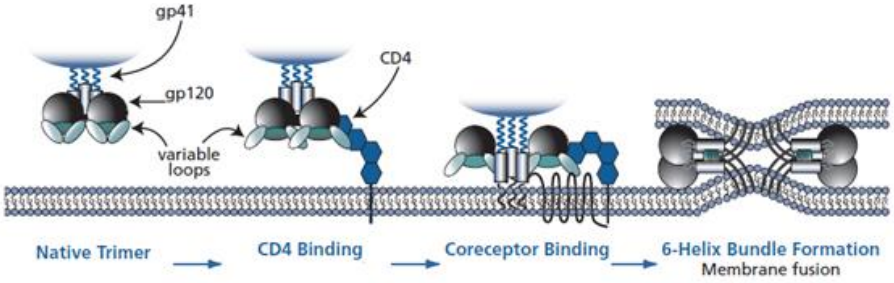
Gp120 is extensively glycosylated, particularly with N-linked high-mannose residues which make up more than 50% of its mass, and contains five variable regions (V1-V5) interspersed with five highly conserved regions (C1-C5) (Doores et al., 2010; Starcich, et al., 1986). The bridging domain is composed of part of the C4 domain and the base of the V1 and V2 stem (Kwong et al., 1998). The carbohydrates form a glycan shield protecting sensitive sites such as receptor-binding pockets, but also present epitopes recognized by some neutralizing antibodies (McCoy & Weiss, 2013). The conserved domains form discontinuous structures which are important for the interaction with gp41 ectodomain and with viral receptors on the host cell (Kwong et al., 1998). Sequence variability in the V domains arose through recombination, point mutations, insertions and deletions. The V1V2 domain is the most variable in loop length, whereas the V3 loop and C2, C3, and C4 domains show relatively little length variation. V3 is important for membrane fusion and coreceptor specificity (the switch from R5 to X4 tropism is driven by V3 mutation). The conserved domains, in particular C1, C3 and C4, are the principle Env determinants that bind CD4 (reviewed in Checkley et al., 2011).

The variability and glycosylation of the gp120 surface probably modulate the immunogenicity of the gp120 glycoprotein (Profy et al., 1990**).**

Infection of the host cell by HIV is initiated by interactions between SU and cell-surface CD4 molecules (Figure 9). SU gp120 confers cellular tropism to the virus by binding to the host amino-terminal of the four immunoglobulin-like domains of CD4, and to a co-receptor (primarily CCR5 or CXCR4), and acts as a trigger for the fusogenic activity of the TM gp41

(Zanetti. Briggs, Grünewald, Sattentau & Fuller, 2006; Turner & Summers, 1999; Kwong et al., 1998). By binding to CD4, gp120 anchors the virus to the cell surface and conformational changes in the envelope glycoproteins are initiated (Doms, 2004). Conformational changes in gp120 exposes a site known as the CD4 induced site (CD4i) and allows the protrusion of V3 loop. Both CD4i and the V3 loop interact with chemokine receptors, being V3 loop the major determinant of R5 or X4 tropism. Co-receptor engagement leads to trimer destabilization and acts as a switch for gp41 to undergo a radical rearrangement that induces the formation of a six-helix bundle that initiates fusion between viral envelope and cell membrane (Weiss, 2013; Colman & Lawrence, 2003). Two heptad repeats (HR1 and HR2) in the ectodomain of gp41 are required for the formation of the six-helix bundle and thus play an essential role in the fusion process (Adamson & Freed, 2008).

Figure 9. Steps in HIV entry into host cells. Adapted from Moore & Doms, 2003.



2.3.2 gp120 HIV-1 glycoprotein as a therapeutic target

The inhibition of HIV entry is a highly promising strategy, and glycoprotein gp120 plays a central role in this process since the CD4–gp120 interaction occurs with high affinity and is an obligate step of the virus replication cycle. As the knowledge on gp120 role in the entry process as well as detailed structural and intimate mechanistic information have increased, the protein has emerged as a promising target for the development of HIV inhibitors (Flores & Quesada, 2013). Due to the interactions that gp120 establishes in the process of entry and fusion, the glycoprotein offers a wide set of different possibilities to interfere with its normal function. For this purpose several molecules acting in different points of the glycoprotein were developed. From unspecific electrostatic interactions established between negatively charged molecules (polyanionic compounds, such as sodium cellulose sulfate (Ushercell®), Carrageenan (Carraguard®), PRO 2000, cellulose acetate phthalate and Aurintricarboxylic acid) and positively charged domains of the protein, to selective binding to sugar residues of the gp120 glycans (carbohydrate-binding agents, such as Griffithsin, Cyanovirin-N, Microvirin, Pradimicin A and Alcian Blue), both precluding efficient attachment, passing through protein-protein interaction inhibition such as CD4 or co-receptor binding, such as

IC9564, a betulinic acid derivative, and tyrosine sulfate mimetics (Lu et al., 2016; Flores & Quesada, 2013; Teixeira, Gomes, Gomes & Maurel, 2011). Nevertheless, although gp120 has a critical role in the virus entry and has an exposed location on viral surface, currently no FDA-approved drug targets directly the HIV-1 gp120 glycoprotein. The search for smaller molecules capable of inhibiting the specific binding of gp120 to CD4 continues, and antibodies, in an attempt to reach targets not accessible to other drugs, are among these molecules (Acharya, Lusvarghi, Bewley & Kwong, 2015).

Despite the overall genetic heterogeneity of the gp120 glycoprotein, the conserved binding site that interacts with CD4 receptor, provides an attractive antiviral target (Teixeira et al., 2011). Rare monoclonal antibodies derived from naturally infected humans and from experimentally immunized animals recognize the CD4-binding site on gp120. As all the diverse HIV strains utilize CD4, some of these antibodies have range as well as potency in neutralizing almost all virus strains (Weiss, 2013).

Antibody responses to the HIV-1 envelope glycoproteins can be classified into three groups: i) binding but non-neutralizing responses, ii) strain-specific responses, and iii) broadly neutralizing responses. The first group includes responses to Env proteins that do not neutralize viruses, even those bearing an Env protein sequence identical to that of the immunizing antigen. The second group includes the responses to Env proteins that neutralize virus in a highly strain-specific manner. In this response regions on gp120 with relatively high sequence variation are the typical target. The third group includes responses to Env proteins that neutralize a broad spectrum of global HIV-1 strains. Such antibodies can act antivirally against a wide spectrum of viruses by targeting relatively conserved regions on the surface of HIV envelope trimer spike (Burton & Hangartner, 2016; Burton & Mascola, 2015).

Until a decade ago, few broadly neutralizing Abs (bnAbs) had been isolated. These defined only three major Env antigenic sites: the CD4-binding site of gp120, an exclusively glycan epitope on the outer domain of gp120 and the membrane-proximal external region of gp41. The development of new techniques of detection and selection of antibodies allowed the isolation of hundreds of bnAbs with markable potency and breadth of reactivity against diverse HIV-1 strains. Moreover, new antigenic sites such as a gp120 V2-glycan site at the apex of the Env trimer, a gp120 V3-glycan site centered on the glycan at Asn332 and an extended region including residues from both gp120 and gp41, were identified (Burton & Mascola, 2015).

Elicitation of and recognition by bnAbs are hindered by the arrangement of spikes on virions and the relatively difficult access to bnAb epitopes on spikes, including the proximity of variable regions and a high density of glycans (Burton & Hangartner, 2016). However, around 30% of HIV-1-infected people develop a broadly neutralizing antibody response against HIV-1 (Euler et al., 2010).

In the past years the scientific community has seen the isolation of many bnAbs of remarkable potency that have shown prophylactic and therapeutic activities in animal models (Burton & Mascola, 2015). VRC1/VRC2 and b12 are examples of monoclonal Abs (mAbs) that have been identified and selected. VRC1 and VRC2 are two potent neutralizing mAbs, with the capacity to neutralize more than 90% of circulating HIV-1 strains, that were identified by sorting HIV-specific patient memory B-cells on rationally designed Env constructs favoring the selection of mAbs that target the CD4 binding site on Env (Wu et al., 2010). b12 recognizes a conformational epitope at the CD4 binding site of gp120 and competes directly with CD4 binding. It was originally selected from scFv phage display libraries and is able to neutralize about 50% of HIV-1 isolates (Binley et al., 2004). These antibodies are helping to guide rational vaccine design and therapeutic strategies for HIV-1 (Burton & Mascola, 2015).
The development of a drug that blocks gp120 could not only function at a therapeutic level but could also prevent viral transmission in a prophylactic manner, and in this way work as the most effective way to prevent HIV-1 infection besides the ultimate goal, which is the development of an HIV vaccine (Hertje, Zhou & Dietrich, 2010). Yet, these novel drugs should be used in combination with conventional retrovirals in HAART in order to inhibit HIV at as many levels as possible and minimize the risk of resistances (Flores & Quesada, 2013).

2.4 Antibodies

2.4.1 Immune system

The immune system is a defensive system whose primary functions are to protect against infectious organism and foreign molecules, and the development of cancer (Zachary & McGavin, 2012). It consists mainly of lymphatic organs, isolated cells, humoral factors, and cytokines that defend the body against virtually any foreign antigen (Zachary & McGavin, 2012; Junqueira & Carneiro, 2008). In general, the immune system works to restore the body's homeostasis by detecting, protecting and eradicating antigens and neoantigens that, respectively, enter or arise in the body (Lopez & Mitchell, 2017). Because effective resistance to infection is critical, the body can’t rely on a single defense mechanism. Thus, to be effective, multiple defense systems linked together to form complex interacting networks must be available. These include physical barriers that exclude invaders, innate immunity that provides rapid initial protection and adaptive immunity that provides prolonged effective immunity (Tizard, 2013).
Physical barriers include intact skin; the “self-cleaning” processes such as coughing, sneezing, and mucus flow in the respiratory tract, diarrhea and vomiting in the gastrointestinal tract, and urine flow in the urinary system; and the presence of well adapted commensal bacteria that can easily out-compete poorly adapted pathogenic organisms (Tizard, 2013).

Innate Immunity is activated immediately when a pathogen overcomes the physical barriers and invades the body, and is directed toward the rapid elimination of the pathogen. It is a network of subsystems that lack any forms of memory, and as a result, each infection episode is treated identically. Therefore, the intensity and the duration of innate responses, such as inflammation, remain unchanged no matter how often a specific invader is encountered. On the other hand, in the adaptive immunity, the more often an individual encounters an invader, the more effective will be the defense mechanism against that organism (Tizard, 2013). The adaptive immune system response involves the proliferation of T and B lymphocytes, a process that is triggered by the binding of antigens (nonself molecules) to cellular receptors (Goldsby, Kindt, Osborne & Kuby, 2003).

A fundamental difference between the innate and adaptive immune systems lies in their use of cell surface receptors to recognize foreign invaders. The cells of innate system use a limited number of preformed receptors that bind to molecules commonly expressed on many different microbes (Tizard, 2013). Unlike receptors on innate immune cells, lymphocytes possess surface receptors (Igs on B lymphocytes and the T-cell receptors on T lymphocytes) that recognize antigens with remarkable specificity (Yatim & Lakkis, 2015). The genes that encode these receptors are not inserted in the germline, as with the defense mechanisms of the innate response (Janeway & Medzhitov, 2002), but are the product of gene recombination during lymphocyte development, a process that generates a very large number of unique antigen receptors by splicing, rearranging, and linking a finite set of adjacent genes (Gearhart, 2004).

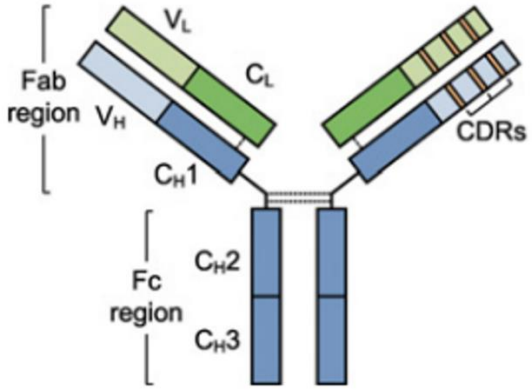
Depending on the origin of the pathogens (external or internal), different defense strategies are required so that the adaptive immune system can be divided into two types of responses. One response is directed against extracellular invaders (humoral response) and the other against intracellular invaders (cellular response). In the humoral immune response the destruction of the invaders is mediated by specific antibodies which result from the rapid proliferation and differentiation of lymphocytes B (Goldsby et al., 2003), whereas in the cell-mediated immune response specialized cells are required to destroy the infected cells since antibodies can´t internalize (Tizard, 2013). The cellular response is mediated by T lymphocytes that express at their surface specific receptors capable of recognizing antigens exposed by the MHC complexes on the surface of antigen presenting cells and, thus, eliciting the immune response. When T lymphocyte receptors bind to the antigens exposed in the MHC complexes, their proliferation and differentiation occurs and leads to effector and memory cells that mediate the cellular response (Goldsby et al., 2003).

Although it takes several days or weeks to become effective, the adaptive immune system is a complex and sophisticated system that provides the ultimate defense of the body (Tizard, 2013).

2.4.2 Characteristics and properties of Antibodies

Antibodies are a class of soluble glycoproteins called immunoglobulins (Ig) that are produced by the immune system in response to the presence of antigens (Tizard, 2013). It is required an immense number of antibodies with different specificities to recognize and bind as many antigens as possible. The genetic information for this repertoire is stored in the B-cell pool of the lymphatic system. The wide repertoire of antigen-binding sites is achieved by random selection and recombination of a limited number of V, D, and J gene segments that encode the variable domains. This process generates the primary antibody repertoire (Tizard, 2013; Male, Brostoff, Roth & Roitt, 2006; Gearhart, 2004). In mammals there are five classes of antibodies known as IgG, IgA, IgM, IgD, and IgE which differ in size, charge, amino acid sequence and carbohydrate content and, therefore, in their functions in the immune system (Male et al., 2006). IgGs are the most abundant immunoglobulins in the blood (Tizard, 2013; Male et al., 2006) and are also the prevalent format of the therapeutic antibody in the recombinant antibodies industry (Aires da Silva, Corte-Real & Gonçalves, 2008)**.**

Figure 10. Basic IgG structure.



The IgG monoclonal antibody is a complex glycoprotein structure consisting of two light chains and two heavy chains, each one comprised of constant (CH/L) and variable (VH/L) regions. The constant regions are responsible for effector functions while the variable regions are involved in binding antigens. Adapted from Ruigrok, Levisson, Eppink, Smidt & van der Oost, 2011.

Its structure consists of two identical light (L) chains and two identical heavy (H) chains (Tizard, 2013). The four chains are connected to each other by covalent and non-covalent bonds which provide a three-dimensional Y-shaped structure with the antigen-binding fragments (Fabs) at the amino-terminal of each chain (Goldsby et al., 2003), linked via a flexible region (hinge) to a constant fragment (Fc) region at the carboxi-terminal (Figure 10). Heavy chains contain a variable domain (VH) and three constant domains (CH1, CH2, and CH3), while light chains contain a variable domain (VL) and a single constant domain (CL).

Between the CH1 and CH2 domains is located the hinge region, which is a flexible segment of the heavy chain that allows the two antigen-binding sites to operate independently (Male et al., 2006). Within each variable region there are three noncontiguous linear intervals of greatest variability, termed complementary determining regions (CDRs), as they can interact with the bound antigen, and are responsible for the specificity (recognition) and affinity (binding) of the antibodies to the antigen. The CDR that have the greatest variation in terms of length and amino acid sequence is the heavy chain CDR3 (HCDR3) (Barbas, Burton, Scott & Silverman, 2001). The sequences between the CDRs are conserved regions referred to as framework residues (FRs) and they act as a scaffold to support the loops of the CDR. While VL and VH form antigen binding sites, CL and CH determine effector functions. Fc region recruits cytotoxic effector functions and it also influences the serum half-life of antibodies (Male et al., 2006).

The L chains of most vertebrates exists in two forms known as kappa (k) and lambda (λ) (Male et al., 2006; Barbas et al., 2001). In humans, the H chains exists in four forms called ɣ1, ɣ2, ɣ3 and ɣ4, that give rise to the four humane subclasses IgG1, IgG2, IgG3, IgG4, with marked differences in their ability to active effector functions (Barbas et al., 2001).

2.4.3. Novel therapeutic antibodies

The detailed knowledge of antibody structure and activity allowed researchers to engineer antibodies in a way that, in the last three decades, they had a dramatic transformation from scientific tools to powerful human therapeutics (Buss, Henderson, McFarlane, Shenton & Haan, 2012), and nowadays they are the fastest growing class of therapeutic agents (Beck, Wurch, Bailly & Corvaia, 2010). The disease areas that these therapeutic antibodies can target have expanded and they are currently utilized as pharmaceuticals for cancer, autoimmune disease, organ transplantation, cardiovascular disease, respiratory disease, ophthalmologic disease and viral disease (Suzuki, Kato & Kato, 2015). In therapies where antibodies are used as therapeutic agents, the goal is to eliminate or neutralize the pathogenic organism or the disease target. For this purpose, therapeutic antibodies can function by three principal mechanisms of action: blocking the action of specific molecules by direct binding; targeting specific cells by directing antibodies with effector functions such as antibody dependent cell mediated cytotoxity and/or complement-dependent cytotoxity towards specific populations of cells; or functioning as signaling molecules by inducing crosslinking of receptors that are, in turn, connected to mediators of cell division or cell death (Brekke & Sandlie, 2003). However, the complex nature of a conventional antibody is sometimes not consistent with efficacy and forms an important obstacle for an immediate development into a therapeutic compound. The long serum half-life associated to a full-length IgG molecule can result in poor contrast in imagine applications and may increase the antibody immunogenicity, the large molecular size decreases the capacity to penetrate

dense tissues, and inappropriate activation of Fc-receptor-expressing cells may lead to toxic effects associated with massive cytokine release (Kobayashi, Choyke & Ogawa, 2016; Holliger & Hudson, 2005; Hudson & Souriau, 2003). The advances that genetic engineering has had in recent decades have allowed the molecular manipulation of antibodies, promoting the development of recombinant molecules and antibody fragments (Figure 11) with fewer negative effects than conventional antibodies (Saerens, Ghassabeh & Muyldermans, 2008).

2.4.3.1 Antibody Fragments

For cytokine inactivation, receptor blockage or viral neutralization, the Fc-induced effector functions are often unwanted (Hudson & Souriau, 2003). The constituent domains of monoclonal antibodies may be separated from each other to provide antibody fragments of different sizes, which retain their original functionality. Initially, they were generated through proteolysis near the disulfide bridges (with enzymes as papain and pepsin) but with the evolution of biotechnology nowadays, it is through genetic engineering techniques that these antibody fragments are obtained. Therefore, smaller antibody molecules such as the Fab, the minimal sized variable fragment (Fv), consisting of the paired variable domains of the H and L immunoglobulin chains, and VL or VH sdAbs may be constructed and have become more attractive as therapeutic agents. The Fv domains dissociate easily because of the lack of covalent or disulphide bridges between the two chains. For their stabilization the VH and VL domains are joined, with a flexible polypeptide linker, into a single-chain Fv (scFv) (Figure 11) (Saerens et al., 2008; Holliger & Hudson, 2005).

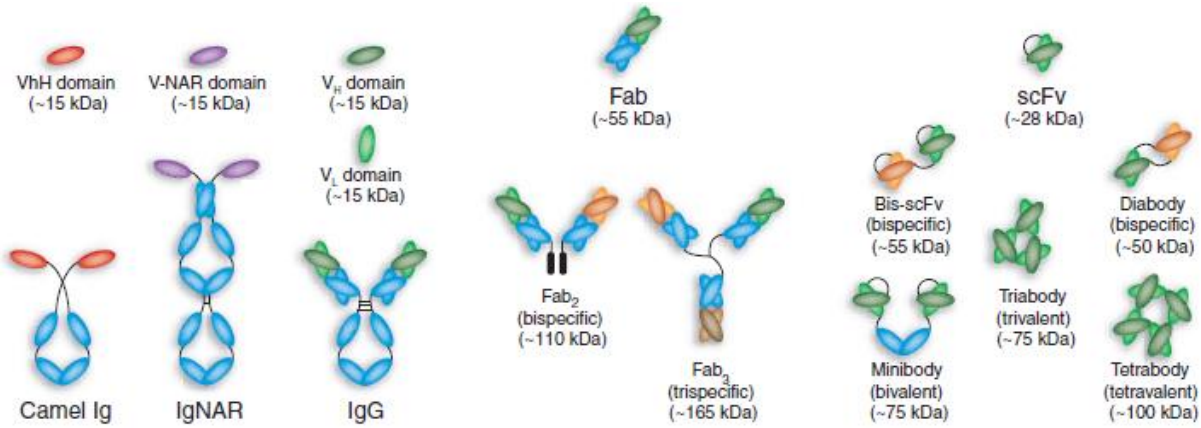
These antibody fragments, because of the antigen-binding VH and VL domains, retain the specificity and affinity of the antibody that gave rise to them, while showing improved pharmokinetics for tissue penetration. In addition to maintaining the same specificity for the target molecule, the costs associated with production are smaller and they have unique characteristics, such as its small size and ease of expression, which allow its application both in terms of diagnosis and therapeutics (Holliger & Hudson, 2005).

2.4.3.2 Single-Domain Antibodies

Although multiple smaller antigen-binding fragments of the immunoglobulin have been developed, sdAbs are the simplest variant of all. sdAbs are monomeric antibody fragments consisting only of the VL or VH regions, presenting reduced dimensions (≈ 15 KDa) compared to the whole antibody (≈150 KDa) and showing several advantages mainly related with their reduced size (Saerens et al., 2008). The diversity with respect to antigen recognition and binding are not affected since they are promoted by the existence of the three CDRs. Thus, CDRs allow the use of sdAbs as individual molecules without the

presence of the remaining glycoprotein chain since they are responsible for recognition and binding to the antigen (Polonelli et al., 2008).

Figure 11. Schematic representation of different antibody formats.



Schematic representation of different antibody formats, showing intact 'classic' IgG molecules alongside camelid VhH-Ig and shark Ig-NAR immunoglobulins. A variety of antibody fragments are represented, including Fab (Antigen-binding fragment), scFv (Single-chain variable fragments), single-domain VH, VhH and V-NAR and multimeric formats, such as minibodies, bis-scFv, diabodies, triabodies, tetrabodies and chemically conjugated Fab multimers. Adapted from Holliger & Hudson, 2005.

There are sdAbs of natural origin and sdAbs that are isolated from complete antibodies. The naturally occurring sdAbs are derived from camelids (camels and lamas) and cartilaginous fish (wobbegong and nurse shark), are H chain antibodies that occur naturally without the L chain, and are referred as VHH and IgNAR, respectively (Figure 11) (Nuttall et al., 2001; Greenberg et al., 1995; Hamers-Casterman et al., 1993). VHH consists of four FRs and three CDRs, while IgNARs contain only two CDRs loops, which makes them the smallest natural antigen-binding site (~12kDa) (Muyldermans, 2001; Greenberg et al., 1995). In both natural sdAbs, CDR3 is longer than the HCDR3 loop in humans or mice, which enables them to reach targets that are not easily recognized by currently available antibody therapies (Holt, Herring, Jespers, Woolven & Tomlinson, 2003; Desmyter, Decanniere, Muyldermans & Wyns, 2001).

sdAbs of non-natural origin are all antibody-derived fragments where VH or VL were isolated by biotechnological methods from antibody repertoires of various species, including human, mice and rabbits.

Since sdAbs are small in size, their genes can be expressed efficiently in bacteria as active, soluble, and robust proteins (Saerens et al., 2008). Besides that, sdAbs usually penetrate solid tissue much more rapidly and efficiently than the full length IgG. However, this size

advantage is countered by a very short serum half-life that eventually could decrease the overall tissue uptake of these fragments (Li & Zhu, 2010). In certain situations, short half-lives are useful in particular when using radiolabelled antibodies, but therapeutic applications generally requires longer half-lives for antibodies to remain in circulation for longer. To increase the serum half-life of antibody fragments, such as sdAbs, several approaches has been explored such as PEGylation, the chemical addition of polyethylene glycol (PEG) to increase the size of the fragments and simultaneously reducing the immunogenicity by coupling of PEG to amino groups on the antibody (Chapman, 2002); and HAS-fusion, fusion of the recombinant antibody to human serum albumin (Muller et al., 2007). Another advantage is the possibility that they have to be genetically modify without losing their original properties, in order to improve features, such as affinity and selectivity, or adapt them to the intended purpose (Saerens et al., 2008).

Since sdAbs lack the Fc domain, the natural effector functions of antibodies are absent and the non-specific uptake in tissues that highly express Fc receptors is low as well as their immunogenicity. Therefore, to generate modified effector functions and to obtain more potent next-generation therapeutics it is necessary to provide the sdAbs with an engineered Fc or with novel accessory effector molecules such as enzymes or toxins, for example (Saerens et al., 2008).

When a conventional IgG antibody is converted into Fabs, scFvs or sdAbs fragments, a decrease in antigen-binding activity due to loss of avidity is usually associated. Yet, this loss in binding activity can be compensated by engineering multivalent antibody fragments (Figure 11). sdAbs have resistance to stringent conditions and strict monomeric behavior that make them suitable as a molecular structure in more complex constructs, being easily designed into multivalent and multispecific formats. Bivalent or bispecific sdAbs, are obtained by the inframe cloning of two identical or two different sdAbs, respectively, spaced with a short linker (Figure 11). Bispecific antibodies whereby each sdAb binds to a different target or to a different epitope on the same target offer a clear advantage over the bivalent, monospecific mAbs. They can be powerful therapeutic agents since the simultaneous blockade of several targets might therefore yield better therapeutic efficacy than inhibition of a single target (Beck et al., 2010; Saerens et al., 2008; Hudson & Souriau, 2003).

The aim of sdAbs is clearly to provide new binding specificities, particularly to targets inaccessible to conventional antigen-binding sites, such as G protein–coupled receptors, enzyme active sites and many viral surface clefts, grooves or buried epitopes, which cannot be reached by conventional IgG or larger antibody fragments (ex: Fab or scFv) (Saerens, et al., 2008; Holliger & Hudson, 2005). This enhances the therapeutic use of sdAbs. The success of the study and application of these proteins is due to the evolution of genetic engineering techniques and to Phage Display technology, an *in vitro* screening approach which allows a rapid selection of the desired antibodies.

2.5 Selection of monoclonal antibodies

2.5.1 Antibody Libraries

Library display has replaced hybridoma technology for the selection of recombinant antibodies by creating large natural and synthetic immune repertoires *in vitro* (Hudson & Souriau, 2003).

In general, an antibody library can be made from the H and L chain gene segments of any animal whose immunoglobulin gene structure and sequences are known (Andris-Widhopf, Steinberger & Barbas, 2001). These genes are amplified from lymphatic tissue or peripheral blood lymphocytes by reverse transcription and polymerase chain reaction (PCR), using specific primers. The PCR fragments are later cut by restriction enzymes, such as SfiI, and cloned into a phagemid vector for Phage Display application. From these libraries, antibodies with specific high affinity can be selected by binding the phenotype to the genotype, thus allowing the simultaneous recovery of the gene encoding the selected antibody (Steinberger, Sutton, Rader, Elia & Barbas, 2003).

There are three types of antibody libraries that can be used: immune, naïve and synthetic libraries. In immune libraries the source of V-genes is the IgG mRNA of immunized animals or human B-cells. The immunization can be intentional (for example, when specific antigen is repeatedly used to vaccinate an animal over the course of several weeks or months), or natural (for example, an individual infected with HIV). These libraries have been used to produce high quality and high affinity antigen-specific antibodies and for the study of biologic antibody responses (Bazan, Całkosiński & Gamian, 2012; Andris-Widhopf et al., 2001; Barbas et al., 2001). The use of immune libraries have the major advantage of generating antibodies that have undergone affinity maturation by the immune system, leading to an increased chance of obtaining high-affinity antibodies, even when the library is relatively small (10^7-10^8 clones) (Andris-Widhopf et al., 2001; Barbas et al., 2001). However, at the same time the immunization of animals is a time consuming process and each antigen requires the construction of a new antibody library. Moreover, there are limitations in the creation of human antibodies libraries for ethical reasons and therefore, they are restricted to individuals who have received approved vaccines or who have been naturally infected or exposed to foreign antigens. Other disadvantages are the impossibility of always predict the immune response to the target antigen and the lack of immune response to antigens as toxins, self-antigens and poor immunogenic antigens (Andris-Widhopf et al., 2001).

In naïve libraries, V-genes are derived from the IgM mRNA of an animal or human that has not been intentionally immunized with a specific antigen (Bazan et al., 2012). IgM antibodies are preferred to IgG because they have not been subjected to antigen selection and therefore are more diverse. The most abundant source of naïve B lymphocytes that express IgMs is the bone marrow, although the spleen is also used. In this approach, the H and L

chain antibody genes are randomly combined to form a library of antibodies that can be used in the selection against almost any antigen. Since there is no need for immunizations, naïve libraries are useful when attempting to obtain human antibodies because medical ethics prevent the intentional immunization of humans with unapproved, unsafe or untested immunogens. Naïve libraries can also offer the advantage of producing antibodies against toxic, self or non-immunogenic antigens such as dose that have highly conserved and limited epitopes that may only be weakly immunogenic (Andris-Widhopf et al., 2001). Nevertheless, in order to successful selection of high affinity antibodies such libraries must be extraordinarily large, typically having 10^9-10^{10} or greater number of unique clones (Moon et al., 2011). Moreover, the potentially limited diversity of the IgM repertoire, the unknown history of the B-cell donor and the tendency to achieve increased cross-reactivity are the main disadvantages of naïve libraries (Azzazy & Highsmith, 2002).

In synthetic libraries, B cells are not involved in antibodies construction. In this library antibody genes are obtained entirely *in vitro* by randomizing the HCDR3 region using oligonucleotide directed mutagenesis or PCR based techniques. Synthetic libraries provide a tool that may be useful when antigen deficiency occurs or the antigen is toxic or immunogenicity occurs and immunization is not possible (Bazan et al., 2012). A main advantage of synthetic repertoires over naïve ones is the potential to control and define the contents, local variability and overall diversity of synthetic libraries (Azzazy & Highsmith, 2002; Knappik et al., 2000).

2.5.2 Rabbit Immunizations

When, in human medicine, antibodies are required for therapeutic purposes, human antibody libraries are the obvious choice. However, due to the ethical challenge of obtaining immunized human B-lymphocytes, it is not always possible to use the human repertoire. Thereby, immunization of animals, such as non-primates, mice, rabbits and chikens has become an alternative (Andris-Widhopf et al., 2001). Mice have become very popular for immunization because of the ease of generation of murine mAbs using hybridoma technology, published in 1975 by Köhler and Milstein. The main advantage of mice immunization include the availability of a wide range of secondary reagents for the detection of mouse antibodies. But sequence variation in the amino terminus of the H chain poses a disadvantage, as it necessitates the use of many PCR primers to get a good representation of the antibody repertoire (Barbas et al., 2001). In addition, murine mAbs are often associated with allergic reactions and the induction of anti-drug antibodies; they exhibit a relatively short half-life in humans compared to human IgG (Ober, Radu, Ghetie & Ward, 2001); and are relatively poor recruiters of effector function which can be critical for their efficacy (Stern & Hermann, 2005). Thus, the main focus of antibody engineering technology development in the last years has been to reduce immunogenicity of murine antibody and to

improve manufacturability (Li & Zhu, 2010). Moreover, hybridoma technology is a very time-consuming and laborious process and therefore different selection strategies were developed since 1975 in order to facilitate the generation of mAbs (Hanack, Messerschmidt & Listek, 2016).

In the form of polyclonal antibodies, the rabbit antibody repertoire has been used in diagnostic applications for decades and is an attractive source for the generation of therapeutic human antibodies (Rader et al., 2000). Rabbits antibody repertoire offer an attractive alternative to the murine repertoire, bypassing the problems associated with the use of mice as the main source of mAbs. Therefore, rabbits, comparing to mice, present a number of advantages for the library approach. First, they elicit a better immune response. Second, very few antibody gene segments are rearranged and accessed during immune responses in rabbits, which reduces the number of PCR primers required for cloning of a repertoire (Barbas et al., 2001). Third, most strategies to generate mAbs are based on the recovery of B cells from spleen, bone marrow and/or blood, which are present in higher quantities in rabbits than in mice due to their larger body size (Feng, Wang & Jin, 2011). Forth, antibody stability is an important property of sdAbs, not only for promoting a good conformation of CDRs for antigen recognition but also for downstream applications such as production and serum half-life. Because rabbit antibodies evolve maturation by somatic hypermutation, the stability of their sdAbs is not dependent on inherent properties of a single VH or VL family but a property that can be subject to evolution (Aires da Silva & Gonçalves, 2008). Fifth, rabbit's antibodies have a long HCDR3, a length distribution more closely related to human antibodies than mouse antibodies (Popkov et al., 2003). This property is often found in potent human anti-HIV gp120 mAbs that have unusual long HCDR3 loops necessary to penetrate the Env glycan shield (Kwong & Mascola, 2012). Besides all this, once rabbits are evolutionarily distant from mice, some epitopes that might not be immunogenic in rodents, can be recognized by rabbit antibodies (Mage, Lanning & Knight, 2006) and these therefore, can be easily selected from rabbit-derived antibody libraries (Aires da Silva et al., 2012). Selection by Phage Display offers the advantage of ready access to antibody sequences and facilitates further *in vitro* optimizations, such as affinity maturation or humanization (to reduce the limitation of immunogenicity inherent to non-human mAbs), once phenotype and genotype are selected at the same time (Aires da Silva et al., 2012; Steinberger et al., 2003) and thereby generates rabbit mAbs as promising reagents for therapeutic applications in humans.

2.5.3. Tissue source

The choice of tissue source is a critical point in the immune library approach. Ideally the selected tissue should be rich in plasma cells that secrete antibodies against the antigen of interest, because, in that way, it will contain the highest levels of specific mRNA. Peripheral

blood is not a good choice of tissue source, because, despite it's possible to observe a great increase in the number of specific antibody-secreting cells shortly after re-exposure to the antigen; at later points this number declines rapidly to a low baseline level. Bone marrow and spleen are a major repository of antibody producing cells and are most frequently used for generation of antibody libraries because, in contrast to what happens in the peripheral blood, there is no need of recent exposure to the antigen to have a high level of specific antibody-secreting cells. The spleen is a good source of post-immunization antibody-producing cells at about 4 to 6 days following antigen boost, but by 11 days following the boost, antigen-specific antibody-producing cells are more prevalent in the bone marrow (Barbas et al., 2001).

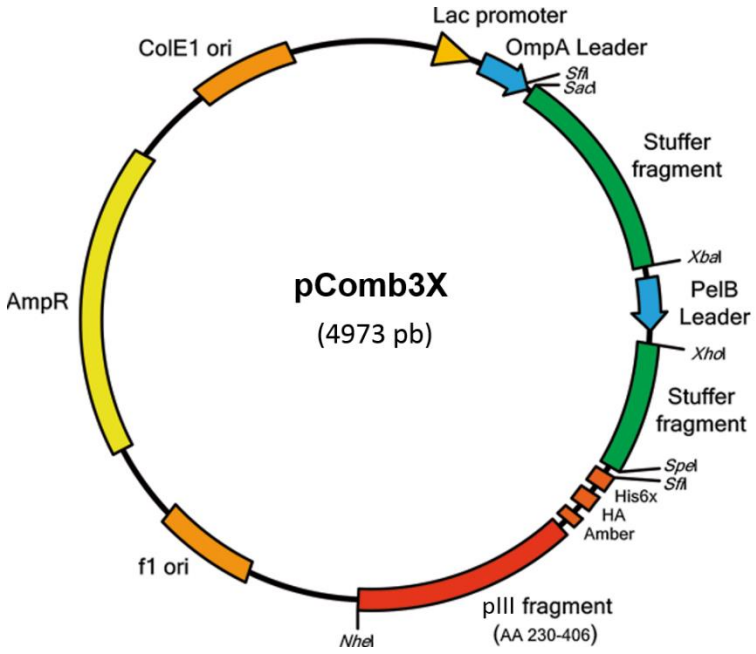
2.5.4. Phage Display technology

Phage Display has been developed as an alternative to traditional hybridoma technology to select diverse antibodies (Pini & Bracci, 2000). Described by George Smith in 1985 (Smith, 1985), Phage Display is a molecular technology that allows the potential of the immune system to be exploited to produce ligands for practically any structure, and its combination with modern protein engineering technology makes it viable to generate antibodies with high affinity and specificity for any diagnostic or therapeutic target (Andris-Widhopf et al., 2001). The genes encoding the antibody variable domains are fused to genes encoding bacteriophage coat proteins. The fused genes can be integrated in bacteriophage particles that also display the heterologous proteins on their surfaces and thus, a physical linkage is established between phenotype and genotype (Smith, 1985). The most commonly used phage protein is the pIII minor coat protein located at the tip of the long, thin filamentous phage M13 (Brekke & Sandlie, 2003). Phage Display vectors can be classified according to the coat protein used for display; whether the protein to be displayed can be fused to all copies of the coat protein or to only a fraction of them; and whether the recombinant fusion is encoded on the phage genome or on a separate genome. They also vary according to the genome used for expressing the coat protein fusion: the genome can range from a wild type (M13) to a modified type that can be propagated as a plasmid under antibiotic selection; to a phagemid (a plasmid that carries the recombinant coat protein gene as well as a phage origin of replication) (Barbas et al., 2001). M13 pIII is often the first choice for Phage Display fusions because of its tolerance for large insertions (Georgieva & Konthur, 2011). However, in phagemid vectors a truncated version of pIII is used for fusion which brings advantages, such as a more efficient display by reducing proteolysis of the fusion protein and reducing the size of the phagemid vector. Moreover, phagemid vectors, compared to phage vectors have other advantages, such as ease of cloning, capacity to accommodate a larger foreign DNA fragment, transformation efficiency, availability of a variety of restriction enzyme

recognition sites convenient for DNA recombination and gene manipulation, and genetic stability under multiple propagations (Qi, Lu, Qiu, Petrenko & Liu, 2012).

The pComb3X vector (Figure 12) is a phagemid vector that is regularly used for Phage Display. Phagemid vectors contains a plasmid origin of replication and a phage-derived origin of replication and, like typical plasmids, an antibiotic-resistance marker is provided to allow selection, but on the other hand, unlike plasmids, the phagemid genomes can be packaged in the phage coat (Barbas et al., 2001).

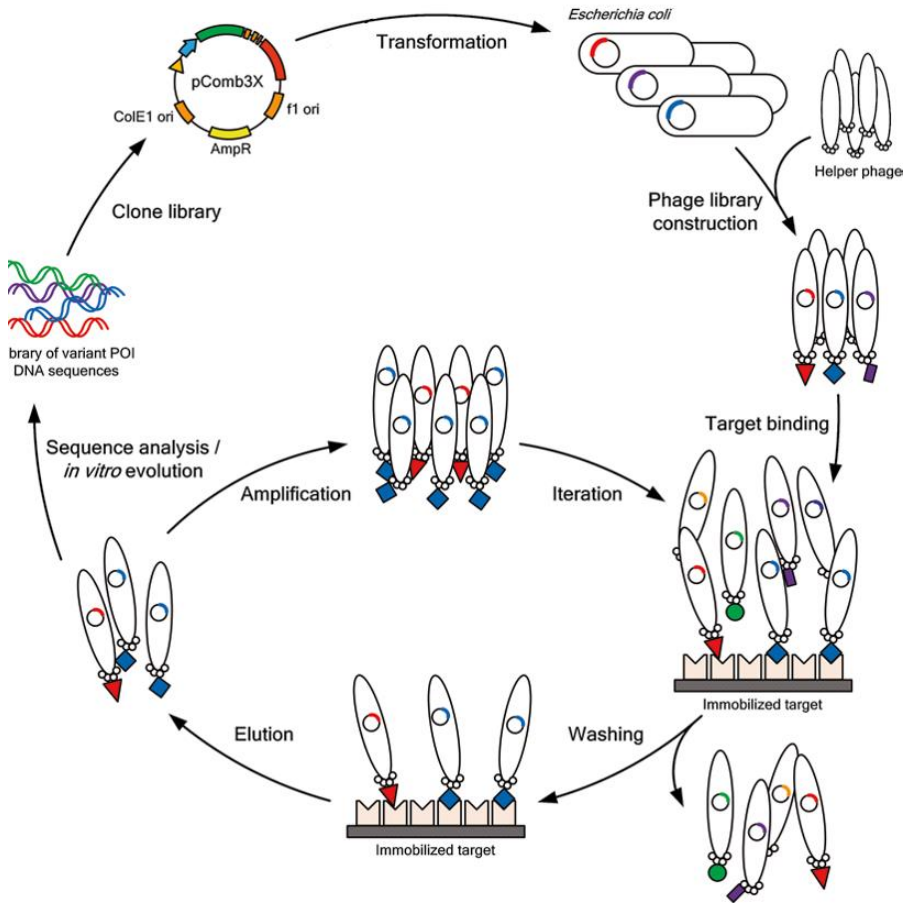
Figure 12. *p*Comb3X phagemid vector.



ColE1 ori – plasmid origin of replication; OmpA leader - facilitates the transport of the fusion protein to the periplasm; *SfiI, SacI, SpeI, NheI, XbaI and XhoI* - restriction enzyme recognition sites; Stuffer fragments – sequence regions bordered by restriction enzyme recognition sites can be removed so that genes of interest can be cloned; His6x and HA – histidine and hemagglutinin tags are used for the purification and detection of recombinant proteins; Ambar - amber stop codon is used to turn-off the expression of the pIII fusion protein; pIII fragment - C-terminal fragment of minor coat protein pIII; f1 ori – phage origin, AmpR- ampicillin resistance gene. Adapted from Levisson, Spruijt, Winkel, Kengen & van der Oost, 2014.

After creating an antibody library, the VH and VL PCR products are cut by restriction enzymes and cloned into the pComb3X vector in frame with the truncated pIII. *Escherichia coli* (*E. coli*) cells are transformed by electroporation with the resulting phagemids and, as pComb3X does not have all the other genes necessary to encode a full bacteriophage, then they are infected with the helper phage (Levisson et al., 2014; Barbas et al., 2001).

Figure 13. Schematic representation of a Phage Display selection round (panning).



A library of DNA fragments, encoding random variants of the protein of interest (POI), is created and cloned into a phagemid vector (pComb3X). E. coli cells are transformed with the obtained phagemid and superinfected with helper-phage to create a library of phages, each displaying a variant of the protein of interest. The resulting library is exposed to an immobilized target molecule and the nonbinding phages are washed away while bound phages are eluted and then amplified by infecting *E. coli cells.* The cycle of selection and amplification process can be repeated as necessary using in each round more stringent washing conditions in order to obtain phages with the displayed protein of interest with the highest target-binding affinity. Adapted from Levisson et al., 2014.

The helper phage provides all of the phage derived proteins and enzymes required for phage replication, and structural proteins that encapsulate both the helper phage and phagemid genomes, “helping” the process of replication and packaging of the phagemid genome. The helper phage contains a kanamycin resistance gene, which along with the ampicillin resistance gene carried by the phagemid, assists in the selection of bacterial cells containing both the helper phage and the phagemid genomes. Therefore, the result is a library of phages, each expressing a mAb on its surface and harboring the vector with the respective nucleotide sequence within (Barbas et al., 2001).

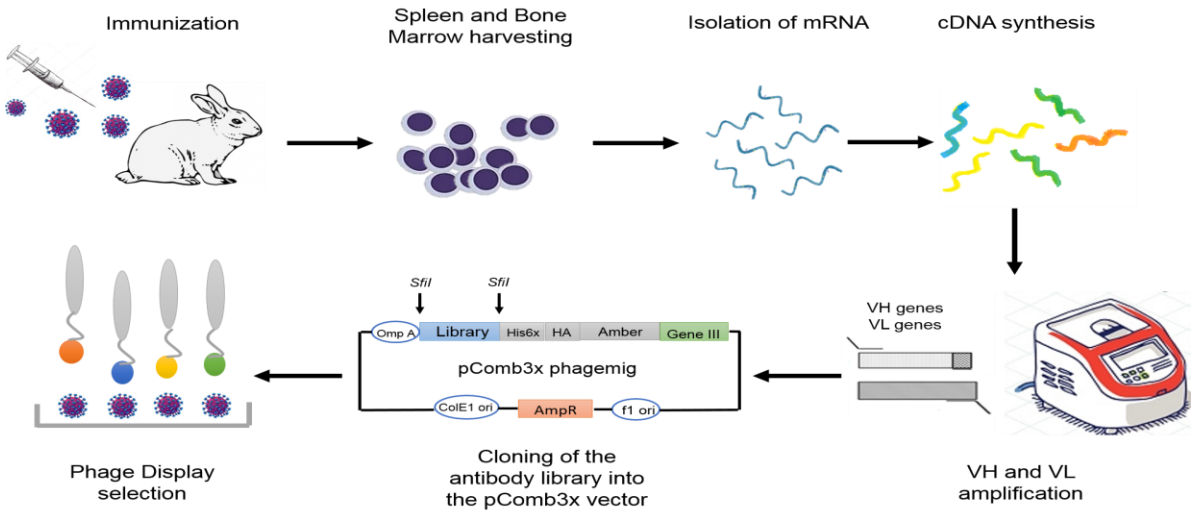
Phages that display antibody fragments can be isolated and amplified by panning. Panning is the antibody selection process in Phage Display where antibody-displaying phage library is incubated with the antigen of interest and nonbinding phage are eliminated by washing. Bound phages are eluted by conditions that disrupt the interaction between the displayed protein and the target molecule and then amplified by re-infecting *E. coli* cells with new addition of helper phage. Selection rounds are subsequently repeated (usually, three to six rounds) using washing steps with increasing stringency, ultimately resulting in a phage population enriched in a limited number of variants with the desired binding affinity and specificity (Figure 13) (Levisson et al., 2014; Breke et al., 2003, Andris-Widhopf et al., 2001; Barbas et al., 2001).

2.6. Objectives

The present work was carried out in the Laboratory of Microbiology and Immunology of the Faculty of Veterinary Medicine of the University of Lisbon. The working group that supported the development of this work investigates and develops single domain antibodies for therapeutics in the areas of oncology, inflammatory and infectious diseases.

This work is integrated in the project PGG/015/2016 supported by Gilead Genese Program - "*Desenvolvimento de anticorpos biespecificos anti-gp120/gp41 para o tratamento da infecção do VIH-1*" whose goal is to develop a recombinant bi-specific antibody that prevents fusion between HIV -1 and the host cell. The aim is to develop a recombinant antibody with a double neutralizing action in the virus entry process. To this end, recombinant antibodies against the viral proteins gp120 and gp41 of HIV-1 will be developed. The group has already developed and selected anti-gp41 antibodies (Cunha-Santos et al., 2016). The aim of the present work is to develop specific rabbit sdAbs against surface gp120 glycoprotein, namely against the epitope responsible for interaction with the host cell CD4 receptor. To achieve the intended goal, initially four rabbits were immunized with a Vaccinia virus prime-gp120 boost regime for 6 months, a project led by Professor Nuno Taveira (Taveira, 2015). Then, in a first phase, VH and VL rabbit immunized antibody libraries construction was performed, by amplification of the different families of the two domains. Gene amplification was performed by PCR from cDNA obtained from mRNA of the spleen and bone marrow harvested of one of the immunized rabbits. Later in the same phase, cloning of the libraries into pComb3X phagemid vector was performed. In a second phase, the specific antibodies against gp120 were isolated using the Phage Display technique. In the future, the identified antibodies will be fused to the already selected anti-gp41 antibodies (Cunha-Santos et al., 2016). The methodology used in the present work is represented in Figure 14.

Figure 14. Schematic representation of the methodology used in the development of the recombinant sdAbs in the present work.



3. MATERIAL AND METHODS

3.1 Rabbit immunizations

Rabbit immunizations were carried out in collaboration with Professor Nuno Taveira from the Center for Molecular Pathogenesis and Research Institute of Medicine (iMed.ULisboa), Faculty of Pharmacy of Lisbon University and Egas Moniz Cooperativa de Ensino Superior and under the Project Gilead Genese (Taveira, 2015). The aim was to immunize rabbits with gp120 glycoprotein so that the iMed.ULisboa research group could obtain serum for the screening of neutralizing antibodies (Calado, 2018) and the research group that supported the development of this work could harvest spleen and bone marrow for the development of sdAbs. All animal care and handling were approved by Ethics and Animal Welfare Committee (CEBEA- Comissão de Ética e Bem-Estar Animal da FMV-UL).

Four New Zealand White female rabbits were primed with 500 µl of $2x10^7$ plaque-forming unit of recombinant Vaccinia virus (VV) expressing gp120 from HIV-1 CRF02_AG clade (VVgp120AG) and boosted three or four times with 500 µl of VVgp120AG supernatant (Sgp120AG) corresponding to 35 µg of the cognate gp120 protein. Immunizations were performed from October 2016 to March 2017, through subcutaneous injections at days 35, 63 and 121 (boost I, II and III) and two of the rabbits (R6 and R8) received an extra boost at day 150 (boost IV). To evaluate the serum specificity and titer, blood was collected, at six time points (T0-T5) during the assay, from the marginal ear vein with a 21-gauge needle and allowed to clot and retract at 37ºC overnight (Table 2). The serum was decanted and clarified by centrifugation at 2500 rotations per minute (rpm) for 15 minutes, and stored at –20ºC, to be used for the tittering of immune serum by ELISA using cognate gp120 protein as antigen, and peroxidase-conjugated goat anti-rabbit antibody (Jackson Immune Research) as secondary antibody. When high serum titer was achieved, a final boost was administered intravenously to R6 and R8. All rabbits were sacrificed on day 154 and the spleen (Sp) and bone marrow (BM) from R6 and R8 were harvested for mRNA isolation.

3.2 sdAbs immune library construction

3.2.1 Isolation of Total RNA

After Sp and BM removal, these were immediately homogenized using a tissumizer homogenizer (Tekmar-Dohrmann) and incubated in 10 ml of Tri reagent (Molecular Research Centre) at room temperature for 5 minutes. Then, 20 ml of Tri reagent (Molecular Research Centre) was added to each sample, and centrifuged at 2500 g for 10 minutes at 4°C. The supernatants were transferred to 50 mL centrifuge tubes and the pellets were discarded.

Table 2. Rabbit immunization schedule.

	T0		T1		T2		T3	T4			T5
	BC	**Priming**	**BC**	**Boost I**	**BC**	**Boost II**	**BC**	**BC**	**Boost III**	**Boost IV**	**BC**
ID	Day 1	Day 1	Day 35	Day 35	Day 63	Day 63	Day 99	Day 121	Day 121	Day 150	Day 154
R5	✓	$VV_{gp120AG}$	✓	$S_{gp120AG}$	✓	$S_{gp120AG}$	✓	✓	$S_{gp120AG}$	-	✓
R6	✓	$VV_{gp120AG}$	✓	$S_{gp120AG}$	✓	$S_{gp120AG}$	✓	✓	$S_{gp120AG}$	$S_{gp120AG}$	✓
R7	✓	$VV_{gp120AG}$	✓	$S_{gp120AG}$	✓	$S_{gp120AG}$	✓	✓	$S_{gp120AG}$	-	✓
R8	✓	$VV_{gp120AG}$	✓	$S_{gp120AG}$	✓	$S_{gp120AG}$	✓	✓	$S_{gp120AG}$	$S_{gp120AG}$	✓

T0-T5 – time points of blood collection; ID- rabbit identification; BC – Blood Collection; VVgp120AG- Vaccinia virus expressing gp120 from HIV-1 clade CRF02_AG; Sgp120AG - gp120 supernatant of cells infected with VVgp120AG. Adapted from Calado, 2018.

To each supernatant, 3 ml of 1-Bromo-3-chloropropane was added and samples were agitated for 15 seconds, incubated for 15 minutes at room temperature and centrifuged again at 17500 g for 15 minutes at 4°C. The upper colorless aqueous phase of each sample was transferred to a new centrifuge tube containing 15 ml of isopropanol. Samples were agitated for 15 seconds and incubated for 10 minutes at room temperature. After that, the samples were centrifuged at 17500 g for 10 minutes at 4°C. The supernatant was carefully removed and discarded and 30 ml of 75% ethanol was added to the pellet, without resuspending it. Centrifugation at 17500 g for 10 minutes, at 4°C, was then performed. Supernatants were carefully removed, and pellets air-dried at room temperature. Finally, each pellet was resuspended in 500 µl of RNase-free water and the RNA concentration and purity was evaluated by measuring the samples absorbance at 260 nm in a NanoDrop™ 2000c Spectrophotometer (Thermo Scientific).

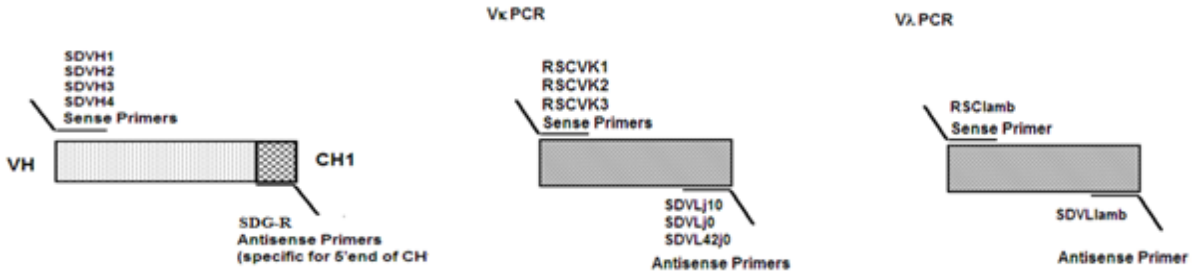
3.2.2 First strand cDNA synthesis

Complementary DNA (cDNA) was synthesized at the thermocycler VWR Collection Thermal Cycler DOPPIO™ (VWR; Radnor) by reverse transcription from total RNA of Sp and BM using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche), according to the manufacturer's instructions. After synthesis, cDNA was quantified using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific) through the measurement of the samples absorbance and it's integrity was evaluated on a 1% agarose gel in TAE 1% by ethidium bromide staining under ultra violet (UV) light with Chemidoc™ XRS+ (Bio-Rad).

3.2.3 PCR amplification of single domains antibody genes

To amplify VH and VL regions from the rabbit's cDNA, PCR at the thermocycler VWR Collection Thermal Cycler DOPPIO™ (VWR; Radnor) was performed with the following conditions: an initial denaturation step, at 98°C for 30 seconds; denaturation for 10 seconds at 98 °C, annealing for 30 seconds at 56 °C and extension for 60 seconds at 72°C, for 30 cycles; and a final extension for 10 minutes at 72°C. For the amplification of the rabbit's VH and VL coding sequences was used, respectively, a four and a ten primer combination (Figure 15). For each reaction, 700 ng of Sp cDNA or 500 ng of BM cDNA was used in a mix with 0.5 µM of Primer Forward and Reverse (20 pmol/µl), buffer 1x (5X), 3 mM of MgCl2 (50 mM), 200 µM of dNTPs (10 mM), 0.02 U/µl of Phusion High Fidelity DNA polymerase (Thermo Scientific) and miliQ water to a final volume of 50 µl. For the amplification of VL lambda family the polymerase used was GoTaq® G2 Flexi DNA Polymerase (Promega Corporation) with the same conditions. Each reaction was evaluated on a 1% agarose gel in TAE 1% by ethidium bromide staining under UV light with Chemidoc™ XRS+ (Bio-Rad). The primers sequences used for the amplification of genes codifying for rabbit VH and VL sdAbs are shown in Table 3.

Figure 15. Primer combination for the generation of VH and VL antibody fragments by PCR.



Different primer combination for the generation of VH and VL antibodies fragments for posterior cloning into the pComb3X system.

3.2.4 Purification of PCR products

With the aim of purifying DNA, PCR products were separated by electrophoresis on a 2% low-melting point agarose gel in TAE 1x buffer. DNA with the expected size was extracted from the gel with a blade scapel and purified with the Zymoclean™ Gel DNA Recovery Kit (Roche), according to the manufacturer's instructions. Then, the recovered DNA was precipitated as described in 3.2.5. DNA concentration was determined using NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific) through the measurement of the samples absorbance.

Table 3. Primers sequences.

VH 5' Sense Primers	**SDVH1-F**	5' GGGCCCAGGCGGCCCAGTCGGTGGAGGAGTCCRGG 3'
	SDVH2-F	5' GGGCCCAGGCGGCCCAGTCGGTGAAGGAGTCCGAG 3'
	SDVH3-F	5' GGGCCCAGGCGGCCCAGTCGYTGGAGGAGTCCGGG 3'
	SDVH4-F	5' GGGCCCAGGCGGCCCAGSAGCAGCTGRTGGAGTCCGG 3'
VH3'Antisense Primer	**SDG-R**	5'CCTGGCCGGCCTGGCCACTAGTGACTGAYGGAGCCTTAGGTTGCCC3'
VL 5' Sense Primers	**RSCVK1-F**	5'GGGCCCAGGCGGCCGAGCTCGTGMTGACCCAGACTCCA 3'
	RSCVK2-F	5'GGGCCCAGGCGGCCGAGCTCGATMTGACCCAGACTCCA 3'
	RSCVK3-F	5' GGGCCCAGGCGGCCGAGCTCGTGATGACCCAGACTGAA 3'
	RSCλ-F	5' GGGCCCAGGCGGCCGAGCTCGTGCTGACTCAGTCGCCCTC 3
VL 3'Antisense Primer	**SDVLj10-B**	5' CCTGGCCGGCCTGGCCTTTGATTTCCACATTGGTGCC 3'
	SDVLj0-B	5'CCTGGCCGGCCTGGCCTAGGATCTCCAGCTCGGTCCC 3'
	SDVL42j0-B	5'CCTGGCCGGCCTGGCCTTTGACSACCACCTCGGTCCC 3'
	SDVLλ-B	5' CCTGGCCGGCCTGGCCGCCTGTGACGGTCAGCTGGGTCCC 3'

Sequence of oligonucleotides used for amplification of genes from rabbit VH and VL sdAbs**.**

3.2.5 Precipitation of PCR products

DNA precipitation with Sodium Acetate was performed in order to concentrate the PCR products. A mixture with 1/10 of the volume of 3 M NaOAc, 2.5 volumes of cold absolute EtOH and 1 µl of glycogen (Roche) was added to the DNA. The mixture was then incubated overnight at -80°C. After incubation, the mixture was centrifuged at 13200 rpm for 1 hour at 4°C. The supernatant was discarded, 1 ml of cold 70% EtOH was added to the pellet for washing and then centrifugation at 13200 rpm for 5 minutes at 4ºC, was performed. The supernatant was removed and the pellet was air-dried and resuspended in 20 µl of miliQ water.

3.2.6 Digestion of vector DNA and PCR products with restriction enzyme *Sfi*I

For pComb3x-SS vector digestion, 20 µg of vector, 120 units (U) of *Sfi* I (10U/ µl) (Thermo Scientific), 20 µl of buffer G (10x) and miliQ water were used to obtain a final volume of 200 µl. The conditions for PCR products digestion are resumed in Table 4. All digestion reactions were incubated overnight at 50°C.

Table 4. Conditions used for VH and VL digestion by *Sfi I* restriction endonuclease.

	VH - Sp	VH - BM	VL - Sp	VL- BM
Purified DNA	4.3 µg	6.2 µg	11.9 µg	10.4 µg
Restriction Endonuclease Sfi I (10 U/µl)	69 U	99 U	191 U	166 U
Buffer G (10x)	30 µl	26 µl	20 µl	20 µl
miliQ water	to obtain a final volume of 300µl	to obtain a final volume of 260µl	to obtain a final volume of 200µl	to obtain a final volume of 200µl

After digestion, vector and PCR products were purified on a 0.8% and 2% low melting point agarose gel, respectively. DNA with the expected size was then removed from the gel and extracted by Zymoclean™ Gel DNA Recovery Kit (Roche). The recovered DNA was precipitated as described in 3.2.5. After precipitation, digested products were quantified using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Scientific) through the measurement of the samples absorbance.

3.3 Phage Display library

3.3.1 Cloning of PCR products into pComb3x vector

In order to clone VH and VL PCR libraries into pComb3x phagemid, a ligation reaction with a vector:insert *ratio* of 1:10 was performed using, for the VH reaction:1.4 µg of vector, 1.6 µg insert, 20 µl of ligase buffer (10x), 5 µl of T4 DNA ligase (BioLabs) and miliQ water to obtain a final volume of 200 µl; and for the VL reaction: 1.4 µg of vector, 1.4 µg insert, 20 µl of ligase buffer (10x), 5 µl of T4 DNA ligase (BioLabs) and miliQ water to obtain a final volume of 200 µl. The ligations were incubated overnight at 16°C. The following day, DNA precipitation with sodium acetate was performed, as described in 3.2.5, and DNA pellet was re-suspended in 20 µl of miliQ water.

Each ligation mix was divided into tree aliquots (5µl, 7.5µl and 7.5µl) and each used to transform 60 µl of electrocompetent *E.coli* ER2738 (Lucigen®) by electroporation. For this purpose, an electrical shock at 1.8 kV, 25 µF and 200 Ω was performed. Immediately after the electroporation, 1 ml of SOC medium was added to each cuvette and successive washes were performed with the same medium to a final volume of 10 ml. The reactions were incubated for 1 hour at 37°C and agitated at 200 rpm. Next, 4,5 µl of ampicillin (100 mg/ml)

was added and the cultures were incubated for one more hour at 37°C and 200 rpm. After incubating, 2 ml of helper phage VCSM13 and 183 ml of pre-warmed SB medium with 92.5 µl of ampicillin (100 mg/ml) and 185 µl of tetracycline (5 mg/ml) were added to the cultures. The cultures were then agitated at 200 rpm for 1.5 hours at 37°C. Finally, 280 µl of kanamycin was added and the cultures were incubated overnight at 37°C with an agitation of 200 rpm.

The next day, the produced phages were recovered by precipitation using 8 g of PEG-8000 and 6 g of NaCl for 1 hour on ice. After that, the phages were centrifuged at 8000 rpm for 20 minutes at 4°C. The supernatant was discarded, the pellet dried by inverting the tubes on a paper towel, resuspended in 2 ml of Phosphate Buffered Saline (PBS) 1x and transferred to a 2 ml eppendorf. To eliminate the remaining bacteria, a final centrifugation was performed at 13200 rpm for 5 minutes. Ultimately, the supernatants with the phages were filtered through a 0.22 µm filter (input).

3.3.2 Confirmation of library insert efficiency and diversity

In order to confirm the library insert efficiency, a sample of the transformed bacteria was plated in agar with ampicillin plates and incubated overnight at 37°C .The next day, PCR was performed to selected colonies. Individual colonies were picked into PCR tubes containing 20 µl of H_2O and incubated for 5 minutes at 94°C in thermocycler VWR Collection Thermal Cycler DOPPIO™ (VWR; Radnor, Pensilvânia, EUA), for bacteria lysis. Afterwards, PCR was performed using 5 µl of colony sample, 0.8 µl of forward primer RSC-F (20 pmol), 0.8 µl of reverse primer RSC-B (20 pmol), 5 µl GoTaq® Flexi Buffer (5X), 1.5 µl of MgCl2 (50 mM), 0.5 µl of dNTPs (10 mM), 0.2 µl of GoTaq® G2 Flexi DNA Polymerase (Promega Corporation) and miliQ water to a final volume of 25 µl. The amplification conditions used were the same used at 3.2.3. PCR products were evaluated on a 1% agarose gel by ethidium bromide staining under UV light with Chemidoc™ XRS+ (Bio-Rad). The primers sequences used are shown in Table 5.

Table 5. Name and sequence of the primers used to confirm the presence of the genes encoding VH and VL sdAbs cloned in pComb3X vector.

Primer	5'-3' Sequence
RSC-F	GAGGAGGAGGAGGAGGAGGCGGGGCCCAGGCGGCCGAGCTC
RSC-B	GAGGAGGAGGAGGAGGAGCCTGGCCGGCCTGGCCACTAGTG

3.3.3 Selection of specific sdAbs against gp120 by Phage Display

For each VH and VL library, four wells of an ELISA plate were used to immobilize gp120 as antigen and three selection rounds (pannings) were performed (Figure 16). At this point Sp and BM were mixed together. To immobilize the antigen, 4 wells were coated with 1 µg/well of antigen in 50 µl of PBS 1x. The ELISA plate was sealed and incubated overnight at 4°C. Next day, the coating solution was shaked out and the wells washed 3 times with 100 µl of PBS 1x. The wells were then blocked by adding 100 µl of PBS/BSA 3% for 1h at 37°C. After incubation the blocking solution was pipeted out, the wells washed 3 times with 150 µl of PBS 1x and 150 µl of freshly prepared phage where added to the blocked wells. The plate was incubated for 2 hours at 37°C. In the first panning, five washing steps were performed; in the second panning, ten washing steps were performed; and in the third panning twelve washing steps were performed. In the washing procedure 150µl of PBS/Tween 0.5% was used and between each step the solution was pipetted vigorously up and down five times and maintained for five minutes before being discarded. After the final washing step the washing solution was discarded and the wells washed one more time just with 150 µl PBS 1x. The solution was removed and 100 µl of freshly prepared 10mg/ml trypsin in PBS 1x was added to each well. The ELISA plate was sealed and incubated for 30 minutes at 37°C. After incubation the phage-trypsin eluates (output) were pipetted vigorously ten times up and down and transferred to a 50 ml Falcon tube. 50 µl of PBS 1x was added to each well and pipetted again vigorously ten times up and down and finally transferred to the same 50 ml Falcon tube The Phage Display conditions implemented in the present project are summarized in Table 6. After each round of panning, phage reamplification was performed. For that, it was necessary to grow *E.coli* ER2738 bacteria from a fresh plate in 20 ml of SB medium containing 40 µl of tetracycline at 37°C at 200 rpm, until an O.D. of approximately 0.6 - 0.7 was reached. Then, to the output phages, 4 ml of bacteria were added and the culture was incubated for 30 minutes at 37°C. After that, 4 ml of pre-warmed SB medium with 1.6 µl of ampicillin (100mg/ml) and 12 µl of tetracycline (5 mg/ml) was added to the culture. The culture was then incubated at 37°C for 1 hour and agitated at 200 rpm. Next, 2.4 µl of ampicillin was added to the culture and incubated in the same conditions for one more hour. After incubation, 1.5 ml of VCSM13 helper phage was added to the culture and incubated for 15 minutes at 37°C. Then, the culture was transferred to a 250 ml Erlenmeyer containing 91 mL of pre-warmed SB medium with 46 µl of ampicillin (100mg/ml) and 184 µl of tetracycline (5 mg/ml). The culture was incubated for 1.5 hours at 37°C, agitated at 200 rpm. Lastly, 140 µl of kanamycin was added and the culture incubated overnight with agitation of 200 rpm at 37°C. The next day, phage precipitation was performed as described at 3.3.1, and then a new round of selection was performed.

Figure 16. Schematic representation of a selection round for Phage Display.

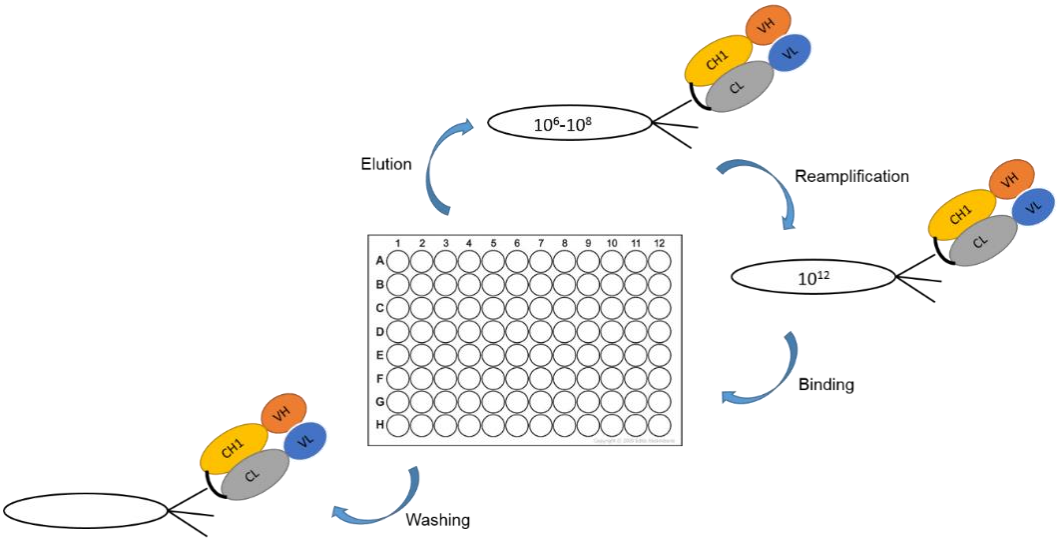


Table 6. Conditions used in each panning.

	1st Panning		2nd Panning		3rd Panning	
	VH	VL	VH	VL	VH	VL
Conditions	1 µg gp120 Wash: 5x PBS/Tween Trypsin- 30 min PBS/Tween – 5 min		0,5 µg gp120 Wash: 10x PBS/Tween Trypsin- 30 min PBS/Tween – 5 min		0,5 µg gp120 Wash: 12x PBS/Tween Trypsin- 30 min PBS/Tween – 5 min	

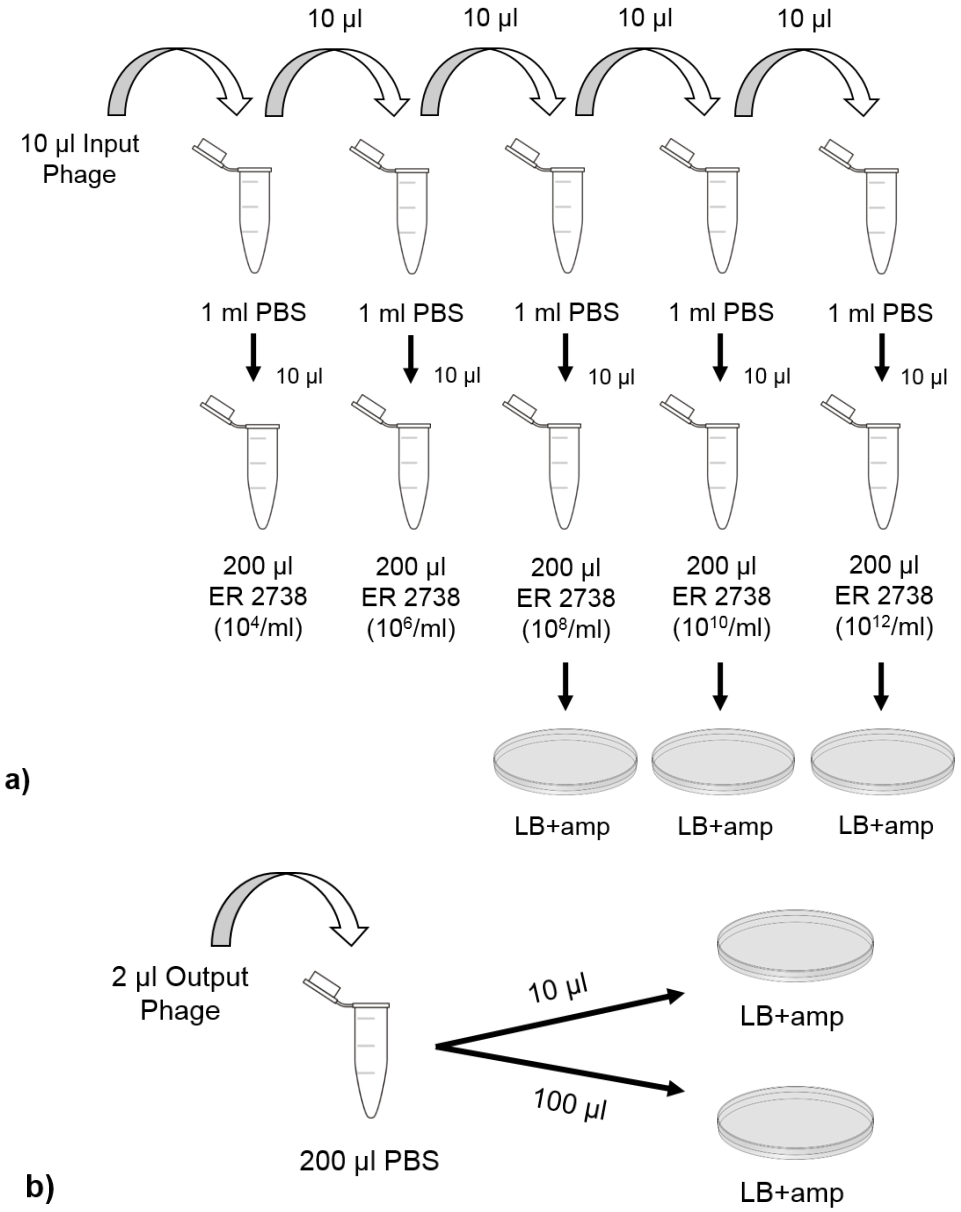
3.3.4 Phage Titration

For input phage titering serial dilutions of 1:10 in PBS 1x was performed (Figure 17-a). Then, 10 µl of each phage dilutions were used to infect 200 µl of exponentially grown *E.coli* ER2738 and incubated for 30 minutes at 37°C. After incubation each culture was plated in LB-agar medium with ampiciline (LB+amp) plates.

For output phage titering, 2 µl of the 4 ml culture was diluted in 200 µl of PBS 1x and 10 µl and 100 µl of this 1:100 dilution were plated on LB+amp plates (Figure 17-b). Plates were incubated overnight at 37°C.

For negative controls, 200 µl of *E.coli* ER2738 was plated on a LB+amp plate and on a LB-agar medium with kanamycin plate. For positive control, 200 µl of *E.coli* ER2738 was plated on a LB-agar medium with tetracycline plate. Plates were incubated overnight at 37°C. Next day, colonies were counted.

Figure 17. Schematic representation of a) input and b) output phage titering.



For each round of selection, input and output phage titering was performed.

3.4. Characterization of sdAbs against gp120

3.4.1. Sequencing of anti-gp120 clones

To ascertain the library diversity, DNA into *pComb3x* was extracted from independent transformants after ligation and transformation. For the purpose, a ZR Plasmid Miniprep™ Kit (ZYMO RESEARCH) was used, according to the manufacturer's instructions.

Sequencing of selected clones was performed by GATC BIOTECH using 10µl of pComb3x ATG primer for each 10µl of DNA of each clone. To translate the obtained nucleic acid sequences to amino acid sequences, ExPASy Bioinformatics Resource Portal was used and, in order to evaluate their homology, the “Vector NTI” software was utilized. Guide tree was built using the Neighbor Joining method and considering a consensus sequence, automatically created by the software after the sequences alignment.

The same methodology was used to characterize the sdABs selected by Phage Display. In this case DNA into pComb3x was extracted from bacterial clones of the output of the third panning of each Phage Display (VH and VL), and then sequencing was performed.

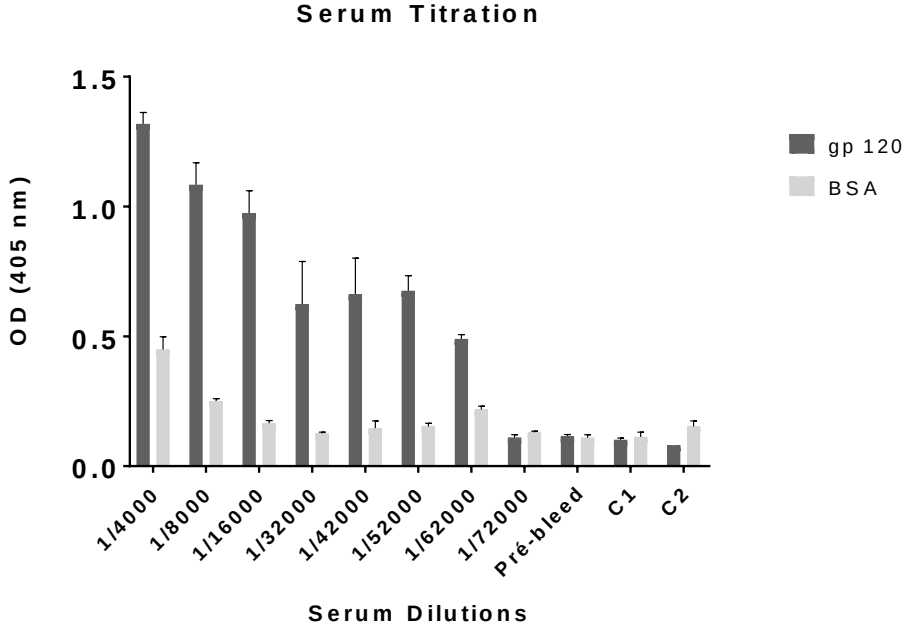
4. RESULTS

4.1 Rabbit immunizations

In this project rabbit derived sdAbs have been explored in order to develop new and potent antibodies against the gp120 HIV-1 glycoprotein. To achieve the intended goal, four rabbits where immunized for 150 days and euthanasied on day 154. However, only the serum, BM and Sp of rabbit R8 were used for this work. Only the biological material of rabbit R8 was used because this was the rabbit that generated antibodies that neutralized the highest percentage of HIV-1 pseudoviruses tested by the iMed.ULisboa team (Calado, 2018).

To evaluate serum specificity and titer, serum from the final bleed (T5) of R8 was analyzed by ELISA. Serum from pre-bleed (T0) served as negative control. The results corresponding to ELISA with pre and final bleed against gp120 are shown in Figure 18. The result shows that the final bleed serum strongly recognized gp120 (with a serum titer of 1:62000*)* contrarily to pre-bleed that did not. This result suggests that the immunizations resulted in a robust immune response, since the serum titer was higher than the negative controls. Therefore, R8 demonstrated a promising immune response against gp120 and was selected to construct the sdAb immune libraries.

Figure 18. Titration of serum antibodies from rabbit R8.



Serum from the immunized rabbit was analyzed for binding to gp120 HIV-1 envelope glycoprotein by ELISA using HRP-conjugated goat anti-rabbit Fc polyclonal antibody as secondary antibody. Serum titer is defined by the highest limiting dilution recognized by the antigen (1:62000). Data were obtained by Abs measurement at 405 nm and values represent the mean of duplicates (n=2) for each condition. C1 and C2 represent negative controls for serum and for serum and secondary antibody, respectively.

4.2 sdAbs immune library construction

4.2.1 Isolation of Total RNA and cDNA synthesis

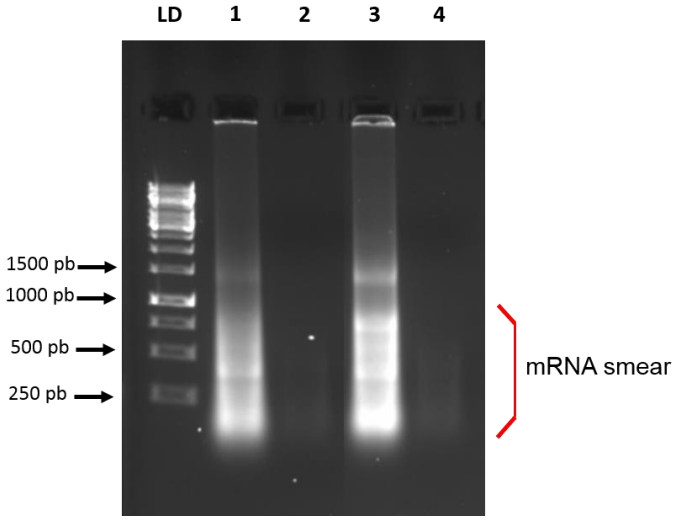
To construct the immunized sdAbs library, mRNA and cDNA obtained from the Sp and BM of rabbit R8 were used. BM and SP were the tissue sources chosen because they have been shown to be rich in plasma cells that secrete antibodies and are, therefore, most frequently used for the generation of immune libraries (Barbas et al., 2001).

The expected yield of total RNA isolated from Sp and BM of one leg of a New Zealand White rabbit is in the range of 6-8 mg (Barbas et al., 2001). It was achieved to isolate a total of 9,8 mg of RNA from Sp and 3,1 mg from BM. Of these, 54 µg of Sp RNA and 36 µg of BM RNA were used to synthesize 41 µg and 29 µg of Sp cDNA and BM cDNA, respectively (Table 7). The integrity of the mRNA and the synthetized cDNA were evaluated in an agarose gel electrophoresis, as shown in Figure 19. Both were integrate and presented the expected profiles (Barbas et al., 2001).

Table 7. Quantification of mRNA and cDNA from the Sp and BM of R8.

	RNA (ng/µl)	cDNA (ng/µl)
Spleen	6466,5 (Total= 9,8 mg)	1534,6 (Total= 41 µg)
Bone Marrow	3122,8 (Total= 3,1 mg)	1585,6 (Total= 29 µg)

Figure 19. RNA and cDNA from rabbit R8.



Samples of RNA extracted and isolated and cDNA synthetized from Sp and BM were eletrophoretically separated on a 1% agarose gel and their integrity evaluated. LD - Ladder 1kb; 1 – RNA Sp; 2 - cDNA Sp; 3 - RNA BM; 4 - cDNA BM.

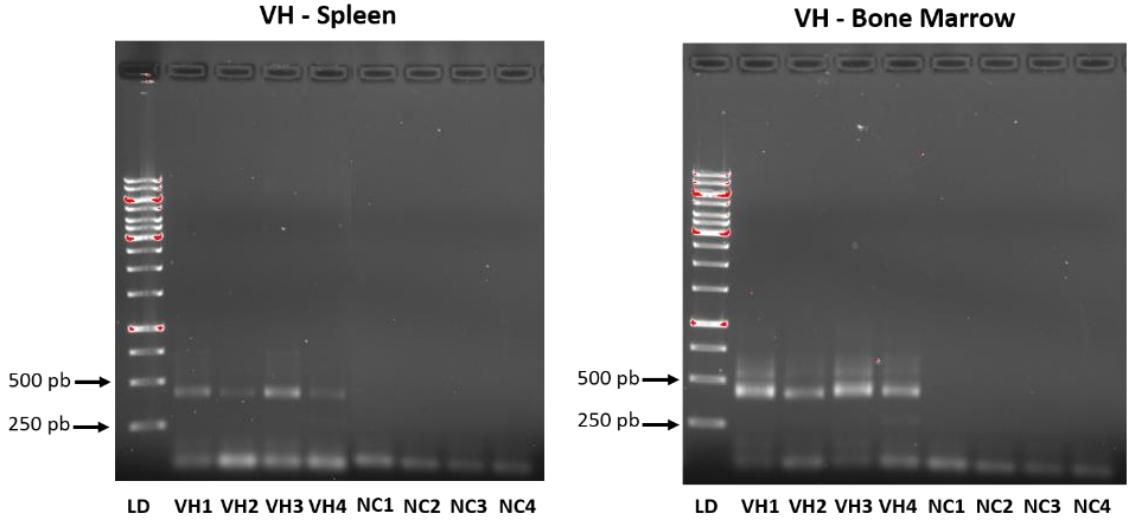
4.2.2 PCR amplification and purification of single domains antibody genes

VH and VL regions were separately amplified from BM and Sp cDNA, using the correct primer combinations and generated, as expected, fragments with 400 bp and 350 bp, respectively (Barbas et al., 2001) (Figure 20 and 21). Several PCRs were performed to achieve a higher diversity and quantity (Table 8).

As shown in Figure 20 and 21, it is possible to verify that all families were successfully amplified, except for Sp lambda family, which results from the combination of RSCλ and SDVLλ primers.

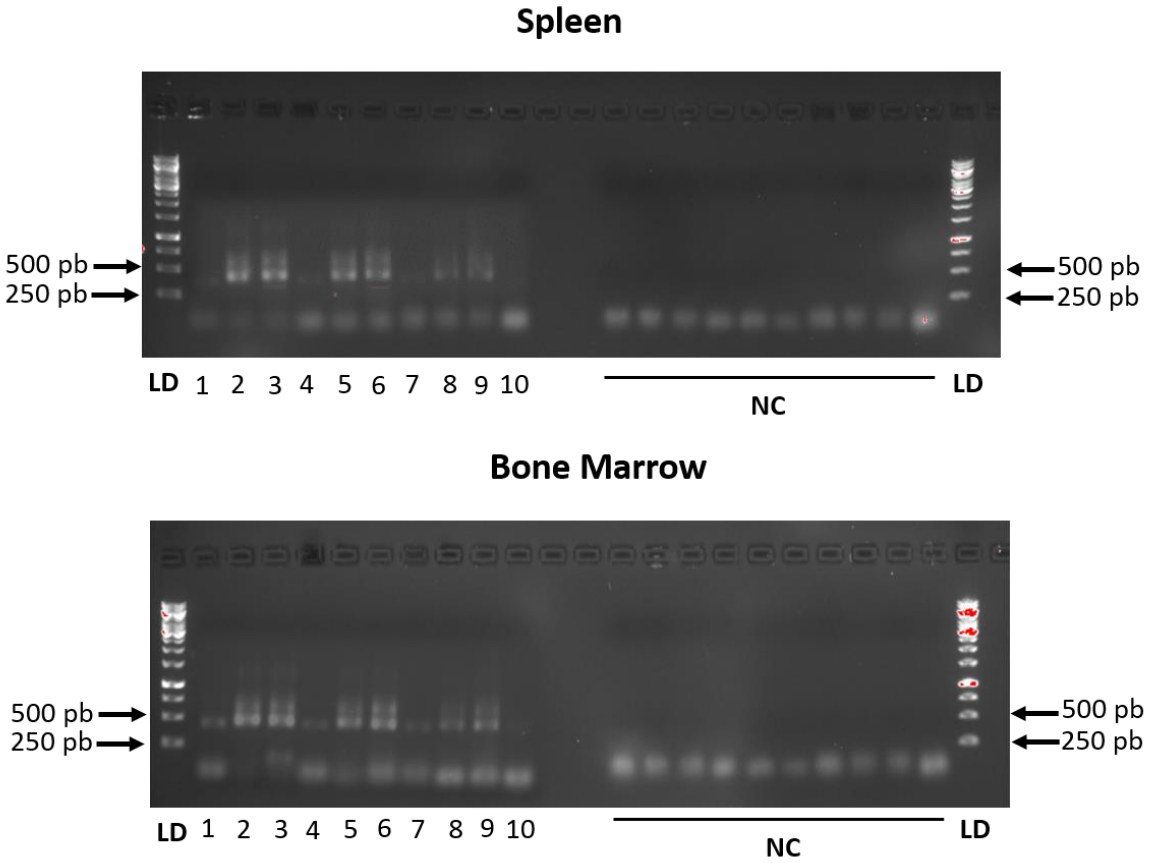
After amplification, PCR products were purified in an agarose gel (Figure 22 and 23) in order to separate the DNA of interest from all other PCR reagents. From the purification of VH Sp, VH BM, VL Sp and VL BM, 4.3 µg, 6.2 µg, 11,9 µg and 20,8 µg of DNA were, respectively, recovered (Table 8).

Figure 20. PCR amplification of VH family from SP and BM cDNA.



After PCR amplification, samples were run on a 1% agarose gel. Amplification of VHs generated fragments with approximately 400 bp. LD - Ladder 1 kb was used as a DNA size control; VH1, *VH2, VH3 and VH4* - primer combination SDVH1+SDG, SDVH2+SDG, SDVH3+SDG and SDVH4+SDG, respectively; NC1, NC2, NC3 and NC4 – negative controls for VH1, VH2, VH3 and VH4, respectively.

Figure 21. PCR amplification of VL family from SP and BM cDNA.

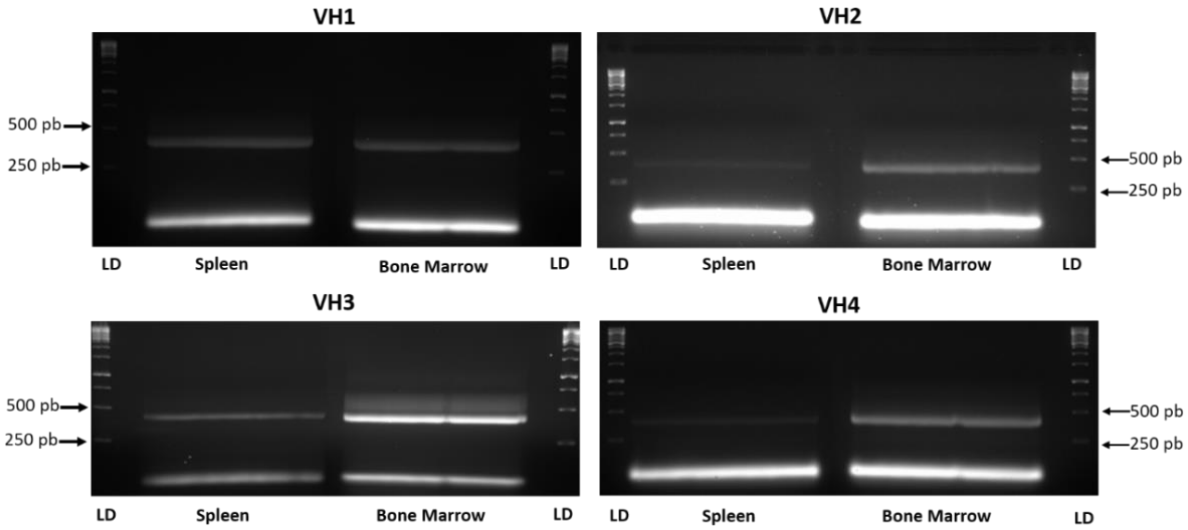


After PCR amplification, samples were run on a 1% agarose gel. Amplification of VLs generated fragments with approximately 350 bp. LD - Ladder 1 kb was used as a DNA size control; 1- RSCVK1+SDVLj10; 2 - RSCVK1+SDVLj0; 3 - RSCVK1+SDVL42j0; 4 - RSCVK2+SDVLj10; 5 - RSCVK2+SDVLj0; 6 - RSCVK2+SDVL42j0; 7 - RSCVK3+SDVLj10; 8 - RSCVK3+SDVLj0; 9 - RSCVK3+SDVL42j0; 10 - RSCλ+ SDVLλ; NC– from left to right, negative controls of: RSCVK1+SDVLj10, RSCVK1+SDVLj0, RSCVK1+SDVL42j0, RSCVK2+SDVLj10, RSCVK2+SDVLj0, RSCVK2+SDVL42j0, RSCVK3+SDVLj10, RSCVK3+SDVLj0, RSCVK3+SDVL42j0 and RSCλ+ SDVLλ.

Table 8. Quantification of VHs and VLs families from Sp and BM of immunized rabbit after amplification and purification.

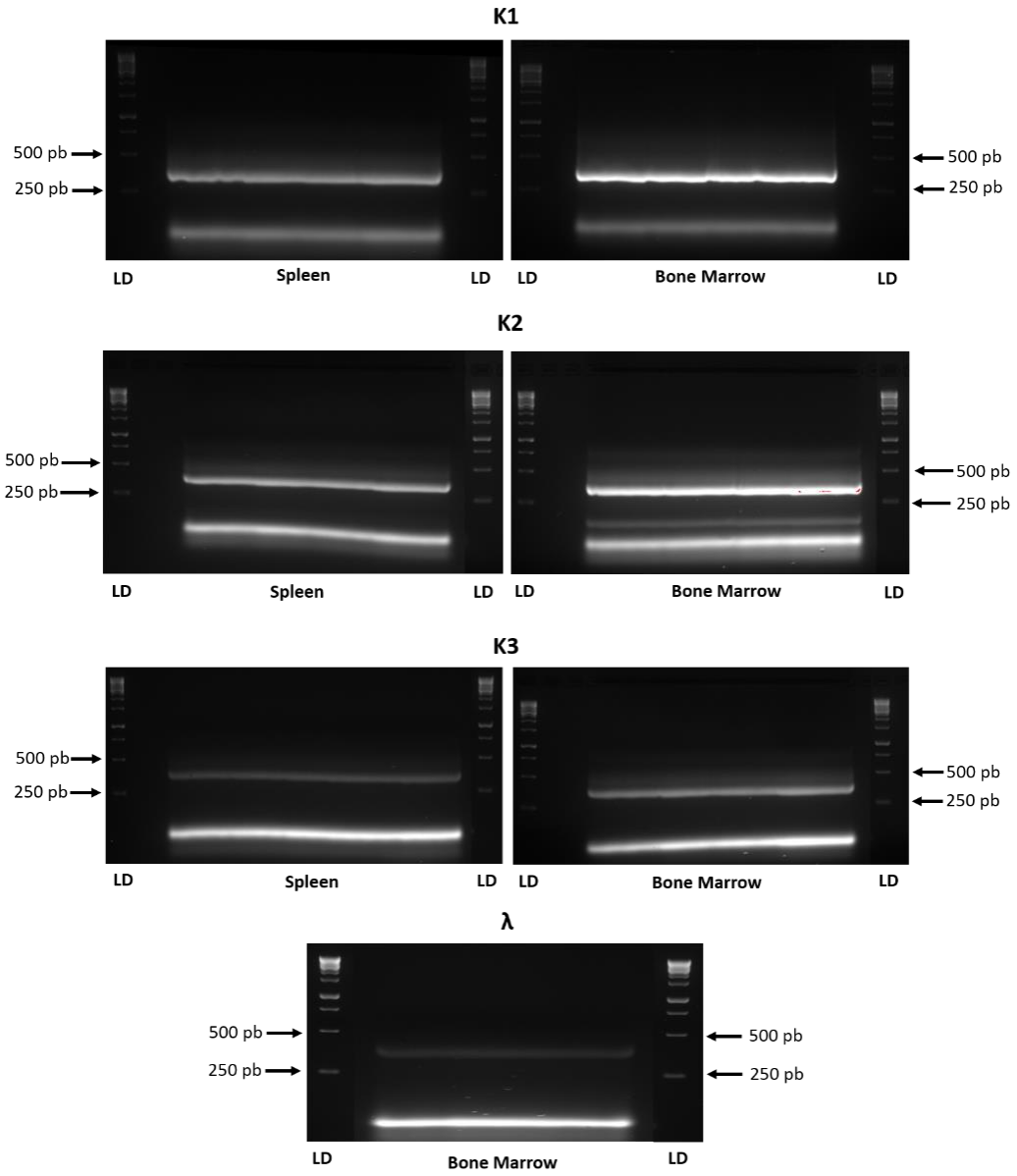
	Family		Quantification (ng)	Total (ng)
Spleen	VH	VH1	1176	4282
		VH2	750	
		VH3	1516	
		VH4	840	
	VL	K1	6630	11918
		K2	3680	
		K3	1608	
		λ	-	
Bone Marrow	VH	VH1	1162	6212
		VH2	2488	
		VH3	1414	
		VH4	1148	
	VL	K1	11960	20838
		K2	4730	
		K3	3250	
		λ	898	

Figure 22. Purification of VH families in a 2% low melting point agarose gel after amplification.



Fragments corresponding to 400 bp were extracted from the gel. LD- Ladder 1kb was used as the DNA size control.

Figure 23. Purification of VL families in a 2% low melting point agarose gel after amplification.



Fragments corresponding to 350 bp were extracted from the gel. LD - Ladder 1kb was used as the DNA size control.

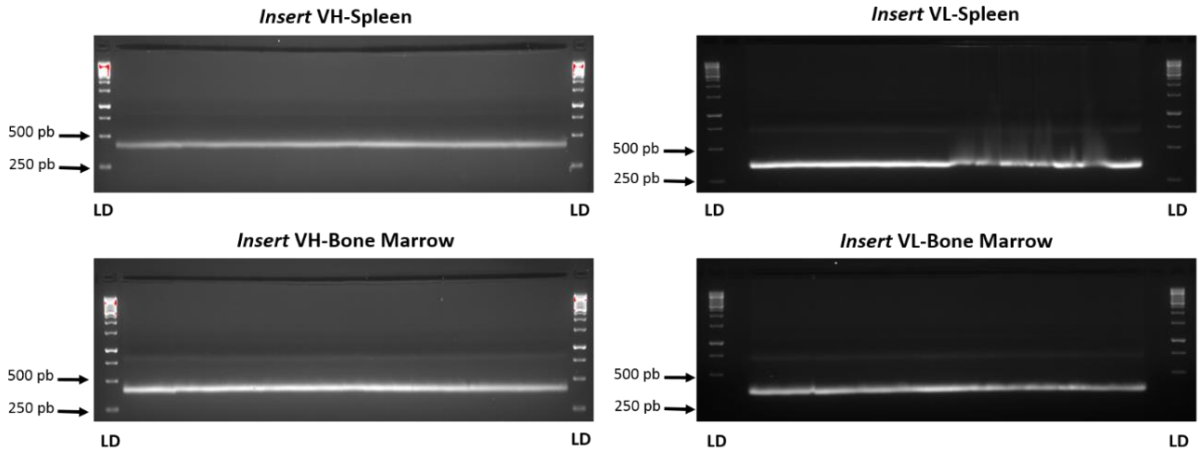
4.2.3 Digestion of vector DNA and PCR products with restriction enzyme *Sfi* I

All PCR products obtained had symmetric *Sfi* I sites on the 5' and 3' ends, which were required for cloning into pComb3x vector. Thermo Scientific SfiI was the chosen restriction enzyme that recognizes GGCCNNNN^NGGCC sites and cuts best at 50°C in G buffer. The purified DNAs were then digested with *Sfi* I restriction enzyme and purified once more

(Figure 24). From the purification, 1.6 µg, 2.9 µg, 4.7 µg and 5.2 µg of DNA were recovered from VH Sp, VH BM, VL Sp and VL BM, respectively.

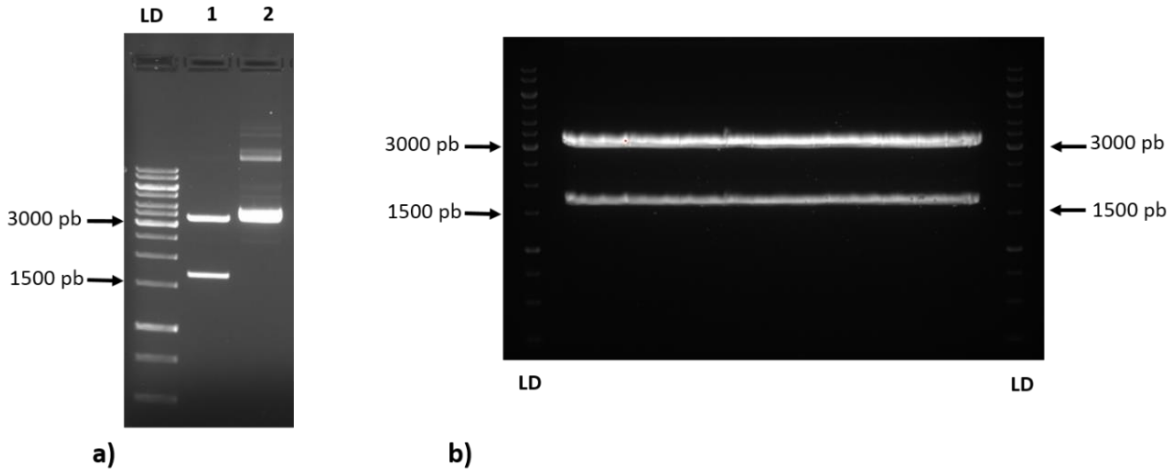
In order to insert the purified DNA into pComb3x phagemid vector, this also had to undergo digestion by Sfi I restriction enzyme (Figure 25-a). After purification (Figure 25-b), 14.2 µg were recovered for latter ligation reactions.

Figure 24. Purification of VH and VL domains, after Sfi I restriction, in a 2% low melting point agarose gel.



The fragments corresponding to 350 bp and 400 bp were extracted from the gel, regarding VL and VH domains, respectively. LD- Ladder 1kb was used as the DNA size control.

Figure 25. Restriction a) and purification b) of phagemid pComb3x-ss, after Sfi I restriction, in a 0,8% low melting point agarose gel.



The stuffer and the restricted phagemid corresponds to the fragments with 1600 bp and 3400 bp, respectively. The latter was extracted from the gel. LD – Ladder 1kb; 1 – Phagemid pComb3x-ss following restrictive digestion by Sfi I; 2 – pComb3x-SS not digested, as a negative control.

4.3 Phage Display library

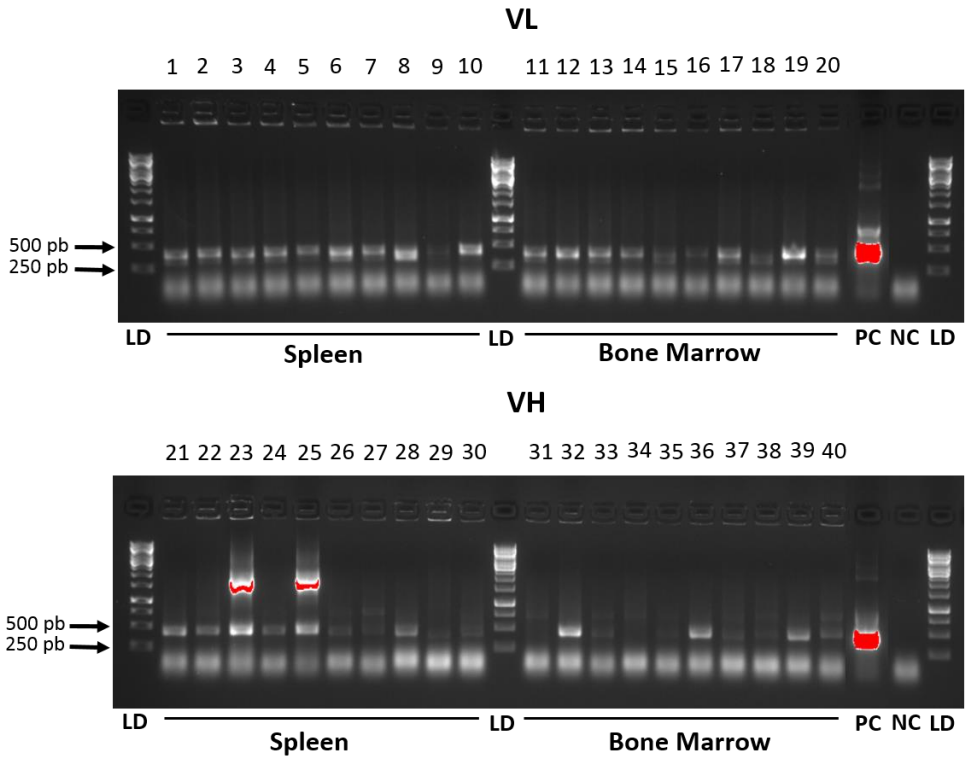
4.3.1 Cloning of PCR products into pComb3x vector, confirmation of library insert efficiency, diversity and clones profile analysis

In order to confirm the libraries efficiency, PCR was performed using 20 clones of each library. A high transformation efficiency is of great importance, since this step is usually the limiting factor for the size of an antibody library (Andris-Widhopf et al., 2001). An efficiency of 100% was obtained in VL library (Figure 26). This means that all transformants had the insert cloned. However, in VH library the efficiency obtained was of 90%.

To determine the quality and complexity of the immune library, the number of independent transformants is very important. It is known that, for libraries derived from immune animals, this number should range from 10^7 to 10^8 (Barbas et al., 2001). The diversity obtained for VH Sp and BM libraries was of $7.5x10^6$ and $1.6x10^6$, respectively. For VL Sp and BM libraries the diversity was of $2.1x10^7$ and $5x10^7$, respectively.

Both libraries were efficiently constructed and are also representative, highly diverse and complex since their diversity is close to 10^7.

Figure 26. Confirmation of transformation efficiency for VH and VL libraries.

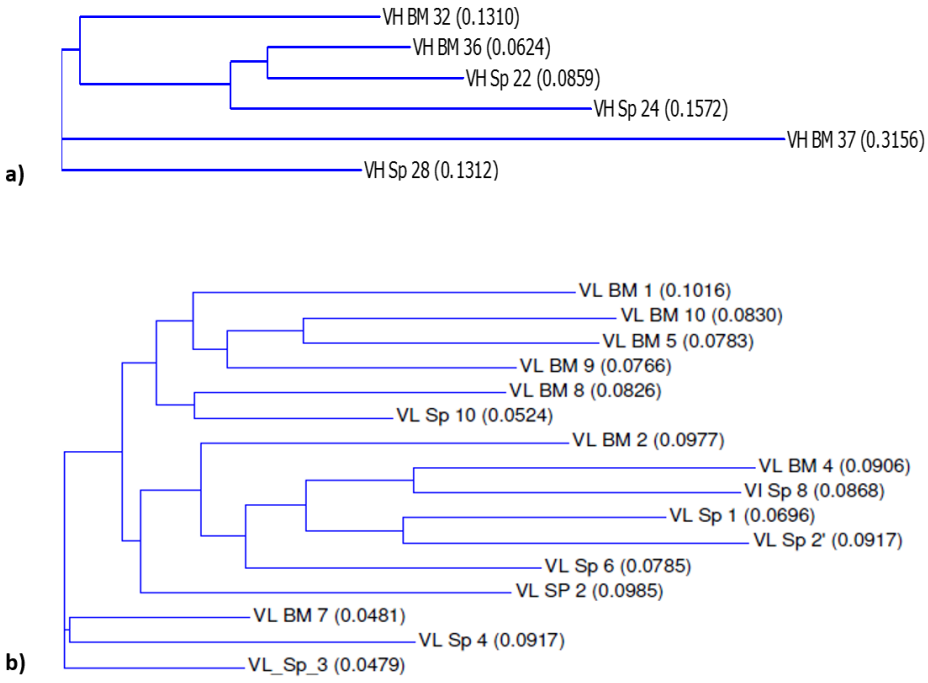


Samples were run and separated on a 1% agarose gel. 1-40 – Clones; LD- Ladder 1 kb; PC- positive control; NC- negative control.

In order to evaluate the profile of the library diversity the selected clones were sequenced and a homology analysis was performed by aligning the obtained amino acid sequences. From the selected clones it was possible to sequence six clones from VH domain and sixteen clones from VL domain. As seen in the Guide tree (Figure 27), all clones are different between them and have different degrees of homology, and the further away a clone is from the root, the greater the difference with the consensus sequence.

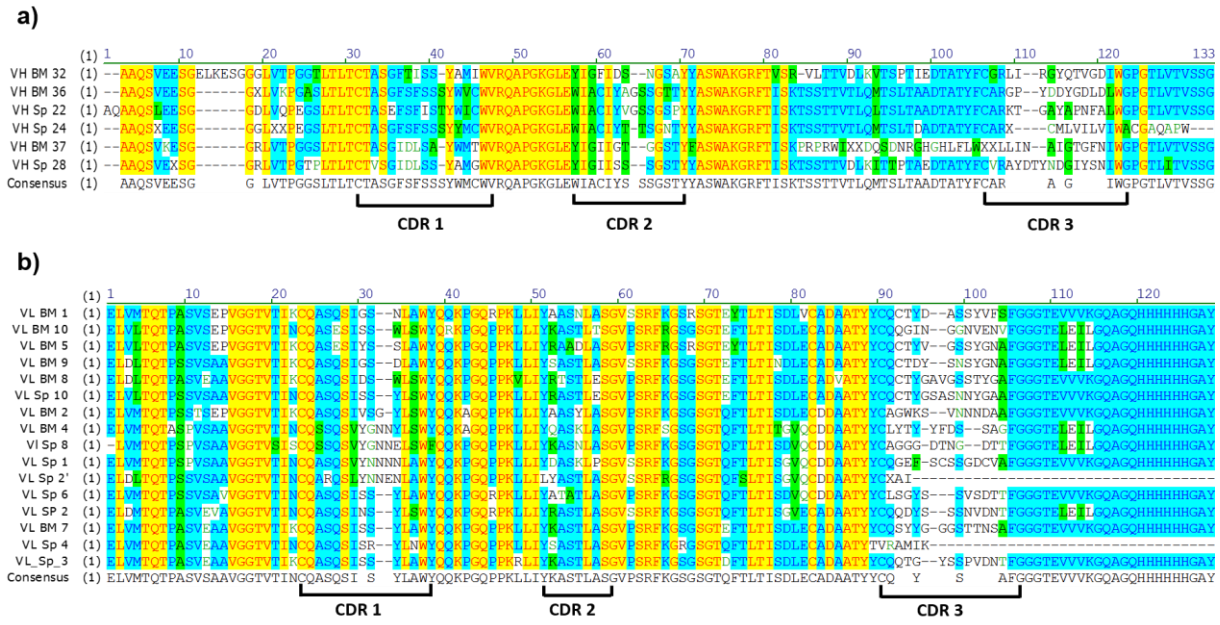
When analyzing the alignment performed, it is possible to identify, by Kabat's nomenclature published in 1991, the CDRs from both VH and VL domains (Figure 28) (Kabat, Wu, Perry, Gottesman & Foeller, 1991). All CDRs are marked by highly conserved FRs to either side, which can be used to determine which residues comprise them. The VL CDR 1 is preceded by a conserved cysteine (C) and followed by a conserved tryptophan (W) and the latter is usually followed by a tyrosine (Y). VL CDR 2 is almost always composed of seven residues and is preceded and followed by a tyrosine and a glycine (G), respectively. VL CDR 3 is preceded by a conserved cysteine and followed by a conserved phenylalanine – glycine (FG) pair. On the other hand VH CDR 1 is preceded by a conserved cysteine and followed by a conserved tryptophan-valine (WV) pair; VH CDR 2 is preceded by a tryptophan and followed by a conserved tyrosine; and VH CDR 3 is preceded by a conserved cysteine, which typically forms a CAR motif, and followed by a conserved tryptophan-glycine pair.

Figure 27. Guide trees.



Homology was obtained based on alignment of amino acid sequences of selected clones from **a)** VH and **b)** VL families before the pannings.

Figure 28. *A*lignment of the sequences obtained for each clone of a) VH and b) VL domains.



Through sequencing analysis, selected clones were identified and grouped by antibody families (Table 9).

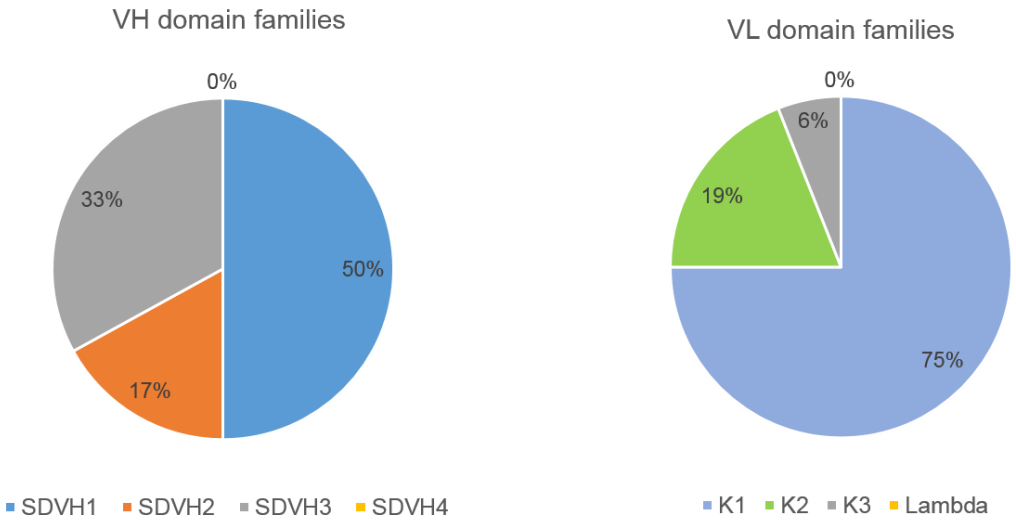
Table 9. Grouping, by antibody families, of the selected and sequenced clones.

VH domain		VL domain	
Antibody family	**Clone identification**	**Antibody family**	**Clone identification**
SDVH1	VH Sp 28 VH BM 32 VH BM 36	**K1/j0**	VL Sp 8 VL BM 5 VL BM 10
		K1/42j0	VL Sp 1 VL Sp 3 VL Sp 6 VL Sp 10 VL BM 1 VL BM 2 VL BM 7 VL BM 8
		K2/j0	VL Sp 2 VL BM 9
SDVH2	VH BM 37	**K1/?**	VL Sp 4
		K2/?	VL Sp 2’
SDVH3	VH Sp 22 VH Sp 24	**K3/j0**	VL BM 4

Once again it is confirmed that the antibody libraries constructed present a great diversity, since clones of different families were identified. In the selected clones of VH domain, SDVH 1 family is the most represented, followed by SDVH 3 and SDVH 2 families. No clone was identified from SDVH 4 family. From VL domain, K1 family was the most represented, K3 the second most represented and K2 the least represented. No clone was identified from lambda family (Figure 29).

Both libraries were efficiently construct and showed a high diversity to be used for antibody Phage Display selection.

Figure 29. Variable domain antibody families of the built immune library.



The charts illustrates the represented percentage of each family in the selected and sequenced clones of both variable domains.

4.3.2 Selection of specific sdAbs against gp120 by Phage Display

4.3.2.1 Input and Output Phage Titering and characterization of sdAbs against gp120

After the libraries construction, selection of specific sdAbs for gp120 was performed by Phage Display, as described in 3.3.3. The selection process was performed separately for VH and VL libraries and, for each one, BM and Sp libraries were mixed together once they had a similar diversity. Three pannings were performed with increasing stringent selection conditions applied throughout the process. In each panning procedure, the antibody displaying phage library (corresponding to the input) was incubated with the antigen of

interest (gp120) and nonbinding phage were eliminated by washing. Binding phage were recovered (corresponding to the output) by disrupting the antigen-antibody complex using trypsin, and then reamplified by infecting E. coli cells. The reamplified phage were used as input for the next panning procedure.

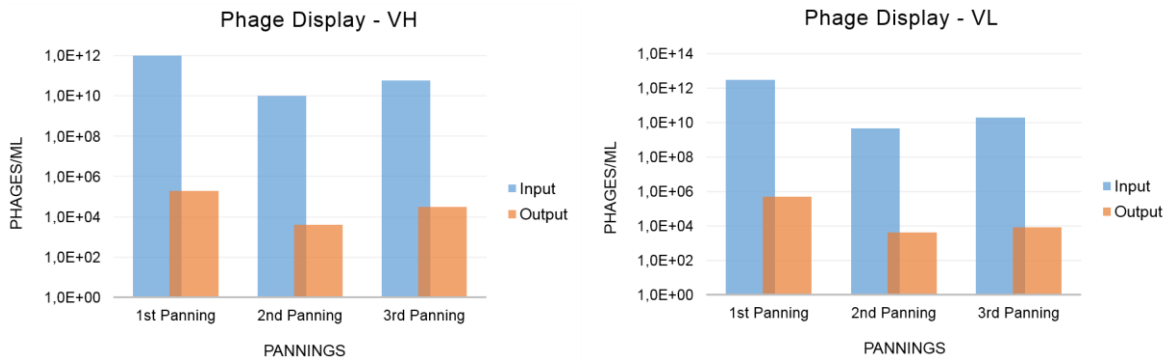
In the three performed pannings there was a lower number of phages in the output titers compared to the input (Table 10 and Figure 30) as expected (Barbas et al., 2001). Besides that, it was also observed a decrease in the output phages from the 1st to the 2nd panning, in which the stringency increased with the increase in the number of washes and the decrease of antigen concentration. Although the stringency also increased from the 2nd to the 3rd panning, the number of obtained phages slightly increased. This is the profile expected for a Phage Display with immobilized antigen and it demonstrates that the conditions implemented were leading to the selection of phages displaying specific antibodies to gp120 HIV-1 glycoprotein (Barbas et al., 2001).

Table 10. Input and Output phage titering.

	1st Panning		2nd Panning		3rd Panning	
	VH	**VL**	**VH**	**VL**	**VH**	**VL**
Input (Phages/ml)	$1x10^{12}$	$3x10^{12}$	$1x10^{10}$	$4,7x10^{9}$	$6x10^{10}$	$2x10^{10}$
Output (Phages/ml)	$2x10^{5}$	$5.1x10^{5}$	$4x10^{3}$	$4x10^{3}$	$3.2x10^{4}$	$8x10^{3}$
Conditions	1 µg gp120 Wash: 5x PBS/Tween		0,5 µg gp120 Wash: 10x PBS/Tween		0,5 µg gp120 Wash: 12x PBS/Tween	

Results obtained from VL and VH domains selection by Phage Display and conditions used in each panning.

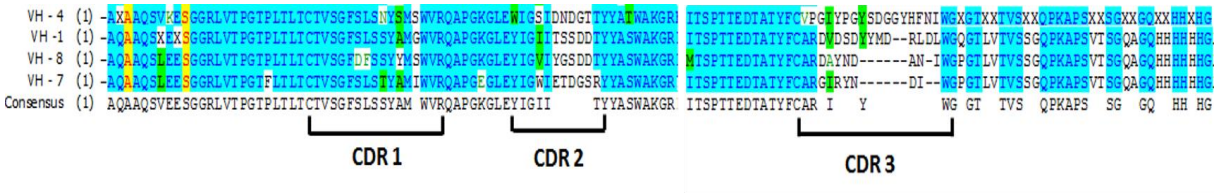
Figure 30. Selection of VH and VL domains by Phage Display.



Charts illustrating the results obtained from VH and VL domains selection by Phage Display.

After the three rounds of selection and with the aim of characterizing the sdAbs obtained, sequencing of the clones obtained from the final output (output of the third panning) of VH and VL Phage Display was performed. Due to errors in the sequencing process, only the complete sequence of four VH clones was obtained (Figure 31). Once again, the CDRs were identified by Kabat's nomenclature.

Figure 31. *A*lignment of the VH Output sequences obtained from the third panning.



5. DISCUSSION

5.1 Rabbit immunizations

The goal of the present work was to develop and select, by Phage Display, specific sdAbs against HIV-1 surface gp120 glycoprotein. For such purpose an immune library from an immunized rabbit was constructed. The choice of an immune library was due to the fact that high quality and high affinity antigen-specific antibodies, derived from natural affinity maturation by the immune system, were desired. The animal model chosen for the construction of the library was the rabbit, as these produce antibodies of high stability and affinity against many antigens that are not immunogenic in mice. It is also known that after humanization of rabbit derived antibodies, they retain both high specificity and affinity for the target (Rader et al., 2000). Moreover, rabbit's long HCDR3 loops is a property necessary to penetrate the Env glycan shield and that has already been found in potent human anti-HIV gp120 mAbs (Kwong et al., 2012) and more recently in a study with four cows which after immunization with the trimer BG505 SOSIP had a rapid elicitation of broad and potent serum antibody responses (Sok et al., 2017). Sok and his colleagues (2017) once again demonstrated that to suit targets somewhat occluded in conserved epitope regions on Env, the antibody repertoires must have long HCDR3 loops. In fact, cows produce antibodies with exceptionally long HCDR3 that can reach lengths of over sixty amino acids long (de los Rios, Criscitiello & Smider, 2015). The CD4 binding site is in a recessed and occluded position on the native trimer, which hinders access by human neutralizing antibodies. The long HCDR3 of antibodies from animal models, such as cows and rabbits, are nonetheless able to access more easily the CD4 binding site on the trimer, therefore making this region immunogenic in the context of the antibody repertoire development. Most of the antibody binding specificity resides within the variable heavy-chain domain and especially within the CDR 3 loops of both variable heavy and variable light chains (Brekke & Sandlie, 2003), therefore the longer the CDR 3 loops, the greater the affinity of the antibodies to their targets, making cows and, in this case, the rabbit an attractive animal model for HIV-1 immunizations.

However, not only is the origin of the antibodies important, but the quality and the nature of the immunogens used in immunizations are essential for the generation of bnAbs against HIV. During chronic infection bnAbs, which are capable of neutralizing heterologous viruses of diverse subtypes, develop after 2–4 years of initial infection in a small portion (10–30%) of HIV-1 infected individuals (reviewed in Ahmed, Tian & Gao, 2017). Due to the high diversity of HIV, it is important that immunogens having the potential to elicit the production of bnAbs during animal immunizations are used for the creation of the immune antibody library. Because of its key role in the virus entry process and since it is the only relevant target for neutralizing antibodies, gp120 offers a mean of HIV infection prevention and so monomeric and trimeric preparations for immunizations have been used, in the attempt to elicit bnAbs.

BG505 SOSIP is a trimeric mimic of the native Env spike that exposes as many bnAbs epitopes as possible and at the same time occludes those for non-neutralizing antibodies (Sanders et al., 2013). It was used for the immunization of the four cows in Sok's and his colleagues (2017) study and showed to rapidly elicit a response of antibodies with a high amplitude of neutralization. Marcelino and his colleagues (2010) investigated the immunogenicity and neutralizing response elicited by novel recombinant envelope proteins derived from HIV-2ALI isolate. For the first time bnAbs against HIV-2 were elicited in mice using a Vaccinia vector-prime C2V3C3 polypeptide boost vaccination strategy. Calado's (2018) PhD thesis main goal was to determine if a similar strategy would elicit the production of bnAbs against HIV-1. Isolates from Angola and Portugal (clades B, C, CRF02_AG and J) were produced and used as immunogens in mice and rabbits. CRF02_AG based immunogens were able to elicit bnAbs against several heterologous HIV-1 viruses in both animal models, however in rabbits the binding and neutralization response was higher. These were the rabbits immunized for the present study. As mentioned in 3.1, the rabbit's immunizations were carried out in collaboration with the working group of Calado (iMed.ULisboa). Since previous studies shown the potential of the same immunogen for the production of bnAbs in mice, the collaboration emerged with the aim of evaluate the quality and ability of the immunogen to elicit bnAbs in rabbits and in parallel to construct an immune antibody library.

For the creation of immune libraries, when the antibody source is an animal it is important to use one with a high serum antibody titer to the antigen of interest. Throughout the immunization protocol, blood samples were taken from rabbits for serologic studies to ensure that the euthanasia of the rabbits was only after higher serum titers were achieved. A high serum titer is presumed to reflect high levels of antibody production and, therefore, higher levels of specific mRNA should be obtainable. In Figure 18 it can be seen that a high serum titer was achieved, which suggests that the immunizations resulted in a robust immune response against gp120 HIV-1 envelope glycoprotein.

5.2 sdAbs immune library construction

To construct the immune antibody library it was necessary to isolate mRNA from rabbit (R8) and produce the respective cDNA. A total of 9,8 mg of RNA from Sp and 3,1 mg from BM was isolated. The mRNA was obtained from the Sp and BM since these are organs with good quantity of plasma cells, which produce antibodies. BM and Sp are both hematopoietic organs responsible, among other functions, for the generation of lymphocytes from immature hematopoietic progenitor cells and for complete maturation of B lymphocytes, respectively (Goldsby et al., 2003). By resorting to both organs, the probability of building a more diverse library was increased. When comparing the electrophoresis images obtained after the amplification and purification of the various antibody families, it is verified that those

corresponding to the bone marrow are represented in greater quantity, since the bands in the agarose gel appear with more intensity (Figure 20-23). This is an expected result and is explained by the function of each organ. When compared to BM and as a final maturation organ of B lymphocytes, the Sp will naturally have a smaller amount of lymphocytes, and consequently a smaller amount of mRNA which translates into a smaller amount of cDNA to amplify.

Due to the numerous advantages described in 2.4.3.2, sdAbs have unique features that may make them the preferred therapeutic format for many applications. Although some studies have shown that VL domains present, in some cases, biophysical properties better than VH domains (such as high expression yield, resistance to aggregation and proteases, stability and high reversibility of thermal unfolding) in this project the genes corresponding to both VL and VH domains were amplified, with the aim of maximizing the diversity of the antibody library (Kim et al., 2014; Hussack et al., 2012; Dubnovitsky et al., 2000).

The amplification of antibody genes by PCR is a crucial part of library construction and primer design will determine the nature of the libraries generated. The VH and VL sdAbs genes were amplified by means of already designed sets of specific oligonucleotide primers (Table 3) in order to amplify all VH and VL known families available in the rabbit antibody repertoire (Barbas et al., 2001). By the analysis of Figure 21 it is possible to see that Sp VL lambda family was not possible to amplify. In fact, when Phusion® High Fidelity DNA polymerase (Thermo Scientific) was used in the PCR reaction, the VL lambda family was not amplified neither from Sp nor from BM. In an attempt to optimize lambda family amplification, a DNA polymerase with less fidelity was used in the PCR reaction. Thus, GoTaq® G2 Flexi DNA Polymerase (Promega Corporation) was used instead of Phusion® High Fidelity DNA polymerase (Thermo Scientific). The fidelity of DNA synthesis with Phusion® High Fidelity DNA polymerase (Thermo Scientific) shows a fifty increase compared to GoTaq® G2 Flexi DNA Polymerase (Promega Corporation). Somatic mutations can introduce changes in the germ-line sequence at any position of the variable region gene. Therefore, there is no guarantee that all antibodies genes in the in vivo repertoire will be amplified. In humans, lambda family is the one that shows more pronounced sequence variation in the first framework region (FR1). Since forward primers are generally designed to correspond to the sense strand at the 5' end of the heavy or light chain genes in FR1, changes in its sequence may lead to the absence of amplification (Barbas et al., 2001). Yet, this does not appear to be the reason for the amplification failure of the lambda family in R8. Once there is amplification with GoTaq® G2 Flexi DNA Polymerase (Promega Corporation), it indicates that there was no somatic mutation in the binding zone of the primers and these were the correct ones used. In rabbit's VL domain antibody repertoire, and especially when it comes to an immunized library, it is known that the lambda family is less expressed than the remaining families. This makes the family sometimes difficult to amplify or even not amplified at all.

Thus, the initial result obtained with Phusion® High Fidelity DNA polymerase (Thermo Scientific) was expected, but by using GoTaq® G2 Flexi DNA Polymerase (Promega Corporation) it was able to optimize the amplification, and in that way making the lambda family contribute to the diversity of the library. However, even with the change of the enzyme, Sp VL lambda family was not amplified most likely due to the poorly expression of the corresponding gene, which makes it impossible to detect mRNA at spleen level.
All other families were successfully amplified (Figure 20 and 21), thus contributing to a good diversity of the immunized libraries constructed.

5.3 Phage Display library

VH and VL immunized antibody libraries were constructed separately. Since Sp and BM libraries in both domains showed diversities within the same order of magnitude, the two were assembled at the beginning of the Phage Display in order to obtain greater diversity and thereby, increase the probability of selecting antibodies against gp120. The diversity obtained in each library was close to 10^7. This value is influenced by the transformation efficiency which, in this case, was quite high. Because both libraries had a high transformation efficiency and a high diversity, it can be concluded that both libraries were efficiently constructed, being highly diverse and complex.
By sequencing the selected clones, before proceeding to the pannings, the diversity of the antibody libraries were again ascertained. The aim was to sequence an equal number of clones from each domain. However, due to errors in the sequencing process itself, sixteen clones from VL domain and only six clones from VH domain where correctly sequenced. Figure 27 and 28 show the profile of the selected clones, and it can be seen that all clones are different, which confirms the diversity of the libraries created. In Figure 28 the obtained sequences were aligned, allowing to identify the CDRs and verify that CDR3 is, in fact, the region that shows more variability among the clones, followed by CDR1, with CDR2 being the most constant region between the selected clones. This was an expected result and reflects the natural maturation that occurred in the rabbit's immune system (Barbas et al., 2001). The sequencing of the selected clones, also allowed to identify which family of antibodies they belong. Table 9 and Figure 29 show that different families were identified and that each domain were represented by three families of antibodies (VH - SDVH 1, SDVH 2 and SDVH 3; and VL – K1, K2 and K3). With the identification of the antibody families, once again it is confirmed that both libraries were efficiently constructed and are representative, as they are greatly diverse.
After the construction of the libraries, the next step was to select, by Phage Display, the antibodies that had specificity for gp120. A stringent selection regime was applied throughout the selection process. For the isolation of specific antibodies for gp120, and as we were working with an immunized library, the amount of antigen immobilized on the plate was

reduced and the number of washings was gradually changed and increased in each selection cycle performed (Table 10). In the case of a naïve library, it would be prudent to keep the concentration of immobilized antigen constant in order to isolate antibodies as specific as possible, since they derive from an unimmunized animal, which implies less affinity. Thus, with the stringent selection regime applied, after the first panning there was a decrease in the number of recovered phages. This indicates that, in the first selecting process, several antibodies with no affinity for gp120 were successfully discarded. After the second panning, the stringency was increased (by decreasing the concentration of immobilized antigen and by increasing twice the number of washes), and a decrease in the amount of recovered phages was again observed. This result shows that the increase in the stringency promoted the elimination of phages that until then had remained bound, but with more unfavorable conditions, they were eliminated due to weak affinity. In the third panning, the concentration of immobilized antigen was maintained, but again the number of washes was increased. Under these conditions there was an increase in the number of phage recovered, which indicates that the obtained phages have high affinity for gp120.

After titering the third and final output, the clones obtained from both domains were sent to sequencing (Figure 31), in order to characterize the antibodies and also to be able to compare their sequence with that of the antibodies of the libraries. The ideal was to sequence the maximum number of clones from each domain. However, once again due to errors in the sequencing process, only four clones from VH domain where correctly sequenced. Figure 31 shows the profile of the sequenced clones, and it is possible to verify that although they correspond to different clones, their sequences are quite similar, with difference above all at the level of CDR 3. When comparing Figure 28 with Figure 31, it is found that, in fact, after the selection process, the antibodies obtained are more similar to each other, with more homogeneity at CDR 1. This result shows that the selection process of Phage Display mimics the process of affinity maturation of the immune system and confirms that, throughout the selection process, antibodies that are increasingly specific for gp120 are being selected.

With these results, it can be considered that the Phage Display was successful, and the exposed antibodies on the surface of the recovered phages, at the end of the selection process, show high binding activity to the target, gp120.

6. CONCLUSION AND FUTURE PERSPECTIVES

Soon after HIV was isolated and confirmed as the cause of AIDS in the eighties, it was widely expected that an effective treatment would be rapidly developed. However, almost forty years have passed and the scientific community is still struggling to develop an effective treatment. The extraordinary diversity of the virus, its capacity to evade adaptive immune responses and the inability to induce bnAbs against HIV-1 represents a barrier to the development of an effective therapy that can overcome the current resistances that the virus has to the various existing therapies. The limitations of currently available drugs and the difficulty encountered to develop an effective vaccine against HIV-1 infection led to the search for new therapeutic strategies for AIDS treatment, including new classes of drugs such as sdAbs which target steps in the viral replication cycle that are not disrupted by currently available drugs. The process of virus entry into the host cell is a key step in the replication cycle of HIV and glycoprotein gp120 has an important role in this process since the CD4–gp120 interaction besides occurring with high affinity is also an obligate step of the virus replication cycle. Therefore, the development of sdAbs which are able to inhibit this stage is extremely promising.

This project aimed to select sdAbs specifics for gp120 from an immunized antibody library. The purpose was to produce sdAbs that, because of their small size, were able to bind more easily to inaccessible regions of the gp120 protein, thereby blocking the binding process and preventing the entry of the virus into the host cell. For this, the potential of rabbit derived sdAbs as therapeutic molecules were explored. VH and VL immunized antibodies libraries were constructed and specific antibodies against gp120 were selected using the Phage Display technique. The rabbit was immunized with an immunogen that was already known to induce the production of bnAbs in mice. Highly diversified antibody libraries were obtained and the profiles attained on the Phage Display were consistent with the stringent conditions applied in each selection, resulting in antibodies recovered with high affinity for gp120.

This work is integrated in a project whose main goal is to develop a recombinant bispecific antibody, with a double neutralizing action in the virus entry process, able to prevent the fusion between HIV-1 and the host cell. We have recently developed an anti-gp41 antibody (F63) that showed to have a broad and potent inhibition activity against HIV-1 and HIV- 2. Here, gp120 sdAbs were selected by Phage Display, but there is still a long way to the development of a specific antibody against gp120, namely against the epitope responsible for interaction with CD4 receptor, which can then be fused to F63 sdAb, and thus form a recombinant bispecific antibody. Therefore, with the results accomplishment in this project, it is necessary to carry out further studies, not only for the characterization of the selected antibodies but also to obtain more data regarding the binding of the same to the target molecule and their capacity to neutralize HIV infection. Thus, it will be important to determine the affinity of the antibodies to gp120 through binding activity studies, and to map the

epitope-paratope interactions in order to determine which sites on the target molecule the selected antibodies bind, and if any bind to the epitope responsible for the interaction with CD4 receptor on the host cell. It will also be important to carry out cellular assays and HIV neutralization studies, in order to verify the ability to inhibit the binding between the viral envelope and the CD4 receptor, thus inhibiting the infection of the cells by the virus. After selection of one or more specific lead candidates antibodies, affinity improvement can be obtained by making point mutations in the hypervariable regions -CDR1, CDR2, CDR3- of the anti-gp120 antibodies in order to increase their affinity.

In the future there will be an increasing use of animal models whose antibody repertoires are more suited to target occluded conserved epitope regions on Env, with the aim of developing new therapies. Due to its small size, easy handling and above all to the advantages characteristics of the antibody repertoire, the rabbit, has been widely used for the development of therapeutic antibodies and in the research of vaccines against all types of diseases. With new research studies, cows, due to the characteristics of their immune system and their extraordinary long HCDR3, seem to have a good potential for the research of new strategies for immunization and development of bnAbs. Therefore, it will be interesting to further explore the immunization of cows, as this may provide an opportunity to rapidly generate antibody with prophylactic and therapeutic properties, which can address disease agents that have evolved to escape human antibody responses, such as HIV. The cat, being an animal that can also be infected by a lentivirus, FIV, which results in an acquired immunodeficiency syndrome resembling HIV infection in humans, is in fact the smallest natural model for the study of lentivirus infections. Due to the enormous structural and pathophysiological similarities between both viruses and their respective infections, the cat has a great potential to be a study model for the development of therapeutic strategies for HIV. The FIV mechanism of entry into the host cell is very similar to the interaction between SU gp120-receptor shown in HIV, since both virus use a primary binding and entry receptor (although different) for infection (Taniwaki, Figueiredo & Araujo Jr, 2013; Elder, Lin, Fink & Grant, 2010). Thus, it would be interesting to apply the same protocol used here to evaluate the immune system response of the cat. Therefore, it could be verified whether, as in the rabbit, bnAbs would be elicited, using the same immunogen. It is also possible to develop new immunogens, derived from FIV Env, and evaluate the response obtained. The fact that cats can be naturally infected with the virus represents an advantage, because the immunizations protocol can be skipped and serum can be directly obtained from FIV positive animals. Thus, the serum can be evaluated for the detection of bnAbs and thus determine which immunogen elicits its production, which can contribute to the development of an effective vaccine. In addition to working as a study model for HIV, applying these techniques

in the cat may increase the knowledge of FIV, making it possible to obtain new molecules that can improve the diagnosis, treatment, and even to revolutionize the prophylaxis of the disease.

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