HIV-2 Chronic Infection

A contribution for the study of viral persistence and viral-host interactions

Susana Cristina Gonçalves Tracana

Thesis to obtain the Master of Science Degree in

Microbiology

Supervisor: Doutor Pedro José Vieira Borga Martins Borrego

Co-supervisor: Professor Doutor Arsénio do Carmo Sales Mendes Fialho

Examination Committee

Chairperson: Professor Doutor Jorge Humberto Gomes Leitão

Supervisor: Doutor Pedro José Vieira Borga Martins Borrego

Member of the Committee: Doutor José Maria Marcelino

September 2018

Acknowledgments

Firstly, my sincere acknowledgments to Doctor Pedro Borrego, for the opportunity, for everything he taught me and for always supporting me, even with all the difficulties. Also, I would like to thank Professor Doctor Nuno Taveira for having received me so well in his research group, as well as for all the support he gave me throughout this work. Both reminded me the importance of patience, hard work and resilience.

I want to thank my co-supervisor Professor Doctor Arsénio Fialho for the availability and orientation.

I would like to thank the PhD student Inês Moranguinho for all the support, for the hours we spend on the BSL3, for the numerous discussions and sharing, for fellowship and friendship inside and outside the laboratory. This master's degree also gave me “uma amiga do norte”.

For all mm140, for their support, friendship, carnivals and tapas nights, particularly: to Doctor Inês Bártolo for all the assistance whenever I had doubts; to PhD student Francisco Martin for his friendship and for all his enthusiasm to help me, even though he is already far from the laboratory; to Doctor João Perdigão for all the material borrowed, for all the support, explanations and mainly for all the patience, in a room full of women; and to the PhD student Marta Calado for the material, friendship and support in everything that I needed during this time. Thank you all very much!

I would also acknowledge Professor Doctor Klaus Strebel for the work material and knowledge, essential in this work.

And last but not least, a special thanks to my parents and my sister for their patience and for believing in me when I said I could do everything at the same time. Also, thank you for helping me pay the tuition fees.

Abstract

The Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are the etiologic agents of Acquired Immunodeficiency Syndrome. HIV-1 is pandemic and HIV-2 occurs mainly in West African countries. In Portugal, 3.3% of HIV/AIDS cases are caused by HIV-2. HIV-2 infection is generally well controlled by the host immune response, being thus considered as an attenuated form of HIV infection. Compared with HIV-1, there are few studies about HIV-2 and consequently the level of knowledge about its characteristics is lower. The aim of this project is to contribute to the study of HIV-2 chronic infection, focusing on features of viral persistence and virus-host interactions. The first goal was the establishment of an *in vitro* cellular model of HIV-2 latency by the production of a recombinant HIV-2 molecular clone expressing the green fluorescent protein (pROD10-EGFP) in Jurkat cells. This recombinant molecular clone was unstable, thus an alternative methodology was applied in view of the production of new molecular clones adaptable to the desired cellular model. The second goal was the analysis of heterologous neutralizing susceptibility of *env* clones from HIV-2 infected individuals. The clones P1-5 and P4-6 were potently neutralized by all HIV-2 sera and these results were correlated to other features associated with a less evolved env. The X4 tropic virus clone showed resistance to neutralization consistence with the disease evolution and with a more evolved env. These preliminary results emphasized the correlation between evolutionary and functional features of the HIV-2 Env and justify further studies.

Key words: AIDS, HIV-2, Model of HIV-2 latency, HIV-2 envelope, Neutralizing antibody response

Resumo

Os vírus da imunodeficiência humana tipo 1 e 2 são agentes etiológicos da Síndrome da Imunodeficiência adquirida. A infecção VIH-1 é pandémica, já VIH-2 ocorre maioritariamente em países da Africa ocidental. Em Portugal, 3,3% dos casos de VIH/SIDA são causados pelo VIH-2. A infecção VIH-2 é geralmente bem controlada pelo sistema imunitário e é considerada uma forma atenuada de infecção VIH. Quando comparado com o VIH-1, existem poucos estudos dirigidos ao VIH-2 e, consequentemente, o nível de conhecimento é menor. O objectivo deste projecto é contribuir para o estudo da infecção cronica do VIH-2: a persistência viral e a interacção vírus-hospedeiro. A primeira meta era o estabelecimento de uma linha celular latente com a produção de um clone molecular recombinante de VIH-2 a expressar proteína fluorescente verde (pROD10-EGFP) em células Jurkat. Este clone recombinante era instável, por isso foi aplicada uma metodologia alternativa, tendo em vista a produção de novos clones moleculares adaptáveis ao modelo celular desejado. A segunda meta era a análise da susceptibilidade à neutralização heteróloga, de clones de *env* de indivíduos com VIH-2. Os clones P1-5 e P4-6 foram neutralizados por todos os soros de VIH-2 e estes resultados foram correlacionados com outras características associadas a um env menos evoluído. O clone de um vírus de tropismo X4 mostrou ser resistente à neutralização, consistente com a evolução da doença do paciente e com um env mais evoluído. Os resultados preliminares realçaram a correlação entre as características funcionais e evolutivas do Env de VIH-2 e justificam mais estudos.

Palavras-chave: SIDA, HIV-2, Modelo de latência de HIV-2, Involucro de HIV-2, Resposta de anticorpos neutralizantes

Table of content

Figure index

Table index

List of abbreviations

ADCC – Antibody-dependent cell-mediated cytotoxicity

ADCMI – Antibody-dependent complement-mediated inactivation

ADCVI – Antibody-dependent cell-mediated viral inhibition

AIDS – Acquired immunodeficiency syndrome

ART – Antiretroviral therapy

CA – Capside

cDNA – Complementary DNA

CDR – Complementarity Determining Region

CMV – Citomegalovírus

CTL – Cytotoxic T lymphocytes

DLS – Dimer Linkage Structure

DMEM – Dulbecco Modified Eagle Medium

DNA – Deoxyribonucleic acid

EGFP – Enhanced Green fluorescent protein

Fab – Fragment antigen-biding

FBS – Fetal bovine serum

Fc – Fragment crystallisable

FcγRs – Fc receptor

GALT – Gut-associated lymphoid tissue

gp – Glycoprotein

HIV – Human immunodeficiency virus

ID50 – Serum dilution that inhibited virus infection by 50%

IFN – Interferons

Ig – Immunoglobulin

IN – Integrase

LB – Luria-Bertani broth

LCMV – Lymphocytic choriomeningitis virus

LTR – Long terminal repeat

MA – Matrix

m.o.i. – Multiplicity of Infection

mRNA – Messenger RNA

msRNA – Multi-spliced RNA

Nabs – Neutralizing Antibodies

NaCl – Sodium Chloride

NC - Nucleocapside proteins

NFAT – Nuclear factor of activated T cells

NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitors

NRTI – Nucleoside Reverse Transcriptase Inhibitors

ORF – Open Reading Frame

PFA – Paraformaldehyde

PI – Protease Inhibitors

PIC – Pre-integration complex

PR – Protease

RNA – Ribonucleic acid

RPMI - Roswell Park Memorial Institute medium

RT – Reverse transcriptase

SIV - Simian immunodeficiency virus

$TCID_{50}$ – Tissue Culture Infective Dose

TGN – Trans-Golgi network

TLR – Toll-like receptors

TNF-α – Tumor necrosis factor α

UNAIDS - Joint United Nations Program on HIV/AIDS

usRNA – Un-spliced RNA

VSV – Vesicular Stomatitis Virus

1 Introduction

1.1 HIV and AIDS history

Following a significant increase of homosexual men with marked immune deficiency and opportunistic and rare infections, in 1981 a new disease was identified: the acquired immunodeficiency syndrome (AIDS) [1,2]. AIDS is characterized as a progressive deterioration of the immune system leading to the acquisition of opportunistic infections [3]. In 1984 the etiological agent of AIDS was established as the Human Immunodeficiency Virus type 1 (HIV-1) [4] wich was isolated for the first time in 1983 from AIDS patients [5]. In 1986, the HIV-2 was isolated for the first time, from patients from Guinea-Bissau and Cape Verde, hospitalized at Egas Moniz Hospital, Portugal, with a clinical picture similar to AIDS and negative tests for HIV-1 [6]. Subsequently, in a Portuguese-French partnership, HIV-2 was confirmed as the second etiological agent of AIDS, alongside with HIV-1 [7]. These viral infections were introduced in the human population by zoonotic transmission of distinct lentiviruses [2,3,8]. Phylogenetic studies have shown that Simian Immunodeficiency Virus (SIV) is the HIV ancestor, and that the passage of the species barrier occurred around 1930 in West Africa, probably as a result of hunting and wounds caused by this activity as well as by the consumption of the contaminated meat [2]. These studies also found that several cross-species transmissions occurred with humans, and each event resulted in the creation of specific groups of HIV. HIV-2 was originated from SIV that infects sooty mangabey (*Cercocebus torgnatus atys* – SIVsm) endemic in West Africa [2,3,9,10]. There were at least nine events that resulted in the HIV-2 groups A to I. Of these, only groups A and B are endemic and the remaining ones were only identified in a single patient (except for group F, which has two strains - F1 and F2, coming from two different individuals) [11]. HIV-1 groups M, N and O derive from SIV that infects chimpanzees (Pan troglodytes troglodytes – SIVcpz) [2,12,13]. The HIV-1 group P is thought to have origin in a cross-species event with gorillas (Gorilla gorila gorila – SIVgor) [14].

Although similar in morphological and genomic organization, these two viruses diverge in genetic and immunological terms. Usually HIV-2 infections have less pathogenic potential than HIV-1 infections [2,3,8]. However, both viruses have high genetic intra-individual and inter-individuals diversity due to a very high replication rate, the lack of reverse transcriptase proofreading activity wich leads to the progressive accumulation of mutations during the replicative cycle, the high recombination rate between viruses that infect the same cell and selective pressure caused by the host immune system [15,16].

1.2 HIV epidemiology

HIV-1 infection is pandemic. On the other hand, HIV-2 infection is prevalent in West African countries such as: Guinea-Bissau, The Gambia, Senegal, Cape Verde, Cote d'Ivoire, Mali, Sierra Leone e Nigeria, as well as countries with historical and socioeconomic ties with them such as

Portugal, former Portuguese colonies (Guinea-Bissau, Angola, Mozambique, Brazil, Goa and Maharashtra) and France [17].

Since 1990, the number of people living with HIV in the world has increased, until around 2002, when stabilization began. In its latest report, UNAIDS estimates 36.7 million people worldwide being infected with HIV [18,19]. The trend towards new cases of HIV infection has been declining. Since 2010, a decrease of 11% has been estimated, and in 2016 the number of newly infected was around 1.8 million people (5000 new infections per day)[18,19]. With the same trend are AIDS-related deaths, which peaked around 2004, and by 2016 1 million people were killed. However 40% of the HIV-infected population does not know their status because they do not have access to HIV tests [18,19]. The vast majority of infections are caused by HIV-1, only about 1-2 million being caused by HIV-2 [20].

In Portugal, in 2016, 261 new cases of AIDS and 1030 new cases of HIV infection were reported, mostly adults and males. Of these new cases, 978 individuals are infected with HIV-1, 34 with HIV-2 and 4 individuals with dual infection. In the same year, 253 deaths occurred in patients infected with HIV. In total, 56,001 cases of HIV infection were reported, of which 3.3% corresponded to HIV-2 infections (1856 individuals) [21].

This global trend towards the reduction of new cases results on the one hand from the effort to prevent infection and the use of prophylaxis in cases of risk, as well as from the increase in the number of people who know their HIV status and live on antiretroviral therapy (ART), that is, with the infection under control, leading to a major reduction in the risk of transmission [22,23]. The number of people living with HIV and under antiretroviral therapy has been increasing in the world. In June 2017 statistics showed that over than 20 million people were on ART, an increase compared to 2000 when there were fewer than one million people living with treatment [19,24]. This corroborates the previous presented data from UNAIDS. Antiretroviral therapy has been reducing AIDS-related deaths. Nonetheless, access to ART is not universal and the prospect for cure or vaccine is limited. Thus, AIDS has remained a threat to public health for decades [1,2].

1.3 HIV structure and genomic organization

HIV-2, as well as HIV-1, are species from the family *Retroviridae*, subfamily *Orthoretrovirinae* and genus *Lentivirus* [25].

Retroviruses are a large and diverse group of viruses whose viral particles are approximately 100nm in diameter. Its genome is organized into a linear positive-sense single-stranded RNA dimer which is maintained by interactions between the 5' ends in a complementary region called Dimer Linkage Structure (DLS) [3,26]. This RNA is highly condensed within the virion due to its association with the nucleocapsid proteins (NC). In HIV-1, the RNA dimer together with the viral enzymes such as protease (PR), reverse transcriptases (RT), integrase (IN) and accessory proteins (Nef, Vif, Vpr and

Vpu or Vpx in HIV-2) are confined into the conic-shaped capside (CA). The capsid is surrounded by a protein matrix (MA) which is enclosed by a lipid envelope with glycoproteins [3,26] (Figure 1.1).

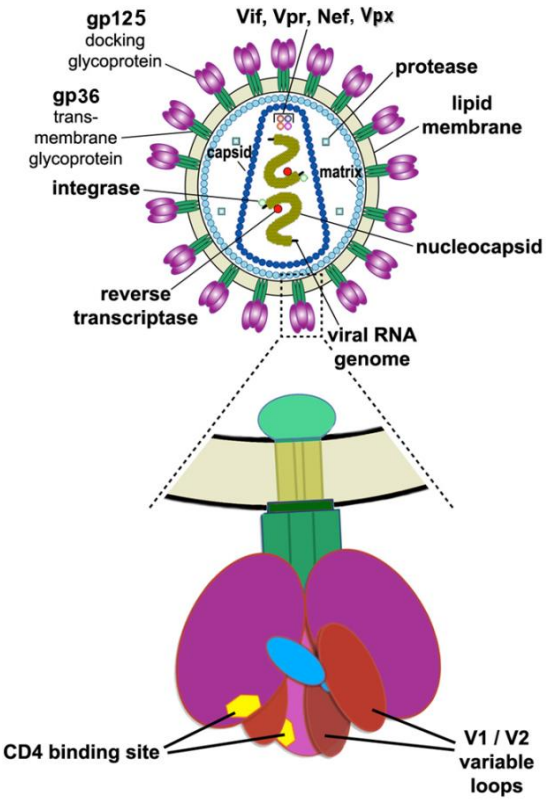


Figure 1.1: Schematic representation of the HIV-2 virion structure with zoom on envelope glycoproteins: transmembrane subunit (gp36) and surface subunit (gp125).
Image adapted from: https://www.fredhutch.org/en/news/spotlight/imports/sieve-analysis-shows-partial-efficacy-of-an-hiv-1-vaccine-tested.html at 26.06.2018.

Viral RNA, upon entering the host cell, is transcribed by the reverse transcriptase into DNA which subsequently integrates into the chromosomal DNA. Thus this viral DNA, called the provirus, serves as a template for the synthesis of viral proteins, as well as for the formation of viral RNA [3,8]. This RNA has several characteristics of a host mRNA such as the 5 'capping and the poly-A tail at the 3' end [3].

HIV-1 and HIV-2, although different in their genetic sequences, are similar in genomic organization (Figure 1.2). The genome of these viruses are flanked at both ends by long terminal repeat (LTR) sequences, the 5'LTR region encodes the transcription promoter of the viral genes. This region is followed by nine genes, in three different frames. Three of the genes encode structural proteins (*gag*,

pol and *env*), two encode regulatory proteins (*tat* and *rev*, two ORFs each) and four encode accessory proteins (*nef*, *vif*, *vpr* and *vpu* in the case of HIV-1 or *vpx* in the case of HIV-2) [3] (Figure 1.2).

The *gag* gene encodes a precursor polyprotein ($Pr55^{Gag}$) which is cleaved by viral protease and gives rise to matrix proteins, capsid proteins, nucleocapsid proteins and C-terminal proteins. These proteins are essential for the assembly and the release of the virus. The *gag* and *pol* genes encode a precursor polyprotein ($Pr160^{GagPol}$) which is cleaved into reverse transcriptase (p51), RNase H (p15), or RT and RNase H (p66), into protease and into integrase. The *env* gene encodes a precursor polyprotein ($Pr140^{Env}$) which is cleaved into surface and transmembrane envelope glycoproteins (gp125 and gp36, respectively, in HIV-2) important for attachment and fusion of the virus to the cell. The *tat* gene encodes a transactivator protein (Tat) and the *rev* gene encodes a RNA splicing-regulator (Rev). Finally, the *nef* gene encodes a negative regulating factor, *vif* encodes a viral infectivity factor, *vpr* encodes a virus protein r and *vpu* encodes a virus protein u (in HIV-1) and *vpx* gene encodes a virus protein x (in HIV-2) [3,8,27,28]. This last protein is partly responsible for the reduced pathogenicity characteristic of HIV-2 [8,29] and appears to have originated in a vpr gene-duplication event [30]. Summary of HIV-2 genes, proteins and correspondent functions in Table 1.1.

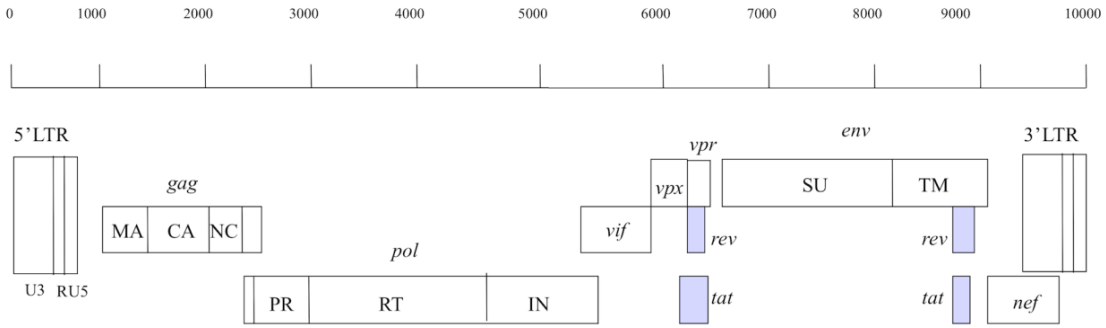


Figure 1.2: HIV-2 genome (9800bp). MA – Matrix proteins, CA – Capside proteins, NC – Nucleocapside proteins, PR – Protease , RT –Reverse Transcriptase, IN – Integrase, SU – Surface subunit, TM – Transmembrane subunit.
Image adapted from Taveira, N. & Martin, F. in Manual sobre SIDA (eds. Antunes, F. & Maltez, F.) 1–11 (Permanyer Portugal, 2018).

Table 1.1: Summary of HIV-2 genes, proteins and their main functions.

Gene	Protein	Functions	References
env	Surface glycoprotein (gp125)	Responsible for the binding of the virus to the host receptor and co-receptors	26,31
	Transmembrane glycoprotein (gp36)	Responsible for the fusion of the viral particle and the host cell; and for downregulation of the host BST-2/tetherin (HIV-1 Vpu-like activity)	26,31
gag	MA	Matrix protein that form the inner membrane	8,32
	CA	Proteins that form the conical capside	
	NC	Proteins that bind to viral RNA, form a complex and promote RNA condensation	
	C-Terminal protein	Protein involve in the release of viral particles	
pol	RT	Transcription of viral RNA in DNA	8
	RNase H	Degradation of RNA of the complex of RNA/DNA during transcription	
	PR	Proteolytic cleavage of Gag and Gag-Pol precursor protein and release of structural proteins and viral enzymes	
	IN	Integration of proviral DNA into host genome	
rev	RNA splicing-regulator	Regulates the nuclear export of non-spliced and partially spliced viral mRNA	8,32
tat	Tat	It is a transactivator of the HIV promoter LTR - Tat protein binds to TAR region of the HIV mRNAs (Tat/TAR transactivation mechanism).	27,32
vpr	Vírus protein r	• Disrupts the cell cycle of HIV-infected cells, leading to degradation of cell cycle proteins, which appears to increase LTR expression • Induces cell differentiation • Assists the transport of the viral DNA to the nucleus • Influence on the reliability of the reverse transcription process when associating with DNA glycosylase (UNG2).	27
vpx	Virus protein x	Required for replication in peripheral blood lymphocytes - integrates the viral DNA and participates	27,33

		in its nuclear import but also has signals to return to the cytoplasm and then integrate the formed virions. In addition, vpx enhances viral infection by neutralizing an host restriction factor (SAMHD1).	
vif	Viral infectivity factor	Binds to antiviral cell factors (cytidine dasaminase-APOBEC3G) and inhibits the incorporation of this factor into viral particles by preventing antiviral activity on reverse transcription.	[27]
nef	Negative regulating factor	• Helps prolong the life span of infected cells by inhibiting apoptosis. • Contributes to the escape of the immune system, reducing the immune defences of the host: ○ removes the CD4 receptor from infected cells, preventing infection by other viruses and further compromising the helper T response; ○ and is responsible for the removal of the MNHC-1 proteins from the surface of the cells to the Golgi complex in order to compromise the detection and destruction of cells infected	[27]

1.4 HIV replication cycle and Latency

The HIV replication cycle is divided in seven steps: 1) binding, 2) fusion, 3) reverse transcription, 4) integration, 5) replication, 6) assembly and 7) budding (Figure 1.3) [34].

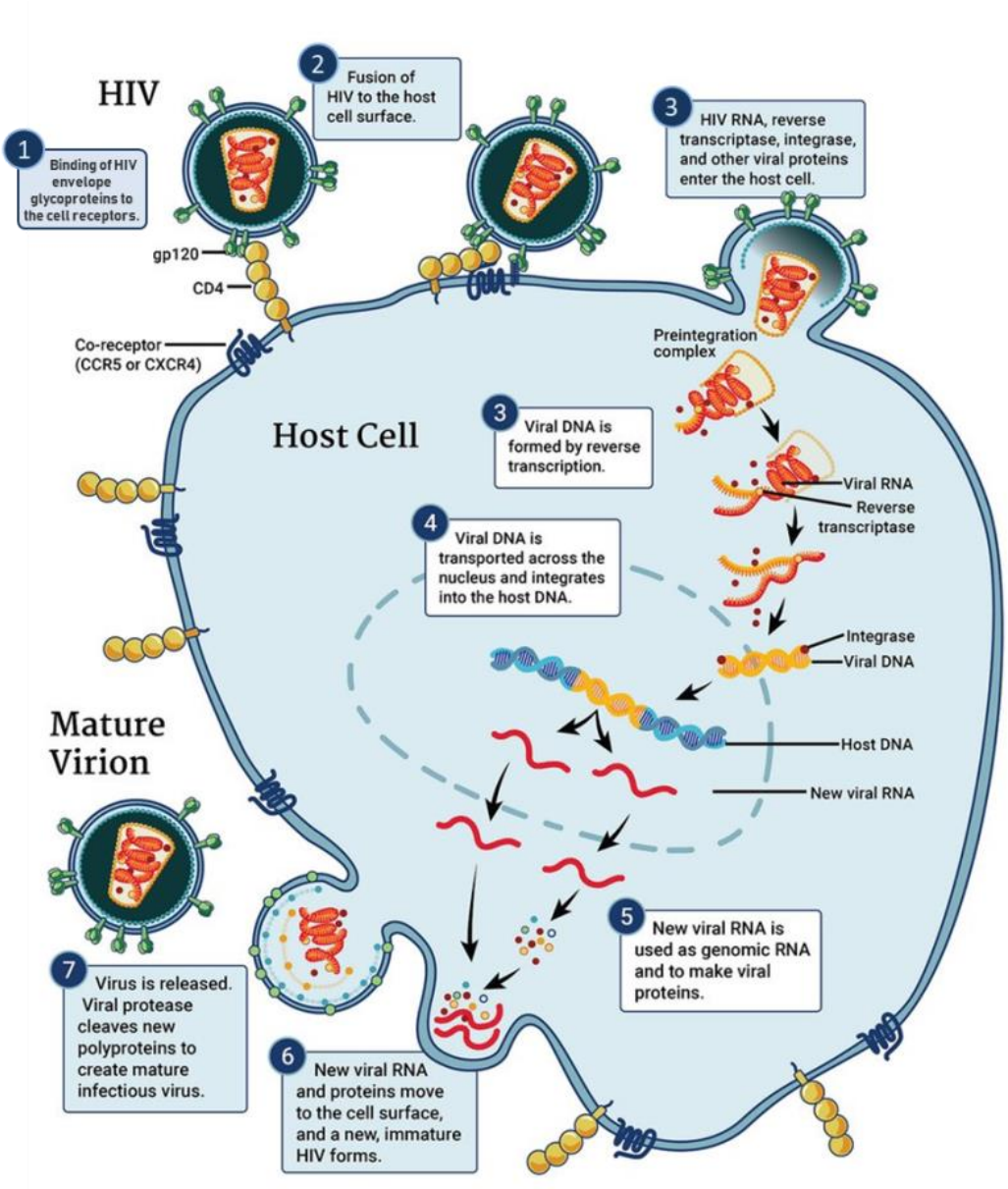


Figure 1.3: Schematic representation of the HIV replication cycle. 1) Binding, 2) Fusion, 3) Reverse transcription, 4) Integration, 5) Replication, 6) Assembly and 7) Budding.
Image adapted from: https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle at 1st January 2018

Firstly, the virus and the cells can attach by cellular attachment factors (for example Lectin) and glycans of the viral envelop glycoproteins [35]. This step is not critical to the binding but can

increase viral infectivity by concentrating viral particles on the cell surface, thereby increasing the chance that Env proteins interact with specific cellular receptor [35].

The replication cycle begins with the binding of the surface subunit of the Env protein to a specific cellular receptor, the immunoglobulin CD4 (present in T lymphocytes, monocytes, macrophages and dendritic cells) [3,26,31,36]. This receptor is necessary in most cases but it is not sufficient to the entry of the virus, allowing only the adsorption of the virus to the cell surface. The interaction between the Env protein and CD4 induces conformational changes in the surface subunit that expose the binding site for a co-receptor necessary to proceed with the infection. The major co-receptors of HIV are the CCR5 and CXCR4, members of the chemokine receptor family [3,26,31,36]. The binding of both CD4 and co-receptor leads to a conformational change of the transmembrane subunit of the Env protein that result in the insertion of the fusion peptide of this subunit into the host cell membrane which leads to the formation of a fusion pore through which the viral capsid is release into the cytoplasm [3,26,31,36].

The uncoating of the virus core precedes the next step of the cycle: the reverse transcription of the HIV-RNA to double stranded linear DNA. This step starts with the synthesis of a single DNA strand (negative strand) using one RNA molecule as template and the tRNA(Lys) as primer. The RNase H subunit degrades the RNA template and the reverse transcriptase subunit synthesizes a positive DNA strand, complementary to the negative strand [27]. The double stranded DNA, matrix proteins, nucleocapsid proteins, integrase, reverse transcriptase and Vpr and Vpx proteins make the pre-integration complex (PIC), which is transported to the nucleus using the cytoplasmatic microtubules network, result of the interaction between the envelope glicoproteins and the co-receptores [27,36,37]. Before being imported into the nucleus, the viral integrase digests the 3' LTR of both DNA strands to create two recessive ends. Then, these ends will be necessary to the integration of the viral DNA into genomic DNA [27]. Depending on several characteristics, this integration may lead to productive infection or latency [27,38].

In case of productive infection, once in the nucleus, the provirus becomes controlled by the host transcriptional machinery [38] and the 5'LTR promotes viral transcription. Hence, three classes of RNA are produced. Initially, it is produced multi-spliced messenger RNA transcripts (msRNA) that encode the regulatory proteins: Tat, Rev and Nef (early transcripts). Then, it is produced un-spliced RNA (usRNA) and single-spliced RNA transcripts (late transcripts). The usRNA are translated into the structural precursor polyproteins $Pr55^{Gag}$ and $Pr160^{GagPol}$ or can be incorporated into virions as genomic RNA. The single-spliced RNA encodes the Env, Vif, Vpr and Vpx proteins [27,39]. The Env glycoproteins are produced in ribosomes bound to the endoplasmic reticulum and the glycosylation occurs in the Golgi apparatus. Then, these proteins are cleaved into the surface and transmembrane glycoproteins and transported to the cytoplasmatic membrane, where the assembly of the viral particles takes place [26,27].

The assembly and the release of the immature virion are mediated by the uncleaved Gag precursor. The incorporation of the RNA virus genome results from interactions between specific RNA sequences near the 5' end (Psi sequences) and specific residues in the nucleocapsid domain of Gag. Release of the new viral particles occurs by budding from the cell membrane, thus acquiring the lipid envelope already containing the Env glycoproteins and some cellular membrane proteins [3,27].

During and after the budding, the polyproteins $Pr55^{Gag}$ and $Pr160^{GagPol}$ are processed into functional proteins by the protease enzyme and several structural rearrangements of the structural components happen, such as the condensation of the capsid and nucleocapsid proteins and the development of the conical shape capsid. Viral particle maturation occurs that turns them into infectious viruses (Figura 1.4) [3,27] .

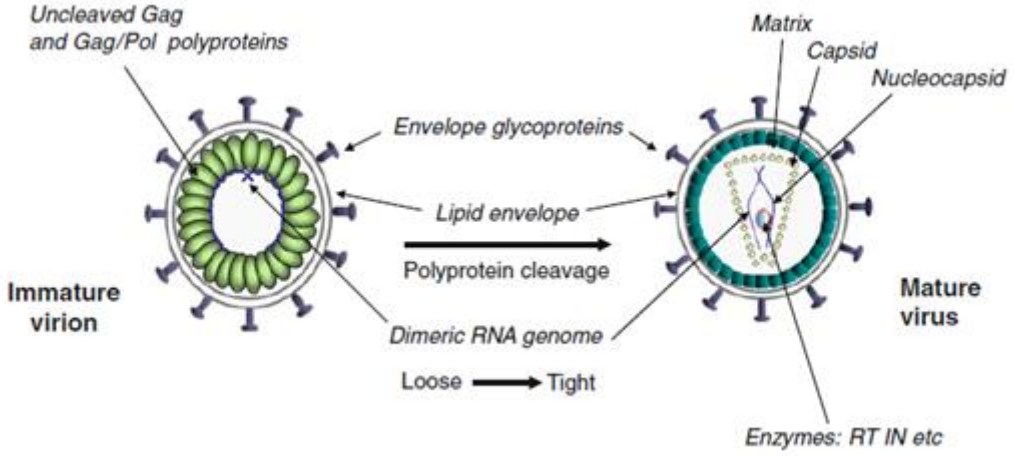


Figure 1.4: Schematic representation of the maturation process of an HIV virion. Cleavage of poliproteins; Condensation of the capside and nucleocapside proteins to the formation of the conical shape capside; and rearrangement of RNA and nucleocapside proteins.
Image adapted from: Lever A.M.L. (2013) Molecular Biology of HIV-2. In: Hope T., Stevenson M., Richman D. (eds) Encyclopedia of AIDS. Springer, New York, NY

1.4.1 Latency

After viral DNA integrates into the cell genome, the infection may become latent. During viral latency, the HIV provirus produces little-to-no viral transcripts, but retains the potential to initiate productive infection at a later time, if reactivated [38]. In latent infection, infected cells do not produce viral proteins and escape the viral cytopathic effects, are ignored by the immune system [38] and escape treatment. Therefore, HIV latency represents a major barrier towards eradication of HIV infection [40].

Several characteristics influence the tendency of HIV-1 and HV-2 for post-integration latency. In HIV-1, latency may occurs after direct infection of resting CD4+ T cells [38] or infection of activated memory CD4+ T cells [38,41], that in the process of transitioning from activated to resting memory CD4+

T cells [42]. There are particular features in HIV-2 infection that promote viral latency. In HIV-2 infection there is a lower rate of T cell activation and enhanced virus-specific immune responses leading to viral control in HIV-2 infections, thereby suppressing T cell responsiveness to activation and leading to viral persistence and latency [42].

Although the proportions of HIV-1 and HIV-2 integrated DNA found in the genome of infected cells are similar, the levels of HIV-2-mRNA are lower when compared to HIV-1. This suggests that the attenuated pathogenesis of HIV-2 can be explained by lower rates of viral replication [43]. The rates of viral replication are dependent on factors such as the proviral integration site, chromatin environment and proviral integration orientation [44,45]. Integration of HIV–cDNA into heterochromatin regions may block the viral transcription, consequence of its poorly accessible nature [46,47]. Although HIV-1 and HIV-2 *in vitro* have similar integration tendencies [48], having preference for integration into coding regions and actively transcribed host genes [44], *in vivo* it is possible to observe a small increase in HIV-2 integration into heterochromatin sites [48]. Also, HIV-2 was found to integrate more often, compared to HIV-1, in the opposite orientation relative to the transcriptional direction of the host genes. Thus, the transcription ability of HIV-2 is further reduced and may contribute to a more latent phenotype [40]. Other transcriptional regulation mechanism is epigenetic DNA methylation. High levels of CpG-rich DNA (CpG islands) methylation are known to prevent the binding of transcriptional machinery. Highly methylated promoter regions leads to more repressed chromatin state and less transcription. HIV-2, as HIV-1, disfavour integration near CpG islands [48].

HIV-2-LTR is less responsive to CD4+ T cells activation signals, than HIV-1-LTR, due to lack of nuclear factor of activated T cells (NFAT) binding site and negative regulatory elements [40,49]. Moreover, the HIV-2-LTR has a duplicated TAR RNA stem-loop structure (in contrast to the HIV-1 single stem-loop structure) that slows down the translation [50]. Both differences may affect basal transcription levels of HIV-2and be correlated to its lower pathogenic phenotype [40]. Overall, these differences suggest that HIV-2 infection has higher tendency for latency than HIV-1 infection [40,43].

Besides post-integration latency, there is a state of pre-integration latency that is common *in vivo* in HIV-1 infection [51]. This second latent form refers to the presence, in host cells, of PIC that will eventually degrade or integrate into the host genome after activation [51,52]. This type of unintegrated HIV-1 DNA can reside near the centromere, for weeks, and may represent a reservoir in slowly dividing or non-dividing cells [53], such as macrophages [54]. However, not much is known about the pre-integration latency in HIV-2 infection [40].

Ex vivo and *in vitro* cellular models have been used to study the mechanisms of HIV-1 latency and infection [46,55,56]. For HIV-2 there are still no *in vitro* models available. Such model may contribute for a better understanding of the mechanisms governing latency of HIV-2, but also offer an important model for the study of many aspects of HIV immunopathogenesis. In addition, future studies may use this tool to develop a culture-based assay to measure the size of latent HIV-2 reservoir [57] and therefore contribute for the clinical treatment assessment and for the development of new-HIV-2

treatment strategies since antiretroviral drugs have been designed for HIV-1 and some of them not provide optimal viral suppression of HIV-2 infection [58,59] .

1.5 HIV-2 Envelope

The HIV-2 envelope consists of a lipid bilayer (originating from the host cell), two highly glycosylated proteins: a surface subunit with 125KDa (gp125) bound by a noncovalent binding to a 36 KDa (gp36) transmembrane subunit and multiple other proteins derived from the host cell membrane (Figure 1.1) [3,26].

The gp125 glycoprotein has between 501 and 515 amino acids and comprises five hypervariable regions spaced by five conserved regions (NH2-V1-C1-V2-C2-V3-C3-V4-C4-V5-C5-COOH). This is the subunit responsible for the initial steps of HIV-cell binding. It binds to the major HIV receptor, the CD4 surface receptor. The CD4 binding site of gp125 comprises amino acids of C2, C3, C4 and V4 regions, and binding to the receptor induces conformational changes by exposing the binding site (loop V3) to the co-receptor. The most common co-receptors are chemokine receptors such as CCR5 or CXCR4 [26], nonetheless, for HIV-2, other equally efficient co-receptors are: CCR1, CCR2, CCR3, and CCR8 [36,60,61]. Based on the coreceptor usage, HIV exhibits different tropisms. In the early stages of infection, HIV generally uses CCR5 as the main coreceptor (R5 tropism), but with the progress of infection, the virus evolves and may exhibit a coreceptor-switch to use the CXCR4 (switch to X4 tropism) [62–64]. The interaction with each of the co-receptors is dependent on the V1 / V2 and/or V3 regions of gp125 [26,65,66]. It has been demonstrated that some HIV-2 isolates may not require binding to the CD4 receptor in which case binding to the co-receptors is most critical to virus binding to the cell [60,67]. This suggests that gp125, unlike gp120 in HIV-1, can spontaneously adopt the CD4-bound conformation which may explain the higher vulnerability of this virus to neutralizing antibodies [68,69].

The glycoprotein gp36 has an extracellular domain, a transmembrane region and an intracytoplasmic domain and is responsible for the fusion of the viral particle to the host cell and for the downregulation of BST-2/tetherin, a cell surface protein that anchors the virus to the cells [26]. The ectodomain starts with a highly conserved and hydrophobic peptide which is important for the fusion of HIV-2 with the host cell [26], an antigenic region against which all HIV-2 infected individuals produce IgG antibodies [70] and a highly conserved Ala residue that binds to BST-2/tetherin [26]. The transmembrane region is highly hydrophobic and its main function is the insertion in the cell membrane [26]. Finally, the intracytoplasmic region is responsible for envelope binding to the protein matrix during the assembly of viral particles [26].

1.6 Transmission

Several human- and HIV-specific determinants are required for efficient viral transmission. The routes of HIV transmission are sexual contacts (vaginal or anal), contaminated blood or blood products (medical injections, blood transfusions or injection drug usage) and mother-to-child

transmission (before, during and after birth or through breast milk). The routes of transmission vary in different parts of the world, but the most common route is the sexual transmission. In some countries, infection with HIV is mainly detected in specific groups of risk, including men-who-have-sex-with-men, injecting drug users, sex workers and the regular partners of such persons [71]. Globally, access to healthcare services and behaviour changes (stop syringe sharing and using condom to a safer sex) are crucial to reduce the risk of HIV infection [72,73].

There is evidence that HIV-1 transmission is directly correlated with the viral load. Studies showed low rates of heterosexual HIV transmission associated to low HIV plasma viral load [74–76] and higher viral load has been shown to be associated with increased levels of mother-to-child transmission, for example [77]. On the other hand, more recent studies emphasize the importance of the concentration of genital HIV-RNA relative to the risk of sexual or mother-to-child transmission due to the possible discordance to plasma concentrations [78].

In case of HIV-2, the transmission occurs less frequently than in case of HIV-1, and this may contribute to the declining prevalence of HIV-2 infection worldwide. Indeed, sexual transmission is four to ten times less frequent than for HIV-1 [11]. This is associated with the lower viral loads observed in HIV-2 infections [43,79], even viremic patients display 30-fold lower plasmatic viral loads than HIV-1 patients [79], although proviral DNA loads are very similar between the two [43,49]. Likewise, mother-to-child HIV-2 transmission is a rare event due to low maternal HIV-2 RNA levels [80].

Other factors that influence the rates of transmission are the effective antiretroviral therapy that decreases HIV-RNA levels [81,82] and lower the risk of transmission [75,83] and genital tract/sexual transmitted infections resulting in inflammation and ulceration of the genital mucosa and can enhance HIV transmission [84].

1.7 Pathogenicity

The HIV infection can be divided in three stages: the acute phase, the chronic phase and AIDS [85].

The acute phase lasts 12 weeks and is characterized by intense HIV replication and massive loss of CD4+ T cells in the absence of an immune response [86]. Little is known about the early events of HIV-2 infection because identifying infected individuals shortly after exposure to HIV is difficult.

When transmission occurs through the blood (transfusions or syringes) or mother-to-child, the virus is probably delivered from circulation by the reticuloendothelial system of the spleen, liver and lungs, with consequent infection of lymphoid tissue [87].

When transmission occurs by sexual contact, less than a week after, CD4+ T cells from genital mucosa are the first targets of HIV replication. These infected cells or virions attached to

dendritic cells migrates *via* draining lymph nodes to the gut-associated lymphoid tissue (GALT) where HIV induces a large depletion of CD4 T memory cells in intestine *lamina propria* [88,89]*.* CD4+ T cell depletion is a combination of direct viral infection, programmed death of both virally infected cells and adjacent uninfected cells and host-derived cytotoxic responses [31,90]. The replication of HIV is followed by the establishment of viral reservoirs in the lymphoid tissue (and GALT), rich in HIV target cells and with reduced drug penetration. Two to four weeks after the transmission, the infected individuals experience acute HIV syndrome with flu-like symptoms, fever, high plasma viremia and lymphadenopathy. During this phase HIV aggressively replicates in the absence of immune response leading to a plasma viremic peak. Then due to the HIV-specific T and B cell mediated immune responses viral load declines spontaneously during several months until a viral set point or steady state. In parallel CD4+ T cells are partially restored. The level of the viral load set point is important to determine the disease progression rate of infected individuals [88]Regarding HIV-2, this viral load set point is usually lower compared to HIV-1 [91]. The lower viral load set point in HIV-2 is a combination of multiple virus- and host immune-related factors such as, the lower viral replication rate leading to fewer viral particle production and less induction of immune activation and higher susceptibility to host humoral immune responses, both of which lead to lower levels of apoptosis of infected cells [92,93].

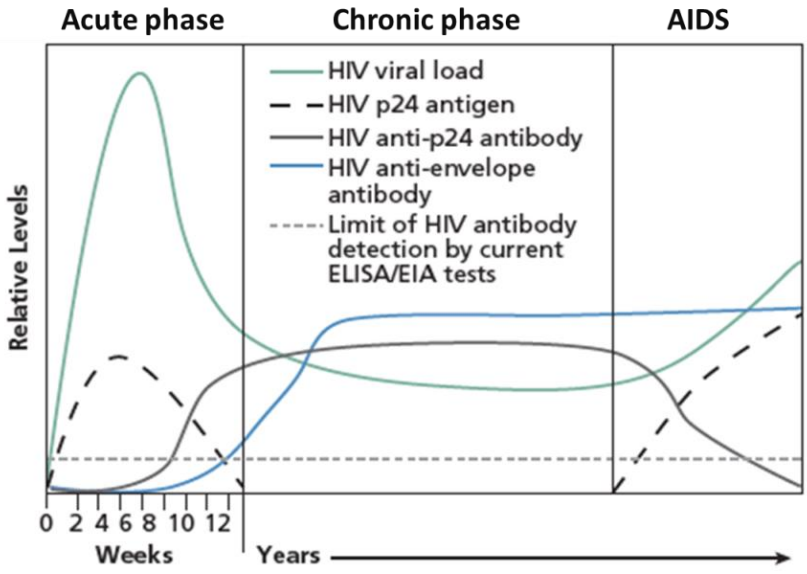


Figure 1.5: Graphic representation of the course of an HIV-1untreated infection. The three stages: Acute phase, Chronic phase and Acquired immunodeficiency syndrome, and repective relative levels of HIV viral load, HIV p24 antigen, HIV anti-p24 antibody, and HIV anti-envelope antibody.
Image adapted from: Daskalakis, D. HIV Diagnostic Testing: Evolving Technology and Testing Strategies. Topics in Antiviral Medicine. 19 (1) 18-22(2011)

Several weeks after HIV transmission, the first immune cell response arise and the “founder virus” evolves. After about 12 weeks of exposer to the virus, autologous neutralizing antibodies appear

and HIV develops neutralization-resistant variants which will drive the emergence of heterologous neutralizing antibodies in some HIV infected individuals [88].

The chronic phase or asymptomatic phase corresponds to clinical latency with low but persistent HIV replication in the viral reservoirs and cells from innate and adaptive immune system become activated by viral proteins and host responses. As mentions before, the major constituent of immune system and the target of HIV are CD4+ T cells. HIV destroys and dysregulates this cells but also induces immunologic dysfunction of CD8+ T cells, B cells, natural killer cells and nonlymphoid cells, increasing cell turnover, activation, differentiation, and homeostatic responses [88,89]. These factors ultimately affect overall immunologic competence. Constant antigen stimulation cause chronic immune activation, cellular exhaustion, senescence, and low renewal potential [88,89]. Therefore, this leads to the acquisition of opportunistic infections like tuberculosis, candidiasis and pneumonia and virus induced tumours such as Epstein-Barr virus related lymphomas, Kaposi's sarcoma and cervical cancer caused by Human Papillomavirus. These diseases mark the onset of AIDS [3,94]. HIV-2 infection is characterized by a lower state of immune activation likely due to low to undetectable levels of plasma viremia, which might account for the slower disease progression, although HIV-2 infection can also leads to AIDS if untreated [95].

In untreated HIV-1 patients, the chronic phase can lasts 3 to 20 years, depending on the rate of disease progression. HIV-1 seropositive untreated individuals with up to 20 years in chronic phase are called long-term non-progressors (2-5% of all HIV-1 positive individuals) and maintain low levels of viremia and elevated CD4+ T cells. Some untreated infected individuals have undetectable levels of viral RNA and are called elite controllers, representing less than 1% of all HIV-1 positive individuals. However, most untreated individuals infected with HIV-1 will progress rapidly to AIDS and death after less than 10 years [96]. In case of HIV-2, approximately 80-90% of untreated patients have very low or undetectable viral loads, being considered long-term non-progressors or elite controllers [40,93,97,98]. For those having undetectable viral loads, mortality caused by HIV-2 infection is probably close to uninfected individuals [97]. The chronic phase of untreated HIV-2 infected individuals usually lasts over 18 years [97] and the immune deficiency is much less severe when compared to HIV-1 infected individuals [99].

1.8 Human responses against HIV-2

1.8.1 Innate responses

When the human immune system detects a pathogenic microorganism activates defence mechanisms to eliminate the infection. The innate immune response, including the epithelial barriers, the complement system, and cells with phagocytic and antigen presenting properties, is the first line of defence against these microorganisms [89].

Essential to the innate immune defences are the pattern recognition receptors that recognise patterns of carbohydrate, protein, or lipid structures that are unique to pathogens (bacteria, viruses, and/or parasites) and not normally produced in human cells [89]. Toll-like receptors (TLR) are one family of these pattern recognition receptors. Viral recognition can be mediated by TLR2/4 recognising viral glycoproteins and TLR9 recognizing DNA as well as TLR7/8 sensing single-stranded RNA and TLR3 sensing double-stranded RNA [100].

The activation of these and other cellular receptors results in the induction of inflammatory stimuli creating a nonspecific antiviral environment. The TLRs potently activate dendritic cells that release high amounts of type 1 interferons (IFNs) and tumor necrosis factor α (TNF-α) with the aim of stopping HIV replication in the infected cells and also promoting the activation of other immune cells to the site of infection. In addition, the activation of dendritic cells and other cells expressing TLRs also promote the release of proinflammatory cytokines [101,102]. A set of cells react to the cytokine cascade and respond to the pathogens. Monocytes, macrophages and dendritic cells phagocyte the antigens, natural killer cells (well preserved during HIV-2 asymptomatic phase of infection [103] and neutrophils destroy the pathogen and infected cells and dendritic cells capture antigens and present them to the adaptive immune responders [102].

The complement cascade is another component of innate immune system. This system induces complement-mediated lysis and tag pathogens for phagocytosis by opsonisation. Also, it induces and maintains the adaptive immune responses and enhance humoral responses [104].

Some host cell intrinsic factors also are involved in the innate immunity against HIV [87]. The replication cycle can be stopped by host restriction factors. TRIM5-α (tripartite motif-containing protein 5α) blocks the HIV uncoating [105] and is capable of limit the HIV-2 replication to certain extent [106]. The APOBEC3G (a member of the cytidine deaminases family) can induce G-to-A hypermutation and degradation of the HIV provirus. However, HIV-1 and HIV-2 Vif proteins can antagonize its action [106]. Another factor involved is tetherin, also called BST2, CD317 or HM1.24. Tetherin (BST-2) is an antigen composed of two cysteine-linked monomers in which both have two membrane-anchored domains [26,107–109]. Thus, anchored simultaneously to the cell membrane and to the viral membrane and thus retain the virus in the cell membrane and consequently prevent the release of viral particles [26,107–109]. This protein is found in the cell membrane but also in the perinuclear region (near the trans-Golgi network – TGN) in the TGN pathway to the membrane [107]. Tetherin is an intrinsic interferon-induced antiviral cellular response that acts against enveloped viruses such as HIV-1, HIV-2 or SIV [26,107–109]. The viruses in turn have anti-tetherin factors, such as Vpu in the case of HIV-1 and Env gp36 in the case of HIV-2 [26,107,110]. These factors antagonize the action of this protein by removing it from the cell surface and sequestering it intracellularly in perinuclear compartments such as those bearing the newly synthesized tetherin. However the mechanism of these viral proteins is independent of the original cellular mechanisms leading to an increase in the release of viral particles by the cell [107].

1.8.2 HIV-specific cellular immune responses

During the acute phase of HIV infection, the host develops cellular and humoral immune specific responses, even though these cannot stop the viral replication [111,112].

T-cells are important in control and pathogenesis of HIV infection [113]. CD8+ T cells, also known as cytotoxic T lymphocytes (CTL), mediate responses against HIV recognizing viral determinants at the surface of infected cells, inducing direct apoptosis and secreting antiviral factors that supress infection, such as pro-inflammatory cytokines [113,114]. The CTL responses against HIV-2 are more polyfunctional compared to HIV-1 infections. This CTL function preservation is thought to account for the slow disease progression in HIV-2 infections [115]. CD4+ T cells recognise viral determinants and respond by proliferating and releasing cytokines (e.g. IL-2). These cells are important in stimulation of innate and adaptive effector cells by activation of CTL and macrophages and in maturation of B cells [87,113]. Since there is a better preservation of the CD4+ T cell counts in HIV-2 infected individuals compared to HIV-1, it is thought that they are important determinants of the attenuated course of HIV-2 infection [116].

1.8.3 HIV-specific humoral immune responses

Humoral immune responses are mediated by antibodies or immunoglobulin (Ig) produced by B cells [117] and can be found in blood and tissue fluids [118]. The Ig is composed by two heavy chains and two light chains. Each Ig is divided in one fragment crystallisable (Fc) and in one fragment antigen-biding (Fab) [118]. The Fc region comprises the constant regions of heavy chains and is responsible for the effector functions [118] such as 1) neutralization of targets; 2) activation of immune cells; and 3) activation of complement system [119]. The Fab region is composed by both heavy and light chain variable regions and support the hypervariable antigen-binding domain or Complementarity Determining Region (CDR) [118]. Antibodies can act like antigen receptors on the surface of B-cells or can be secreted into the extracellular space [119]. According to their Fc fragment, antibodies can be classified in IgM, IgD, IgG, IgA and IgE [118] and antigen specificity is determined by the antigen binding domain [119].

Naïve B cells (before antigen exposure) express IgM and IgD on their surface. Upon antigen contact, maturation of B cells is induced by CD4+ T cells causing isotype switch to IgG, IgA or IgE. Each isotype determines the effector function. The antigen specificity is determined by somatic hypermutations in the CDR and clonal selection based on antigen affinity [118]. Proliferation, somatic hypermutation and affinity maturation occur when B cells migrate into the B cell follicle to form the germinal centers [120].

During HIV infection, IgM, IgG and IgA are produced [121]. Similar to HIV-1 [122,123], in HIV-2 infection memory B-cell depletion occurs associated with CD4+ T cell decrease, despite low viremia load [124,125]. In HIV-2, specific antibody potency increases in direct association with this memory B-cell

exhaustion and depletion [125]. In both infections, B-cell depletion is not fully recovered with antiretroviral treatment [122].

In HIV-1 infection, hiperactivation of B-cells in association of immune activation [126] increases cell turnover and differentiation of B-cell in plasmablasts leading to higher levels of immunoglobulin isotypes (hypergammaglobulinemia polyclonal) in plasma [126,127]. However, the frequency of HIV-specific B cells is low [126], which may be explained by the presence of plasmablasts too early in infection [128]. Ultimately, this leads to expansion of several aberrant B-cell population, B-cell exhaustion and immunological failure [123,127,129].

In HIV-2 infection, B-cell activation is less pronounced, relative to HIV-1 infection, and hypergammaglobulinemia only occurs, though to a lesser extent, with the decrease of CD4+ T cells, in late stage disease. However, this hypergammaglobulinemia is not polyclonal since it affects only IgGs [117,127]. IgG1 and IgG2 are the predominant responding antibody subclasses. In most chronically infected HIV-2 patients, IgG1, IgG3, and IgA responses are generated against gp36 and the C2V3C3 region in gp125 [70]. Although still to be confirmed, this C2V3C3 region may exert an immunosuppressive activity on CD4+ and CD8+ T cells, which may account for the lower rates of immune activation and CD4+ T cell loss in most HIV-2 infected patients [70]. Increasing IgG1, IgG3 and IgA levels are associated to the decrease of the immunosuppressive function of this region and lead to CD4+ T cell loss of chronically infected patients [70]. More studies on the impact of HIV-2 infection on B-cells homeostasis and antibody production need to be done, in particular in the acute phase of infection[70].

1.8.3.1 Antibody responses against the envelope glycoproteins

The initial antibody response to HIV-1 can be detected one week after infection. These antibodies mainly form immune complexes that do not seem to have impact in viral replication suggesting that they are not responsible for the initial decline in plasma viral load [121].

1.8.3.1.1 Non-Neutralizing Antibodies

Non-neutralizing antibodies, which are able to bind to HIV antigens present at the surface of cells and recruit other cells from the innate immune system (B cells, natural killer cells, dendritic cells, neutrophils and macrophages) that have an Fc receptor (FcγRs) emerge in the first two-three weeks of infection [130]. The effector function of these non-neutralizing antibodies can act by antiviral mechanisms such as: 1) antibody-dependent cell-mediated cytotoxicity (ADCC); 2) antibody-dependent cell-mediated viral inhibition (ADCVI); 3) antibody-dependent complement-mediated inactivation (ADCMI) and 4) antibody-dependent cellular phagocytosis (Figure 1.6) [130].

1.8.3.1.2 Neutralizing Antibodies (Nabs)

Neutralizing antibodies (Nabs) act by binding to cell-free virus and preventing the virion to infect the host cell [131]. Autologous neutralizing antibodies are antibodies that bind to envelope

glycoproteins of contemporaneous cell-free HIV (infecting strain) [132] (Figure 1.6) and appears months after infection [88] targeting the variable loops of HIV-1-Env gp120 [121]. However, autologous Nabs exert selective pressure and the virus evolves to escape neutralization, thus new antibodies arise, on a cycle of neutralizing antibodies production and viral escape [133]. Viral escape, that limit or block the access of antibodies to conserved neutralizing epitopes, is mediated by several features of Env such as: carbohydrate shielding and shifting, conformation masking, steric occlusion, temporary epitope exposure and non-functional envelope spikes [134].

In HIV-1 infection, few individuals develop antibodies able of neutralize heterologous isolates and this usually take 2 to 4 years to develop. This suggests that chronic antigen exposure and antibody affinity maturation are key factors for the ability of neutralization [133]. In contrast, most HIV-2 infected patients develop potent autologous and heterologous neutralizing antibody response [64,68,135]. It is thought that the better control of the neutralizing antibodies on HIV-2 replication may be due to factors such as, the lower potential (due to less sites) for gp125 glycosylation, resulting in more exposed neutralizing domains and the more open conformation of gp125 in the envelope complex [69].

Studies about antibody neutralization in HIV-2 present some limitations regarding: the number of patients, the representability of the full spectrum of disease (different viral load and CD4+ T cell counts to associate to antibody titters), the use of adapted strains or primary isolates and the non-standardization of the assays used [65,68]. Thus, direct comparison between the studies remains difficult. Rocha et al [125] found that the potency of these antibodies has strong direct association with memory B-cell exhaustion. Rodriguez *et al* [136] demonstrate that heterologous neutralizing antibody responses, in HIV-2 infection, are generally broad, but of a lower potency compared to HIV-1. They also found that Nab titters were directly associated with viral load on both HIV-1 and HIV-2. More recently, Kong *et al* [135] confirmed that, neutralizing antibody responses in HIV-2 infected individuals were both broad and potent, but in their study these responses were not significantly associated with CD4+ T cell count or viral load. They also found that neutralization escape was rare. Özkaya Sahin et al [137] also confirmed that neutralizing antibodies were both broader and more potent in HIV-2 infected patients, relative to HIV-1 patients, and found no association with viral load in HIV-2 infected patients; in their study association of Nab response with viral load was only present in HIV-1 infection. Similarly, de Silva et al [68] reported the presence, in HIV-2 infected individual, of potent autologous and heterologous neutralizing antibodies, with no association with viral load. In addition, they found no clear association between the Nab response and the clinical outcome of infection. Finally, they found that higher Env sequence diversity tended to be associated with higher resistance to neutralization antibodies. On the other hand, Marcelino *et al* [64] demonstrated that X4 tropic viruses which emerge in late stage disease patients having low CD4+ T cell counts and positive plasma viremia are much more resistant to neutralizing antibodies than R5 isolates. Overall, the results demonstrate that most HIV-2 infected patients produce broad and potent neutralizing antibodies against R5 isolates regardless of viral load. Resistance to antibody neutralization is usually associated with X4 viral tropism and major changes in V3 sequence and conformation [64].

Regarding the acute infection, Rocha et al [138] studied the neutralizing antibody response and molecular and phenotypic evolution of HIV-2 in the first years of infection in two children. They show that a potent Nab response is elicited very early after HIV-2 infection and that the HIV-2 envelope evolves at a high rate in the first years of infection. R5 to X4 tropism switch, nucleotide and amino acid diversity in V1 and V3 and convergence of V3 to a β-hairpin structure were related with rate of escape from Nab response, suggesting that Nabs have a major impact on the rapid molecular and phenotypic evolution of the viral envelope in acute and early stages in HIV-2 infection. Thus, the higher rate of evolution is consistent with a better immune control [138].

As mentioned before, IgG1, IgG3, and IgA responses are generated against HIV-2-Env. IgG1 is the predominant anti-Env antibody and IgG3 can also be found in significant concentrations [70]. In addition, it was also found that plasma IgA may have potent neutralizing activity against HIV-2 (in contrast to HIV-1 infection) [139].

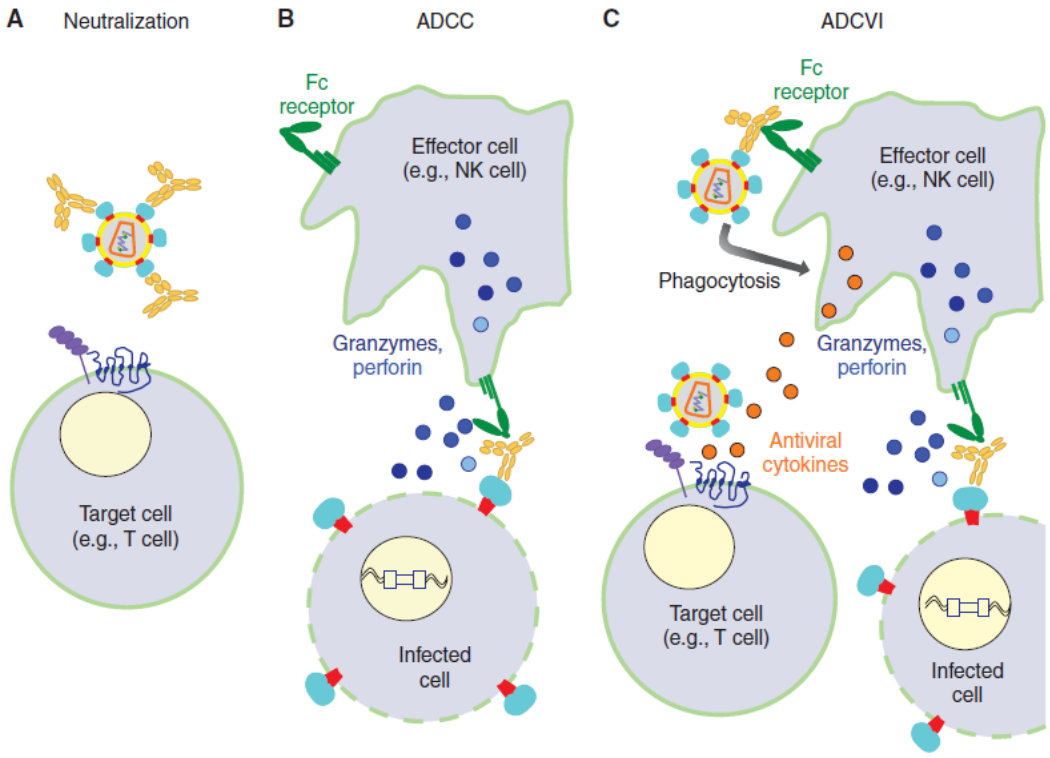


Figure 1.6: Schematic representation of the mechanism of action of neutralizing and non-neutralizing antibodies. A: Neutralizing antibodies bind to envelope glycoproteins of cell-free HIV. B: Antibody-dependent cellular cytotoxicity leads to the killing of infected cells. C: Antibody-dependent cell-mediated virus inhibition, lead to reduced virus production and virus inhibition by antiviral cytokines.
Image adapted from: Overbaugh, J. & Morris, L. The Antibody Response against HIV-1. Cold Spring Harb. Perspect. Med. 1, 1–17 (2012).

2 Aims and work plan

The general aim of this project was to provide a better description of the mechanism underlying HIV-2 chronic infection, namely virus persistence and virus-host interactions. To achieve this aim the following specific objectives were defined:

1. The establishment of an *in vitro* model of HIV-2 latency. The work plan to achieve this goalrequired: 1) production of a recombinant HIV-2 molecular clone that expresses the green fluorescent protein (GFP); and 2) the production of a latently infected cell line containing a full-length integrated HIV-2 genome. This cell line would be the first HIV-2 latency model allowing for the research of the mechanisms governing latency of HIV-2.
2. Characterization of the susceptibility of the HIV-2 envelope to neutralizing antibodies. The work plan to achieve this goal required:1) amplification of a panel of *env* sequences obtained from HIV-2 primary isolates; 2) production of Env-pseudotyped viruses; and 3) analysis of the susceptibility of Env-pseudotyped viruses to heterologous neutralizing antibodies. This work was expected to provide a more detailed picture of the interaction of the HIV-2 envelope with neutralizing antibodies.

3 Materials & Methods

3.1 Env Sequences

Chen et al, in 2016, produced and analysed 35 different *env* gene clones originated from 7 HIV-2 infected patients from Portugal [140]. Using phylogenetic analysis it was found that all clones belong to HIV-2 group A and all different sequences from each patient were 96 to 99% conserved (amino acid sequences). Between patients, the variation was around 25%. It was shown that 50% of the *env*-isolates tested antagonize BST-2/tetherin *(*i.e., have *Vpu-like* activity) at different levels. Also, in the same patient different *env* sequences were found with and without this activity [140]. Chen et al [140] also found no correlation between the ability of Env to infect cells and the ability of Env to antagonize BST-2/tetherin, except for P4-6 (one of the *env*-sequences used in this work) and P4-11, both with the highest Vpu-like activity and the highest Env-function of the panel [140]. All clones studied have Vpu-like activity and consequently greater virus release. However, all of them have this activity at different levels, with P1-5 being the clone with the lowest Vpu-like activity, i.e. the lowest virus release rate produced on HeLa cell line [140]. One of the clones with the highest rate of virus release, and consequently higher Vpu-like activity, is the clone P3-11, followed by P2-7 and P4-6 clones. It is also known that P1-5, P2-7 and P4-6 use the co-receptor CCR5 and P3-11 uses the co-receptor CXCR4 [140].

A total of 35 plasmids with different *env* genes were studied. These plasmids derive from pCM10 and have a hemagglutinin (HA) tag, as described [140] (Table A: – supplementary data). For the current study, the plasmids containing the *env* genes were obtained from Klaus Strebel in filter papel. Plasmids were extracted by immersion in sterile water, and transformed into One Shot™ TOP10 Chemically Competent E. coli (ThermoFisher). Cell clones were grown in Luria Broth with ampicillin and the plasmids were extracted by midiprep protocol (NZYMidiprep kit from NZYTech®).

3.2 Human Sera

For this study, 5 serum samples from patients from Cape Verde were used for heterologous neutralization assays. Each serum was heat-inactivated at 56^{o}C/30 minutes before neutralization. The characteristics of patients are summarized in the table 3.1.

Table 3.1: Characteristics of HIV-2 infected patients from Cape Verde and respective Serum ID.

Patient ID	Sampling date	Date of Birth	Time Since Infection (months)	Type of infection	CD4+ T cell count/ul	Clinical Stage CDC
HAN 2	30/06/2014	11/06/1964	21	HIV-2	656	1
HAN 4	07/07/2014	21/3/1956	33	HIV-1	608	1
HAN 20	29/07/2014	18/11/1960	3	HIV-2	617	1
HAN 37	25/08/2014	26/12/1974	2	HIV-2	405	2
HAN 43	22/09/2014	06/12/1967	10	HIV-2	121	3

CDC - Centers for Disease Control and Prevention (HIV infection stages based on age-specific CD4 T lymphocyte count - for 6 years through adult infected individuals: Stage 1- ≥500; Stage 2- 200-499; Stage 3- <200) [141]; w/o - without; na - non applicable; AZT - zidovudine; 3TC - lamivudine; LPV/R - lopinavir

3.3 Plasmids

The following plasmids were used: *pcDNA™3.1 V5-His-TOPO* (Invitrogen, to amplification of the CMV promoter); *pROD10* (plasmid expressing an infectious-molecular clone of HIV-2 strain ROD [142]); *pSG3.1 Δenv* (plasmid expressing an infectious molecular clone of HIV-1 strain 3.1 with the *env* gene deleted [143]) obtained from the NIH AIDS Reagent Program; RODenv8 (clone 8 of the original ROD-*env* sequence); *pSM119* (plasmid expressing HIV-1 *env* gene); *pVSV* (plasmid expressing the Vesicular Stomatitis Virus envelope gene).

3.4 Cells

For cloning procedures, the cells used were One Shot™ TOP10 Chemically Competent E. coli (ThermoFisher) and Stellar™ Competent Cells for Infusion Clontech® cloning.

For cell culture procedures, three types of cells were used: 293T cell line for Env-pseudotyped production; TZM-bl cell line for luciferase assay; Jurkat E 6-1 cell line for infection and flow cytometry analyse. All cells were obtained from the NIH AIDS Reagent Program.

3.5 Cell culture media

Luria-Bertani LB broth was used for cloning procedures in bacteria. Liquid LB contained 10g/L of Tryptone, 5g/L of Yeast Extract, 5g/L of NaCl; solid medium required the addition of 15g/L of Agar. The final volume of the solution was adjusted with H_2O and sterilized by autoclaving for 20 min at 121^{o}C.

Dulbecco Modified Eagle Medium (DMEM) (ThermoFisher) supplemented with 10% fetal bovine serum (FBS) (ThermoFisher) was used for the cell culture of 293T cell line and TZM-bl cells. the medium used was. Jurkat cells were cultured in Roswell Park Memorial Institute medium (RPMI) (ThermoFisher) supplemented with 10% FBS.

3.6 Production of HIV-2 ROD-GFP molecular clone

For the production of the recombinant HIV-2ROD molecular clone containing the Green Fluorescence Protein (HIV-2ROD-GFP) the *EGFP* sequence was first amplified by Polymerase Chain Reaction (PCR) from the plasmid *pLEGO-G* [144] and then cloned into the plasmid *pcDNA™3.1 V5-His-TOPO* as per the manufacturer instructions (Invitrogen). This generated the plasmid *pcDNA3.1-EGFP.* This plasmid was used to amplify the CMV promoter-EGFP cassette. Table 3.2 describes the set of primers designed to amplify the *EGFP gene*, the *CMV-EGFP* cassette and later the *Kozak-EGFP* cassette. Three of the four set of primers were also used to the cloning step using the InFusion Clontech® method, with the objective of improvement of the cloning success rate.

Table 3.2: Sequence primers and respective amplified sequences (from pcDNA3.1-EGFP) to subsequent InFusion Clontech® cloning.

Sequence Primers (Forward/Reverse)	Amplified gene or cassette (plasmid of origin)
5'-TACTAGCATTCGTATGGTGAGCAAGGGC-3' 5'-GATTGAATGCTTTACTTGTACAGCTCGTCCAT-3'.	EGFP (pLEGO-G)
5'-TATGGCGTACCCACGTGTATGGTGAGCAAGGGC-3' 5'-TGCATTTTTCCACGTGTTACTTGTACAGCTCG-3'	EGFP (pcDNA3.1-GFP)
5'-ATGGCGTACCCACGTGGACATTGATTATTGA-3' 5'-TGCATTTTTCCACGTGTTACTTGTACAGCTCG-3'	CMV-EGFP (pcDNA3.1-GFP)
5'-TATGGCGTACCCACGTGTGCCACCATGGTGAGCAAGGGC-3' 5'-TGCATTTTTCCACGTGTTACTTGTACAGCTCG-3'	Kozak-EGFP (pcDNA3.1-GFP)

The fragments were cloned with the InFusion Clontech® kit, as per the manufacturer instructions, in the proportion 2:1 (fragment:plasmid) into *pROD10*. The plasmid was previously digested with the BsaAI digestion enzyme in 10% CutSmart® Buffer, for 1 hour at 37°C (Neb England Bio Labs).

3.7 Virus Production

For the production of the viral particles with clones of HIV-derived vectors, 293T cells ($2.5x10^5$ cells/mL) were plated in 6 well plates with 2mL of supplemented DMEM. Twenty-four hours later the cells were transfected with 4µL jetPRIME® (Polyplus) reagent and 2µg of total DNA in a 3:1 proportion, respectively: *pROD10-EGFP* or *pROD10-CMV-EGFP* or *pSG3.1 Δenv* and a plasmid with an HIV-2 *env* gene or *pSM119* or *pVSV*. The medium was changed 4 hours later and the supernatants were harvested 48 hours later.

3.8 Tissue Culture Infective Dose 50% (TCID50) Assay

To determine the infectious titer of the viruses, the procedure performed was the TCID50 assay, which determines the amount of virus needed to infect 50% of the exposed cells. TZM-bl cells

(10^4 cells/mL) were plated in a 96 well plate in 100µL supplemented DMEM medium. Twenty-four hours later the cells were infected in serial dilutions in supplemented DMEM medium with DEAE-Dextran (26,4 µg/ml). The negative control – Mock was made with 200µL of supplemented DMEM medium, also with DEAE-Dextran. TZM-bl has luciferase gene (LUC) that are expressed with presence of TAT (HIV regulatory-protein) [145]. Luciferase is an enzyme that emits light when luciferin is converted to oxyluciferin [146]. The higher the luminesce value the better is the pseudovirus ability of infection. . Luciferase activity was quantified 48 hours later on a luminometer (TECAN) using the One-Glow Luciferase Assay System (ThermoFisher).

3.9 Fluorescence microscopy

The construction of the HIV-2ROD-GFP vectors was confirmed by fluorescence microscopy in 293T cells. 293T cells transfected with the vectors were trypsinised (Trypsin from ThermoFisher), 48 hours later, from the wells and fixated with 500µL of paraformaldehyde (PFA) for 15 minutes. Cells were transferred to microscope slide glass and observed under the fluorescence microscope.

3.10 Flow Cytometry

Jurkat E6.1 cells ($5x10^5$) were infected with the viruses produced from 293T cells transfected with pROD10-EGFP and pROD10-CMV-EGFP at a m.o.i. of 0.1, by spinoculation. This procedure enhances the infection rates. Jurkat E6-1 cells were subjected to spinoculation for 90 minutes at 500xg, at room temperature, in 12-well plate with 1 mL supplemented RPMI and 4µg/mL Polybrene. After 48 hours, prior to flow cytometry analysis, cells were fixed in PFA, as described above. A total of 5,000 to 10,000 live cell events were analysed [46,147,148] in guava® Flow Cytometry easyCyte™ Systems. Cell supernatants without virus were used as negative controls.

3.11 Neutralizing assay

The neutralizing activity of antibodies present in the each serum was analysed using a luciferase gene reporter assay in TZM-bl cells as described [149]. Briefly, TZM-bl cells (10^4 cells/mL) were plated in a 96 well plate in 100µL supplemented DMEM medium to adhere overnight. On a 96 round well plate, serial dilutions (dilution factor 2, starting in dilution 1:40) were made with each serum and supplemented DMEM medium with DEAE-Dextran (26,4 µg/ml). 100µl of virus supernatant (corresponding to 200 TCID50) were added to each well and the plate was incubated 1 hour at 37°C. All 200µl of serum and virus were transferred to the cells as well as only medium and cells control and medium and virus control. Luciferase activity was quantified 48 hours later on a luminometer (TECAN) using the One-Glow Luciferase Assay System (ThermoFisher).

3.12 Neutralization percentage calculation

The neutralizing activity represents the percentage of infection inhibition of a virus relative to each serum dilution. To calculate the neutralizing activity, the background luminescence value (cells

and medium) was removed from each infection read (luciferase assay results to each pseudovirus). The neutralization percentage of each serum relative to each pseudovirus (inhibition percentage) was calculated by:

Equation 1: Equation for the calculation of inhibition/neutralization percentage. Antibody positive infection value – value of infection of each virus with each serum dilution; Antibody negative infection value – value of infection of each virus in only medium (Adapted from [64]).

$$neutralizing\ \% = \left(1 - \frac{antibody\ positive\ infection\ value}{antibody\ negative\ infection\ value}\right) \times 100$$

3.13 Calculation of the serum dilution that inhibited virus infection by 50% (ID50)

ID50 was estimated by the sigmoidal dose–response (variable slope) equation in Prism version 5.0. Nonspecific inhibition was assessed by testing all HIV-2 isolates against HIV-negative plasma and all plasma samples against pSG3Δenv pseudotyped with pSM119 envelope and pseudotyped with VSV envelope [138].

4 Results & Discussion

4.1 *In vitro* model of HIV-2 latency

4.1.1 Production of a recombinant HIV-2 molecular clone that expresses the green fluorescent protein

The first step to establish the HIV-2 latent cell line was the production of a recombinant HIV-2 molecular clone expressing the enhanced green fluorescent protein (GFP) under the control of the HIV promoter in the 5' long terminal repeat (LTR) (Figure 4.1 A) [46]. In parallel, a recombinant HIV-2 molecular clone was made with GFP under the control of the Human cytomegalovirus (CMV) promoter (Figure 4.1 B). This construct was used as a positive control for the cell line construction because in this construction GFP is constitutively expressed.

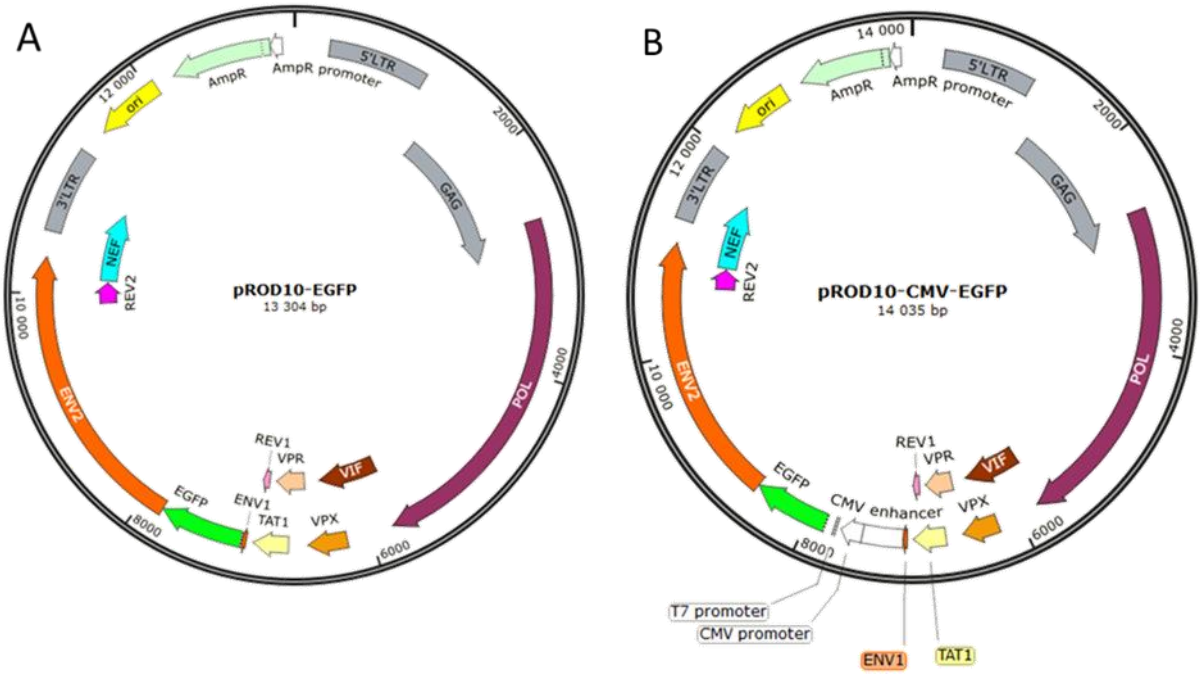


Figure 4.1: Map of the recombinant molecular clones produced in this work. A: Plasmid map for *pROD10-EGFP*; B: Plasmid map for *pROD10-CMV-EGFP*

Upon digestion of the *pROD10* plasmid, the EGFP sequence was cloned into the open reading frame of the *env* gene of the HIV-2 ROD (Figure 4.2 A). This construction was intended to disrupt the viral Env and ensure the expression of GFP when HIV-2 is integrated into the genome and is expressed. The purpose of an *env*-defective molecular clone was to restrict the analysis to a single

infection cycle. The same was made with the CMV-EGFP cassette (Figure 4.2 B). Two positive clones of each construction were chosen randomly (pROD10-EGFP clones 5 and 15; pROD10-CMV-EGFP clones 18 and 44). The plasmid constructions were confirmed by enzymatic digestions with BsaAI enzyme.

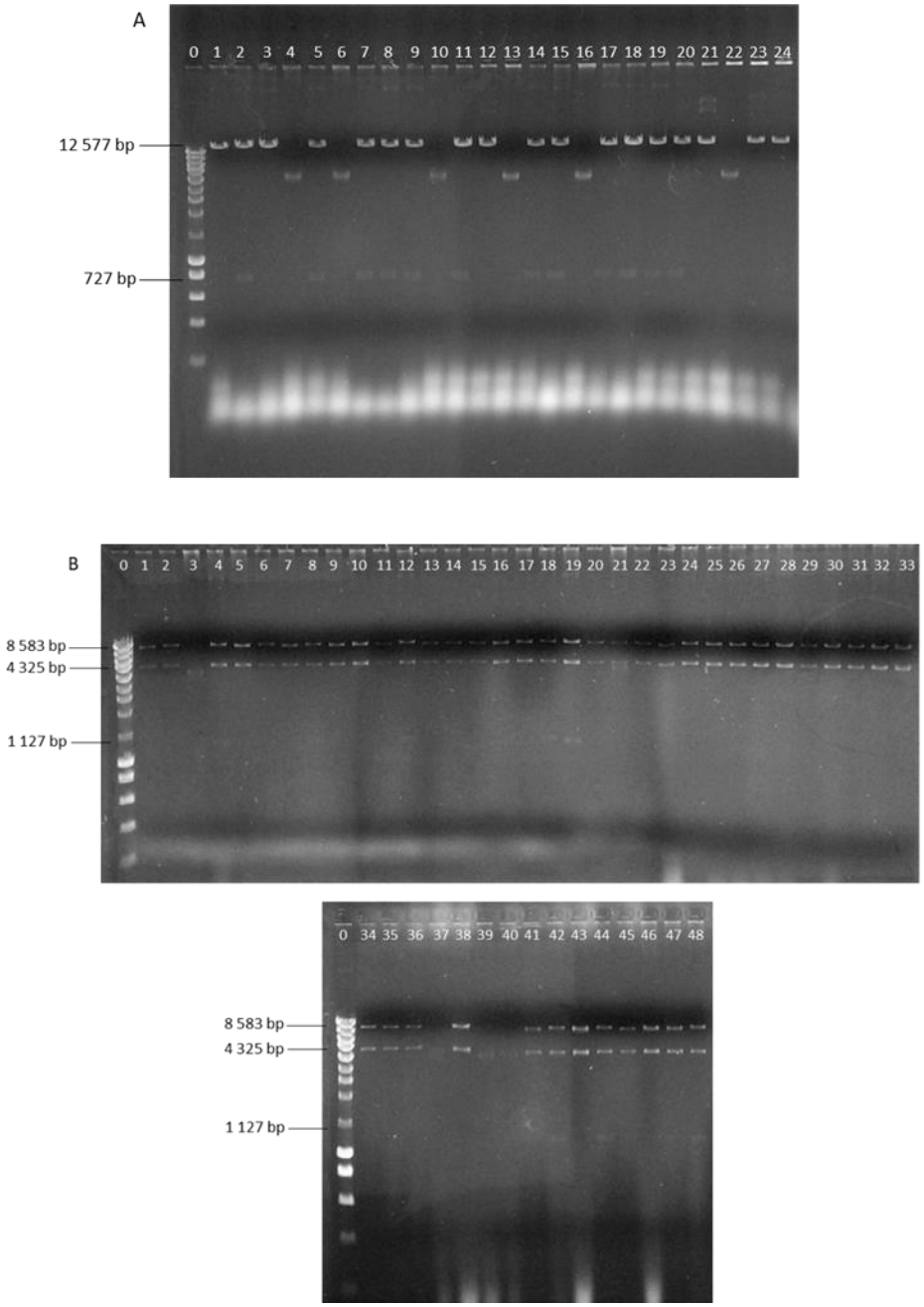


Figure 4.2: Electrophoretic agarose gel with DNA of plasmid constructions. A: Electrophoretic agarose gel of DNA extraction and digestion with BsaAI enzyme from 24 colonies that result of the cloning of pROD10 plasmid with GFP. 0 – NZYDNA Ladder III ; 1, 3-4, 6, 12-13, 16, 21-24 – Negative colonies ; 2, 5, 7-9, 11, 14-15, 17-20 – Positive colonies. The chosen clones were the 5 and 15. B: Electrophoretic agarose gel of DNA extraction and digestion with HindIII enzyme from 48 colonies that result of the cloning of pROD10 plasmid with CMV-GFP sequence. 0 – NZYDNA Ladder III ; 1-3, 5-11, 13-17, 20-41, 43, 46 – Negative colonies ; 4, 12, 18-19, 42, 44-45, 47-48 – Positive colonies. The chosen clones were the 18 and 44.

4.1.2 Virus production from the recombinant molecular clones

The chosen Env-defective molecular clones (pROD10-EGFP clones 5 and 15; pROD10-CMV-EGFP clones 18 and 44) were used to produce Env-pseudotype viruses in 293T cells. Since they were *env* defective, it was necessary to perform a co-transfection with a second plasmid expressing the *env gene*. Two *env* sequences were tested, one was the clone 8 of the original ROD-*env* sequence (RODenv8) and the other was an *env* sequence isolated from infected patient. This *env* sequence was chosen due to its high infectivity in previous works [140] (More information below).

To confirm the production of viruses, the transfected 293T cells were analysed under the fluorescent microscope after cell fixation. In the Figure 4.3 A and B it is represented the fluorescent cells infected with pROD10-EGFP clone 15 and pROD10-CMV-EGFP clone 44, respectively. All four clones were fluorescent confirming the correct expression of the *GFP*.

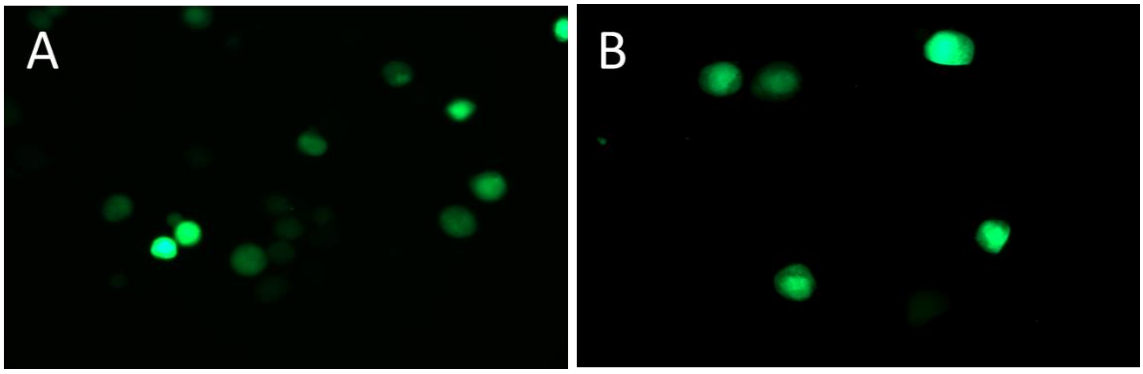


Figure 4.3: Fluorescent microscopy image of cells expressing EGFP. A: Image from fluorescence microscope of 293T cells transfected with the clone pROD10-EGFP 15. B: Image from fluorescence microscope of 293T cells transfected with the clone pROD10-CMV-EGFP 44.

The production of infectious Env-pseudotyped viruses was tested by adding 100 microliters of cell supernatant to TZM-bl cells and measuring the *de novo* production of the Tat protein with luciferase assay. The results are expressed in Table 4.1 as luminescence values. The best clone chosen to continue the work was pROD10-EGFP 15. As a positive control clone pROD10-CMV-EGFP 44 was chosen. These experiments indicated that the best Env expressing plasmid was HCC6-2-7HA.

Table 4.1: Luciferase assay results from TZM-bl cells infected with the infectious Env-pseudotyped viruses.

Conditions	Luminescence (Relative Light Units)			Mean
Mock 1 [a] (Negative Control)	6205	5765	4373	5448
pROD10-CMV-EGFP 18 | RODenv8	22625	23902	33791	26773
pROD10-CMV-EGFP 44 | RODenv8	47773	28956	35494	37408
Mock 2 [a] (Negative Control)	5391	5063	4977	5144
pROD10-CMV-EGFP 44 | HCC6-2-7HA	174189	187647	200982	187606
Mock 3 [a] (Negative Control)	4065	3562	4729	4119
pROD10-EGFP 5 | RODenv 8	8650	11232	13053	10978
pROD10-EGFP 5 | HCC6-2-7HA	59886	140994	352767	184549
pROD10-EGFP 15 | RODenv 8	5966	12240	19099	12435
pROD10-EGFP 15 | HCC6-2-7HA	398087	630556	799121	609255

The conditions was represented with both co-transfected plasmids (recombinant pROD10 | Env plasmid).
[a] Mock 1, 2 and 3 are negative control for each test.

4.1.3 Infection of Jurkat cells

Jurkat cells E6.1 were infected with pROD10-EGFP clone 15 pseudovirus and analysed by Flow cytometry towards the optimization of the infection and subsequent production of the latent cell line. The rational for this assay is the following: cells efficiently infected, with HIV-DNA integrated into the cell genome and actively replicating, would produce GFP and consequently be fluorescence when excited; cells efficiently infected, but under latency, would have the provirus but the replication would not be active, thus GFP would not be expressed; cells not efficiently infected would also be GFP negative. A schematically representation of the rational is represented in Figure A in Supplementary data. The objective would be to select GFP negative cells by fluorescence activated cell sorting for a subsequent cell culture assay based on clonal expansion, that would allow to separate the latently infected cells from uninfected cells or from the infected but not productive cells, upon exposure to viral reactivation factors [46]. These steps are schematically represented in the Figure 4.4.

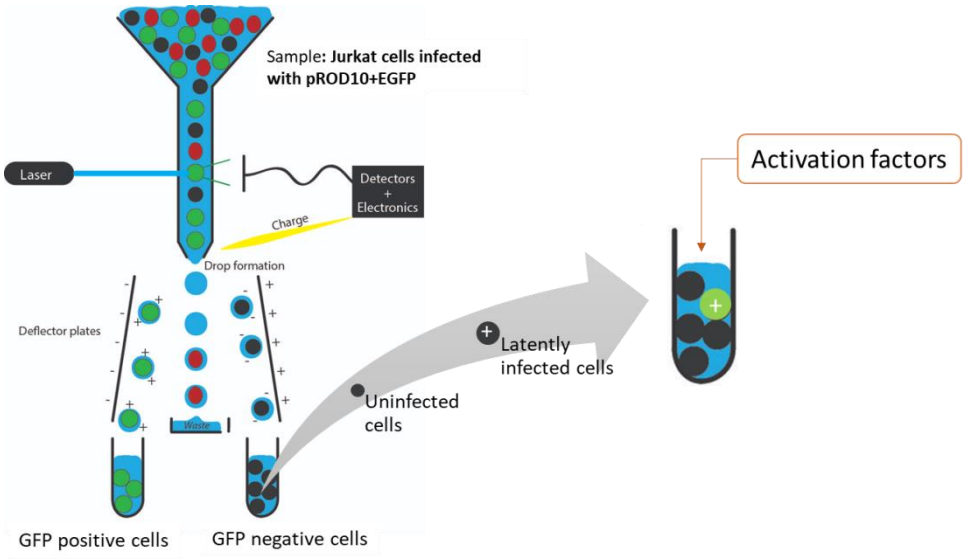


Figure 4.4: Schematic representation of fluorescence activated cell sorting and subsequent exposure of GFP negative cells to viral reactivation factors. Image adapted from http://www.unifr.ch/pathology/en/facs.

Jurkat cells infected with pROD10-EGFP and the positive control (pROD10-CMV-EGFP) showed fluorescence at the mock level, *ie* only background fluorescence, being considered GFP negative. According to the literature, the events leading to latency have low frequency (1% of the cells) [46], therefore cells were considered not infected. The results were not statistically analysed since they are negative (raw data in figure A, B and C in supplementary data). Two hypotheses of explanation were placed: 1) inefficient infection or 2) problems with the integrity of pseudoviruses or even problems with the integrity of the plasmids. The infection was repeated but the same negative outcome was achieved.

Therefore, to confirm the integrity of plasmids and pseudovirus, the plasmid pROD10-EGFP was transfected in 293T cells without *env*-gene-plasmid and the cell supernatants were collected. The 293T cells transfected were fixed and analysed under the fluorescent microscope to confirm the production of GFP. The supernatants were also used to infect TZM-BL cells and the infections were analysed by Luciferase Assay (as previously described). Since the GFP sequence disrupted the *env* gene, it was expected that the supernatants would not be infectious. However, values of luminescence were more than 10 times the values from wells with only cells and medium (mock), being considered that the transfection produced viral particles, contrary to the expected. The results are expressed in Table 4.2 as luminescence values. In addition, the results from fluorescence microscopy were negative to GFP production. Globally, these results suggest that the recombinant molecular clone is unstable and not efficient for the subsequent assays intended to obtain the HIV-2 latent cell line.

Table 4.2: Luciferase assay results of pROD10-EGFP transfection in 293T cells.

Conditions	Luminescence (Relative Light Units)			Mean
Mock [a] (Negative Control)	2981	2814	3346	3047
pROD10-EGFP	75048	39409	195710	103389

[a] Mock is negative control of infection

4.2 Study of HIV-2-*env* characteristics

4.2.1 Virus production

For the study of HIV-2-*env* characteristics, namely its susceptibility to neutralizing antibody response, it was made, first, amplification of a panel of env sequences obtained from HIV-2 primary isolates and production of Env-pseudotyped viruses.

Firstly, 293T cells were co-transfected with the recombinant clone pROD10-CMV-EGFP and all patient *env* clones to form pseudoviruses. All Env-pseudotyped viruses were tested for their ability to infect cells with the Luciferase assay in TZM-BL cell line. However, the problem with the plasmid (as reported above) leads to uncertainty about the integrity of pseudoviruses formed, due to the possibility of both patient-Env and ROD10-Env are present.

Thus, as alternative strategy, Env-pseudotyped viruses were produced by co-transfection of 293T cells, but now with a well-known HIV-1 backbone pSG3.1 Δenv and eight patient *env* clones chosen because were positive for the infection screening made by Chen et al [140]. The supernatants were then used to infect TZM-BL cells in single-round infectivity assay. This way, four plasmids from different patients (highlighted in orange in table 4.3) were chosen due to their high level of infection in TZM-bl cells.

Table 4.3: Luciferase assay results for evaluation of infection capacity of pseudovirus produced with each *env*-gene plasmid.

Conditions	Luminescence (Relative Light Units)		Mean
Mock 1 [a] (Negative Control)	2371	2928	2650
HCC1-1-5HA (P1-5)	142443	136924	139684
HCC6-2-7HA (P2-7)	735535	796633	766084
HCC10-3-11HA (P3-11)	197533	164780	181157
HCC19-4-6HA (P4-6)	41036	43757	42397
Mock 2 [a] (Negative Control)	2600	2285	2443
HCC6-2-3HA (P2-3)	6958	7059	7009
HCC6-2-6HA (P2-6)	1988	2064	2026
HCC19-4-1HA (P4-1)	8265	8438	8352
HSMNC8-2HA (P8-2)	3951	4332	4142

[a] Mock is negative control (cells and medium) for each test

4.2.2 Neutralization assays

To assess the susceptibility of these Envs to heterologous neutralization envelope pseudoviruses were tested against the sera from five HIV patients from Cape Verde, a country at the center of HIV-2 epidemic, where HIV-1 and HIV-2 co-circulate [150,151]. Four sera from patients infected with HIV-2 and one patient misdiagnosed with HIV-2, but who was actually infected with HIV-1 (Serum 4) were used in the neutralization assays, performed in TZM-bl single-round infectivity assay [64,152]. The susceptibility of HIV-2 to neutralizing antibodies has been associated with the rate and pattern of molecular and phenotypic evolution of HIV-2-*env* [138]. An *env* with a lower molecular and phenotypic evolution and origin from an R5 tropic virus is commonly more susceptible to neutralization [138].

Specificity controls for these experiments were pseudovirus with the Vesicular Stomatitis Virus (VSV) envelope and HIV-1. Both virus controls were tested with the serum at the lowest dilution (1:40), and were not neutralized, demonstrating the specificity of the process (Table B- Supplementary data).

Pseudoviruses formed with Env clones P1-5, P2-7 and P4-6, were potently neutralized by all but, serum 4 (HIV-1 infected patient) (Figure 4.5 and Table 4.4).

Table 4.4: ID50 calculated from neutralizing assays

	ID50				
	Serum 2	Serum 4	Serum 20	Serum 37	Serum 43
HCC1-1-5HA (P1-5)	263018	nn	302786	899909	23246
HCC6-2-7HA (P2-7)	2764925	nn	1228465	3899423	183437
HCC10-3-11HA (P3-11)	nn	nn	nn	nn	nn
HCC19-4-6HA (P4-6)	65307	nn	28483	164715	5163

nn - non neutralized

P1-5 pseudovirus was the most susceptible to antibody neutralization. A dilution of 1:2.048x 10^4 of serum samples 2, 20 and 37 neutralized 95%, 99% and 100%, respectively, of P1-5 replication. For serum 43, a dilution of 1:5.12x10^3 neutralized 82% of P1-5 (Figure 4.5 and Figure E – Supplementary data). The ID50 calculated was 1:2.63018x10^5 for serum 2, 1:3.02786x10^6 for serum 20, 1:8.99909x10^5 for serum 37 and 1:2.3246x10^4 for serum 43. P1-5 viruses, a R5 tropic virus [140] is more susceptible to neutralization then the results are consistent with Chen hypothesis that the ability to antagonize BST-2 is a more recent function of HIV-2 [140], as this clone would correspond to a less evolved *env* since: 1) it had origin in a patient with a more recent diagnosis year (Table 4.5) [140]; 2) has a lower Vpu-like activity [140] and 3) a high susceptibility to heterologous neutralization. In addition, this patient has 50% of his *env* clones without Vpu-like activity, and those with this feature with very low Vpu-like activity [140].

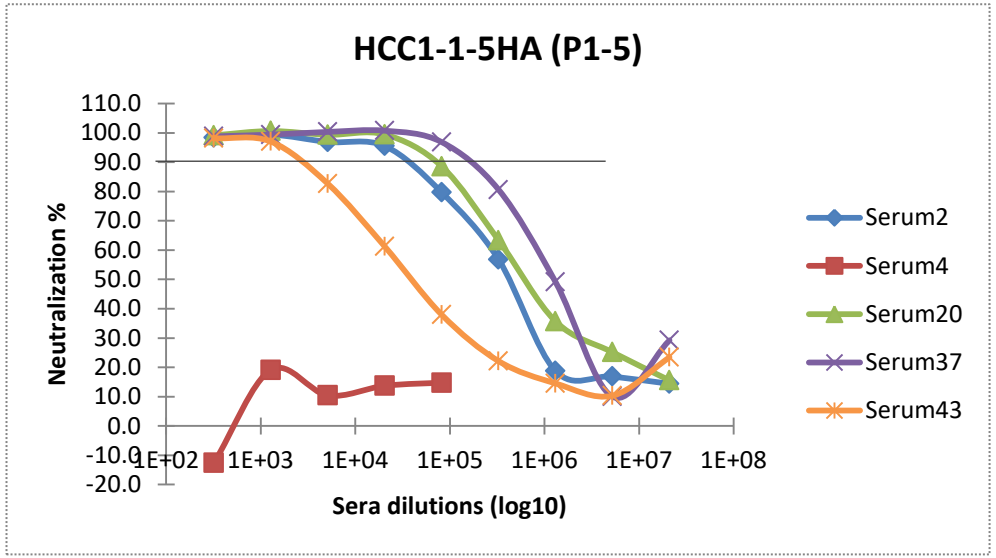
Table 4.5: Summarized characteristics of HIV-2 infected patients and respective *env* clones selected. Adapted from Chen, C.-Y. et al. Antagonism of BST-2/Tetherin Is a Conserved Function of the Env Glycoprotein of Primary HIV-2 Isolates. J. Virol. 90, 11062–11074 (2016).

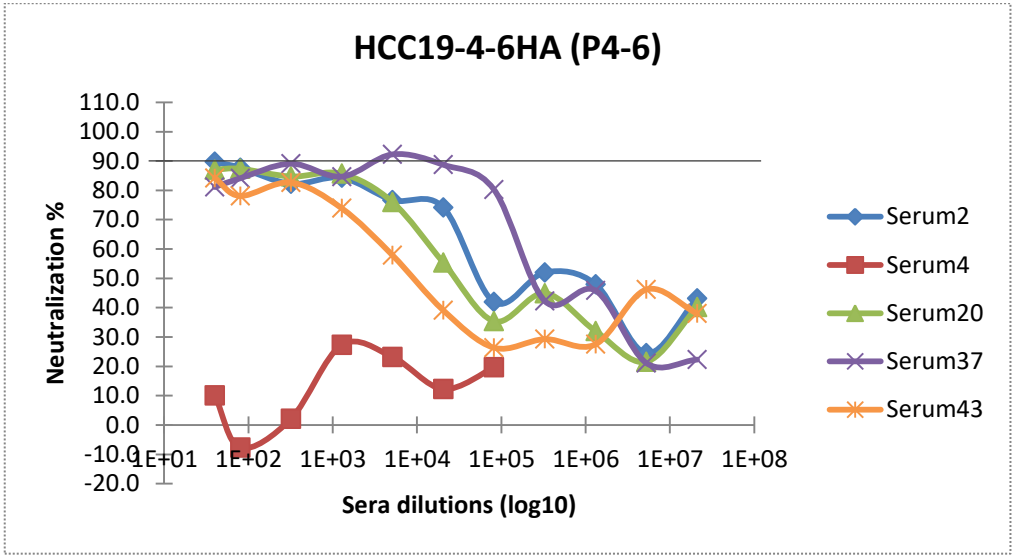
Patient	Gender	Diagnosis	HIV RNA copies/mL	CD4+ T cell count/ul	Sample collection
P1 (HCC1)	F	2001	<200	308	2003
P2 (HCC6)	F	1992	<200	615	2003
P3 (HCC10)	M	1996	160559	48	2003
P4 (HCC19)	F	2003	<200	175	2003

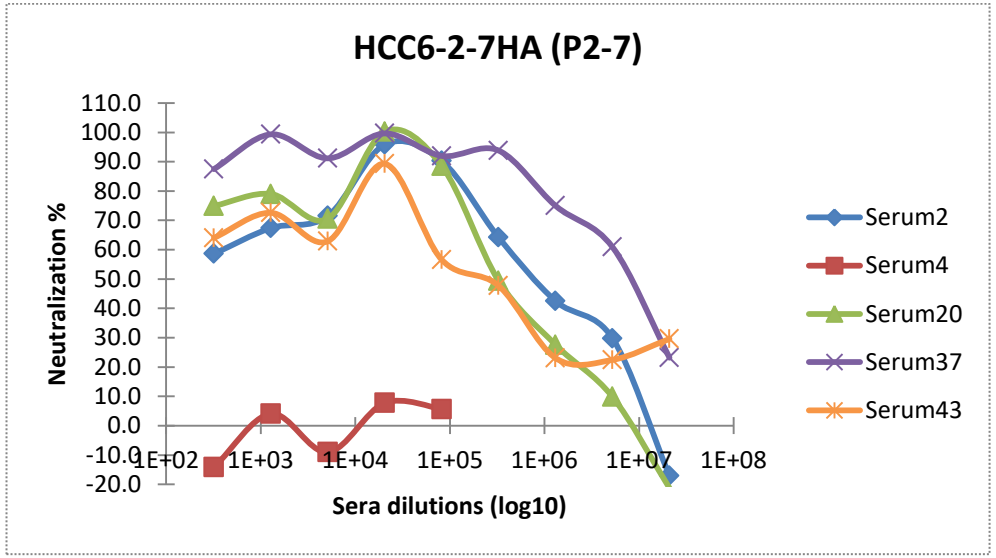
The P4-6 showed to be less susceptible to neutralization than P1-5, since at the lowest dilution (1:40) none of the five HIV-2 sera achieved more than 89% of neutralization (Figure 4.5 and Figure E – Supplementary data). In addition, the ID50 calculated corroborate this affirmation (Table 4.4). It seems to be a more evolved HIV-2-*env* than P1-5, due to the highest Vpu-like activity of *env* clones of patient 4 (from 50% Vpu-like activity positive clones) [140] as well as due to the lower susceptibility to neutralization observed in these neutralizing assays. Moreover, patient 4' diagnosis was later than patient 1 and his CD4+ T cell count was lower [140] (Table 4.5), suggesting that he was infected before that patient 1. Potent neutralizing antibodies were associated with low CD4+ T cell count [153] and consequently to the virus evolution to escape [133].

The neutralization results for the P2-7 clone were the most difficult to analyse, taking into account the variation of neutralization percentage even at higher dilutions (Figure 4.5). ID50 was calculated for that four sera but they are inconclusive (Table 4.4). Nonetheless, it is possible to infer that the viruses produced with this *env* are susceptible to neutralization by the four sera from patients infected with HIV-2. However, it would be essential to repeat the screening of sera dilutions in order to assess the potency of neutralization and enable comparison.

The only clone with X4 tropism (P3-11) showed resistance to neutralization (Figure 4.5). The viral shift of R5 tropism to X4 tropism is common with the ongoing of infection, X4-tropic viruses are usually associated with low CD4+ T cells counts as was the case of this patient (Table 4.5) [64,138,154]. Thus, patient 3, who is the patient with the lowest CD4+ T cell count and diagnosed seven years before the time of sampling [140] (Table 4.5), is at an advanced stage of infection where virus neutralization resistance (X4 tropism) is common [64,138]. It is expected that this clone would be more resistant to neutralization than the other *env*-clones analysed. Additionally five of the seven *env* clones of this patient have Vpu-like activity and this activity is high, consistent with disease evolution [140].







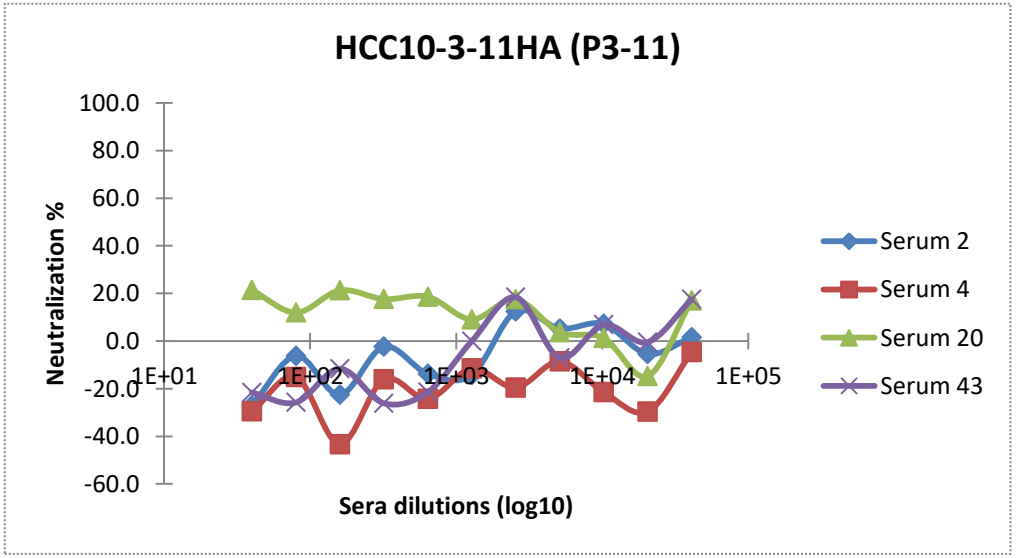


Figure 4.5: Relationship between HIV sera dilutions and neutralization percentage of the four HIV-2-*env* clones (P1-5, P2-7, P3-11 and P4-6) analysed. HCC1-1-5HA (P1-5): the most susceptible to neutralization; highlighted correlation of sera dilutions to 90% and 10% of neutralization. HCC19-4-6HA (P4-6): highlighted the correlation of sera dilutions to 90% (all sera above) and 10% of neutralization. HCC6-2-7HA (P2-7): viruses produced with this *env* are susceptible to neutralization by the four sera from patients infected with HIV-2. HCC10-3-11HA (P3-11): no significant results were obtained due to the poor reproducibility of infection/neutralization results.

5 Final remarks

Even though the HIV-2 latent cell line results suggest that the recombinant molecular clone (pROD10-EGFP) is unstable and is not efficient for the subsequent assays intended, several hypotheses were considered for production of the recombinant clone. The first one and already in progress was the production of a recombinant HIV-2 molecular clone, this time with the Kozak consensus sequence immediately before the EGFP sequence, since Kozak sequence acts as a enhancer for translation [155] and may improve the production of the fluorescent protein. The presence of this sequence already proved efficiency in the case of HIV-1 with the plasmid pNL4.3 Δenv EGFP [156]. Other difference in this recombinant clone is the inactivation of the restriction sites after cloning using the InFusion protocol, in order to prevent re-ligation of the plasmid without the insert (Figure 4.4).

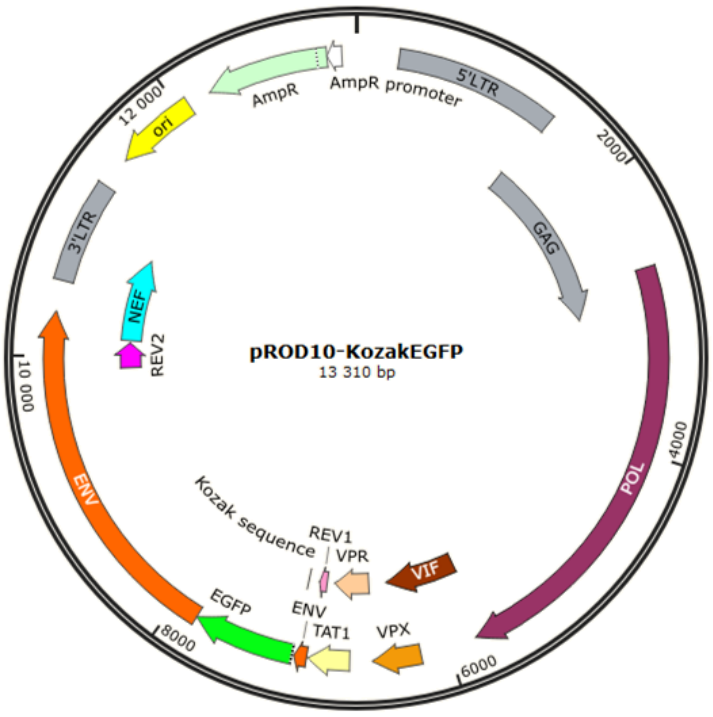


Figure 5.1: Plasmid map for pROD10-KOZAK-EGFP

Other hypotheses could be: 1) the choice of other enzyme(s) and hence another GFP sequence insertion site, however maintaining the inactivation of the *env* gene; 2) the inactivation of the *env* gene by random mutagenesis (by PCR for example) and posterior insertion of the EGFP sequence in a site that does not affect the remaining HIV-2 genes or 3) the replacement of the *env* gene by the EGFP gene by enzymatic digestion and cloning (as described in [51]) A problem common to all hypotheses is that the sites currently available for enzyme restriction are not ideal for the purposes.

All existing restriction sites would have to be re-evaluated and probably produced new sites adapted to the purposes. For example, the creation of restriction sites to remove the *env* sequence not affecting the remaining HIV-2 genes.

Regarding the second goal of this project, these preliminary results showed to be consistent with previous evidence concerning the relationship of Vpu-like activity and the evolution of *env*. They emphasize the correlation between evolutionary and functional features of the HIV-2 Env, namely the genetic sequence, cell tropism, susceptibility to antiviral cell restriction factors and susceptibility to humoral neutralization. These results justify further study reaffirming this correlation. Firstly, an increase in the number of samples from *env* clones and sera from HIV-2 infected patients and purification of sera to safeguard the interference of drugs (ART) would be required, to ensure viable comparative analyses of heterologous neutralization responses of the different *env* clones intra- and inter-individuals. As well as a relationship with the Vpu-like activity information of each one.

It would also be interesting explore the amino acid sequence-differences of the binding sites of BST-2/tetherin to HIV-2-Env for a better understanding between mutations leading to the presence or absence of BST-2 antagonism and the neutralizing antibody response. The *Vpu-like* activity is sensible to single-amino-acid changes such as ROD10/ROD14 naturally occurring T598A mutation [157]. As well as the T568I mutation between two env sequences of the patient 4 (P4-7 and P4-11) [140]. Both mutations involved in a conformational change of the gp36 to allow a decrease of affinity between BST-2/tetherin and the env glycoprotein [140]. However, binding is essential but is not enough to Vpu-like activity [140,158]. In HIV-1 specific mutations in Vpu increase the susceptibility of the virus to immune humoral responses [159]. Notably, in infection by lymphocytic choriomeningitis virus (LCMV), BST-2 is important in shaping the anatomical distribution and adaptive immune response against persistent viral infection *in vivo* [160], it would be interesting to study this effect of BST-2 on HIV-2 infection, considering the imunopathogenesis and disease progression of this infection.

6 References

1. Curran, J. W. Pneumocystis Pneumonia - Los Angeles. *Morb. Mortal. Wkly. Rep.* **45,** 729–733 (1996).

2. Sharp, P. M. *et al.* Origins of HIV and the AIDS Pandemic. *Cold Spring Harb. Perspect. Med.* 1–22 (2011). doi:10.1101/cshperspect.a006841

3. Freed, E. & Martin, M. *Fields Virology*. *Fields Virology* (Wolters Kluwer, 2013). doi:9781451105636

4. Gallo, R. C. *et al.* Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science (80-.).* **224,** 500–503 (1984).

5. Barré-Sinoussi, F. *et al.* Isolation of a T-Lymphotropic Retrovirus from a Patient at Risk for Acquired ImmuneDeficiency Syndrome (AIDS). *Science (80-.).* **220,** 868–871 (1983).

6. Clavel, F. *et al.* Isolation of a New Human Retrovirus African Patients with AIDS from West. **248,** (1986).

7. Clavel, F. *et al.* Human immunodeficiency virus type 2 infection associated with AIDS in West Africa. *N. Engl. J. Med.* **316,** 1180–1185 (1987).

8. Seitz, R. Human Immunodeficiency Virus (HIV). *Transfus. Med. Hemotherapy* **43,** 203–222 (2016).

9. Gao, F. *et al.* Human infection by genetically diverse SIVsm-related HIV-2 in West Africa. *Nature* **356,** 133–135 (1992).

10. Lemey, P. *et al.* Tracing the origin and history of the HIV-2 epidemic. *Proc. Natl. Acad. Sci.* **100,** 6588–6592 (2003).

11. Visseaux, B., Damond, F., Matheron, S., Descamps, D. & Charpentier, C. Hiv-2 molecular epidemiology. *Infect. Genet. Evol.* **46,** 233–240 (2016).

12. Feng Gao, Elizabeth Bailes, D. L. R. Origin of HIV-1in the chimpanzee Pan troglodytes troglodytes. *Nature* **397,** 1178–1183 (1999).

13. Korber, B. Timing the Ancestor of the HIV-1 Pandemic Strains. *Science (80-.).* **288,** 1789–1796 (2000).

14. Plantier, J.-C. *et al.* A new human immunodeficiency virus derived from gorillas. *Nat. Med.* **15,** 871–872 (2009).

15. Taveira, N. & Martin, F. in *Manual sobre SIDA* (eds. Antunes, F. & Maltez, F.) 1–11 (Permanyer Portugal, 2018).

16. Santoro, M. M. & Perno, C. F. HIV-1 Genetic Variability and Clinical Implications. *ISRN Microbiol.* **2013,** 481314 (2013).

17. Campbell-Yesufu, O. T. & Gandhi, R. T. Update on human immunodeficiency virus (HIV)-2 infection. *Clin. Infect. Dis.* **52,** 780–787 (2011).

18. UNAIDS. Trend of new HIV infections. (2017). Available at: http://aidsinfo.unaids.org/. (Accessed: 20th April 2018)

19. UNAIDS. Global HIV & AIDS statistics — 2018 fact sheet. (2018). Available at: http://www.unaids.org/en/resources/fact-sheet. (Accessed: 20th April 2018)

20. Gottlieb, G. S., Raugi, D. N. & Smith, R. A. 90-90-90 for HIV-2? Ending the HIV-2 epidemic by enhancing care and clinical management of patients infected with HIV-2. *Lancet HIV* **5,** e390–e399 (2018).

21. Martins, H. C. *Infeção VIH e SIDA: a situação em Portugal a 31 de dezembro de 2016*. (2017).

22. National Institute of Allergy and Infectious Diseases. New HIV Infections Drop 18 Percent in Six Years. (2017). Available at: https://www.hiv.gov/blog/new-hiv-infections-drop-18-percent-in-six-years. (Accessed: 20th April 2018)

23. Direcção-Geral da Saúde Lisboa - Ministério da Saúde. *Programa Nacional para a infeção VIH/SIDA e Tuberculose*. (2016).

24. UNAIDS. The Global HIV/AIDS Epidemic. (2017). Available at: https://www.hiv.gov/hiv-basics/overview/data-and-trends/global-statistics. (Accessed: 20th April 2018)

25. ICTV. International Committee on Taxonomy of Viruses (ICTV). (2002). Available at: https://talk.ictvonline.org/taxonomy/p/taxonomy-history?taxnode_id=20164256. (Accessed: 2nd January 2018)

26. Taveira, N. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–6 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_35-1

27. Taveira, N., Borrego, P. & Bártolo, I. in *Manual sobre SIDA* (ed. Antunes, F.) 31–54 (Permanyer Portugal, 2011).

28. Leitner, T. *et al. HIV Sequence Compendium 2018*. (Theoretical Biology and Biophysics Group, 2018).

29. Vicenzi, E. & Poli, G. Novel factors interfering with human immunodeficiency virus-type 1 replication in vivo and in vitro. *Tissue Antigens* **81,** 61–71 (2013).

30. Ueno, F. *et al.* Vpx and Vpr proteins of HIV-2 up-regulate the viral infectivity by a distinct mechanism in lymphocytic cells. *Microbes Infect.* **5,** 387–395 (2003).

31. Kirchhoff, F. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–9 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_60-1

32. Lever, A. M. L. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–11 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_43-1

33. Strebel, K. HIV Accessory Proteins versus Host Restriction Factors. *Curr. Opin. Virol.* **3,** 692–699 (2013).

34. U.S. Department of Health and Human Services. The HIV Life Cycle. (2017). Available at: https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/19/73/the-hiv-life-cycle. (Accessed: 2nd January 2018)

35. Karsten, C. & Pöhlmann, S. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–10 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_61-1

36. Azevedo-Pereira, J. M. in *Manual sobre SIDA* (ed. Antunes, F.) 13–30 (Permanyer Portugal, 2011).

37. Shah, V. B. & Aiken, C. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–9 (Springer New York, 2014). doi:10.1007/978-1-4614-9610-6_67-1

38. Chavez, L., Calvanese, V. & Verdin, E. HIV Latency Is Established Directly and Early in Both Resting and Activated Primary CD4 T Cells. *PLoS Pathog.* **11,** 1–21 (2015).

39. Massanella, M. & Richman, D. D. Measuring the latent reservoir in vivo. *J. Clin. Invest.* **126,** 464–472 (2016).

40. Saleh, S., Vranckx, L., Gijsbers, R., Christ, F. & Debyser, Z. Insight into HIV-2 latency may

disclose strategies for a cure for HIV-1 infection. *J. virus Erad.* **3,** 7–14 (2017).

41. Siliciano, J. D. & Siliciano, R. F. A long-term latent reservoir for HIV-1: Discovery and clinical implications. *J. Antimicrob. Chemother.* **54,** 6–9 (2004).

42. Cavaleiro, R., Sousa, A. E., Loureiro, A. & Victorino, R. M. M. Marked immunosuppressive effects of the HIV-2 envelope protein in spite of the lower HIV-2 pathogenicity. *Aids* **14,** 2679–2686 (2000).

43. MacNeil, A. *et al.* Direct Evidence of Lower Viral Replication Rates In Vivo in Human Immunodeficiency Virus Type 2 (HIV-2) Infection than in HIV-1 Infection. *J. Virol.* **81,** 5325–5330 (2007).

44. Schröder, A. R. W. *et al.* HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110,** 521–529 (2002).

45. Jordan, A., Defechereux, P. & Verdin, E. The site of HIV-1 integration in the human genome determines basal transcriptional activity and response to Tat transactivation. *EMBO J.* **20,** 1726–1738 (2001).

46. Jordan, A., Bisgrove, D. & Verdin, E. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO J.* **22,** 1868–1877 (2003).

47. Lewinski, M. K. *et al.* Genome-Wide Analysis of Chromosomal Features Repressing Human Immunodeficiency Virus Transcription Genome-Wide Analysis of Chromosomal Features Repressing Human Immunodeficiency Virus Transcription †. *J. Virol.* **79,** 6610–6619 (2005).

48. MacNeil, A. *et al.* Genomic Sites of Human Immunodeficiency Virus Type 2 (HIV-2) Integration: Similarities to HIV-1 In Vitro and Possible Differences In Vivo. *J. Virol.* **80,** 7316–7321 (2006).

49. Popper, S. J. *et al.* Low plasma human immunodeficiency virus type 2 viral load is independent of proviral load: low virus production in vivo. *J Virol* **74,** 1554–1557 (2000).

50. Soto-Rifo, R. *et al.* Different effects of the TAR structure on HIV-1 and HIV-2 genomic RNA translation. *Nucleic Acids Res.* **40,** 2653–2667 (2012).

51. Pierson, T. C. *et al.* Molecular Characterization of Preintegration Latency in Human Immunodeficiency Virus Type 1 Infection. *J. Virol.* **76,** 8518–8531 (2002).

52. Zhou, Y., Zhang, H., Siliciano, J. D. & Siliciano, R. F. Kinetics of Human Immunode ciency Virus Type 1 Decay following Entry into Resting CD4. *J. Virol.* **79,** 2199–2210 (2005).

53. Zamborlini, A. *et al.* Centrosomal pre-integration latency of HIV-1 in quiescent cells. *Retrovirology* **4,** 1–7 (2007).

54. Kelly, J. *et al.* Human Macrophages Support Persistent Transcription From Unintegrated HIV-1 DNA. *Virology* **372,** 300–312 (2008).

55. Hakre, S., Chavez, L., Shirakawa, K. & Verdin, E. HIV latency : experimental systems and molecular models. *FEMS Microbiol* **36,** 706–716 (2012).

56. Folks, T. M. *et al.* Characterization of a promonocyte clone chronically infected with HIV and inducible by 13-phorbol-12-myristate acetate. *J. Immunol.* **140,** 1117–1122 (1988).

57. Churchill, M. J., Deeks, S. G., Margolis, D. M., Siliciano, R. F. & Swanstrom, R. HIV reservoirs: What, where and how to target them. *Nat. Rev. Microbiol.* **14,** 55–60 (2015).

58. Mendoza, C. D. *et al.* Antiretroviral treatment of HIV-2 infection. *Future Virol.* **12,** 457–468 (2017).

59. Brun-Vezinet, F., Matheron, S. & Descamps, D. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–9 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_52-1

60. Reeves, J. D. *et al.* CD4-independent infection by HIV-2 (ROD/B): Use of the 7-transmembrane receptors CXCR-4, CCR-3, and V28 for entry. *Virology* **231,** 130–134 (1997).

61. Azevedo-Pereira, J. M., Santos-Costa, Q., Mansinho, K. & Moniz-Pereira, J. Identification and characterization of HIV-2 strains obtained from asymptomatic patients that do not use CCR5 or CXCR4 coreceptors. *Virology* **313,** 136–146 (2003).

62. Shen, H. S. *et al.* HIV coreceptor tropism determination and mutational pattern identification. *Sci. Rep.* **6,** 1–11 (2016).

63. Visseaux, B. *et al.* Molecular determinants of HIV-2 R5-X4 tropism in the V3 loop: Development of a new genotypic tool. *J. Infect. Dis.* **205,** 111–120 (2012).

64. Marcelino, J. M. *et al.* Resistance to antibody neutralization in HIV-2 infection occurs in late stage disease and is associated with X4 tropism. *Aids* **26,** 2275–2284 (2012).

65. Shi, Y. *et al.* Evolution of human immunodeficiency virus type 2 coreceptor usage, autologous neutralization, envelope sequence and glycosylation. *J. Gen. Virol.* **86,** 3385–3396 (2005).

66. Santos-Costa, Q., Lopes, M. M., Calado, M. & Azevedo-Pereira, J. M. HIV-2 interaction with cell coreceptors: amino acids within the V1/V2 region of viral envelope are determinant for CCR8, CCR5 and CXCR4 usage. *Retrovirology* **11,** 1–17 (2014).

67. Reeves, J. D. *et al.* Primary Human Immunodeficiency Virus Type 2 (HIV-2) Isolates Infect CD4-Negative Cells via CCR5 and CXCR4: Comparison with HIV-1 and Simian Immunodeficiency Virus and Relevance to Cell Tropism In Vivo. *J. Virol.* **73,** 7795–7804 (1999).

68. de Silva, T. I. *et al.* Potent Autologous and Heterologous Neutralizing Antibody Responses Occur in HIV-2 Infection across a Broad Range of Infection Outcomes. *J. Virol.* **86,** 930–946 (2012).

69. de Silva, T. I., Cotten, M. & Rowland-Jones, S. L. HIV-2: the forgotten AIDS virus. *Trends Microbiol.* **16,** 588–595 (2008).

70. Marcelino, J. M. *et al.* Envelope-specific antibody response in HIV-2 infection: C2V3C3-specific IgG response is associated with disease progression. *Aids* **22,** 2257–2265 (2008).

71. Cohen, M. S. *et al.* The spread, treatment, and prevention of HIV-: evolution of a global pandemic. *J. Clin. Invest.* **118,** 1244–54 (2008).

72. Direcção-Geral da Saúde Lisboa - Ministério da Saúde. *Infeção VIH, SIDA e Tuberculose em números – 2014*. *Ministério da Saúde - Governo de Portugal* (2014).

73. Bezerra, L. M. D. *Global report : UNAIDS report on the global AIDS epidemic 2010*. *Urban Research* **3,** (2010).

74. Quinn, T. *et al.* Viral load and heterosexual ransmission of human immunodeficiency virus type 1. *N. Engl. J. Med.* **342,** 921–929 (2000).

75. Wilson, D. P., Law, M. G., Grulich, A. E., Cooper, D. a & Kaldor, J. M. Relation between HIV viral load and infectiousness: a model-based analysis.[Erratum appears in Lancet. 2008 Nov 22;372(9652):1808]. *Lancet* **372,** 314–320 (2008).

76. Castilla, J. *et al.* Effectiveness of highly active antiretroviral therapy in reducing heterosexual transmission of HIV. *J Acquir Immune Defic Syndr* **40,** 96–101 (2005).

77. Garcia, P. M. *et al.* Maternal levels of plasma human immunodeficiency virus type 1 RNA and the risk of perinatal transmission. *N. Engl. J. Med.* **341,** 394–402 (1999).

78. Coombs, R. W. *et al.* Genital HIV-1 RNA Quantity Predicts Risk of Heterosexual HIV-1 Transmission. *Sci. Transl. Med.* **3,** 77ra29 (2011).

79. Popper, S. J. *et al.* Lower human immunodeficiency virus (HIV) type 2 viral load reflects the difference in pathogenicity of HIV-1 and HIV-2. *J. Infect. Dis.* **180,** 1116–1121 (1999).

80. O'Donovan, D. *et al.* Maternal plasma viral RNA levels determine marked differences in mother-to-child transmission rates ofHIV-1 and HIV-2 in The Gambia. *Aids* **14,** 441–448 (2000).

81. Vernazza, P. L. *et al.* Potent antiretroviral treatment of HIV-infection results in suppression of the seminal shedding of HIV. *Aids* **14,** 117–121 (2000).

82. Zhang, H. *et al.* Human Immunodeficiency Virus Type 1 in the Semen of Men Receiving Highly Active Antiretroviral Therapy. *N. Engl. J. Med.* **339,** 1803–1809 (1998).

83. Porco, T. C. *et al.* Decline in HIV infectivity following the introduction of highly active antiretroviral therapy. *AIDS* **6,** 81–88 (2004).

84. Manuscript, A. *et al.* Interactions of HIV, Other Sexually Transmitted Diseases, and Genital Tract Inflammation Facilitating Local Pathogen Transmission and Acquisition. *Am. J. Reprod. Immunol.* **65,** 308–316 (2012).

85. U.S. Department of Health and Human Services. The Stages of HIV Infection. (2018). Available at: https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/19/46/the-stages-of-hiv-infection. (Accessed: 18th July 2018)

86. Daskalakis, D. HIV diagnostic testing: evolving technology and testing strategies. *Top. Antivir. Med.* **19,** 18–22 (2011).

87. Espada de sousa, A. & Victorino, R. in *Manual sobre SIDA* (ed. Antunes, F.) 55–78 (Permanyer Portugal, 2011).

88. Moir, S., Chun, T.-W. & Fauci, A. S. Pathogenic Mechanisms of HIV Disease. *Annu. Rev. Pathol. Mech. Dis.* **6,** 223–248 (2011).

89. Mogensen TH, Melchjorsen J, Larsen CS & Paludan SR. Innate immune recognition and activation during HIV infection. *Retrovirology* **7,** 54 (2010).

90. Akbas, F. The Calm before the Storm. *J. Finance* **71,** 225–266 (2016).

91. Andersson, S. *et al.* Plasma viral load in HIV-1 and HIV-2 singly and dually infected individuals

in Guinea-Bissau, West Africa. *Arch. Intern. Med.* **160,** 3286–3293 (2000).

92. Peterson, K. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–6 (Springer New York, 2014). doi:10.1007/978-1-4614-9610-6_39-1

93. Rowland-Jones, S. Protective immunity against HIV infection: lessons from HIV-2 infection. *Future Microbiol.* **1,** 427–433 (2006).

94. Antunes, F. in *Manual sobre SIDA* (eds. Antunes, F. & Maltez, F.) 50–54 (Permanyer Portugal, 2018).

95. Leligdowicz, A. *et al.* Direct Relationship between Virus Load and Systemic Immune Activation in HIV-2 Infection. *J. Infect. Dis.* **201,** 114–122 (2010).

96. Poropatich, K. & Sullivan, D. J. Human immunodeficiency virus type 1 long-term non-progressors: The viral, genetic and immunological basis for disease non-progression. *J. Gen. Virol.* **92,** 247–268 (2011).

97. van der Loeff, M. F. S. *et al.* Undetectable plasma viral load predicts normal survival in HIV-2-infected people in a West African village. *Retrovirology* **7,** 2–11 (2010).

98. Ariyoshi, K. *et al.* Plasma RNA viral load predicts the rate of CD4 T cell decline and death in HIV-2-infected patients in West Africa. *Aids* **14,** 339–344 (2000).

99. Hoffmann, C. & Rockstroh, J. K. *Hiv 2015/16*. (Medizin Fokus Verlag, 2015).

100. Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *Int. Immunol.* **17,** 1–14 (2005).

101. Chang, J. J. *et al.* Differential regulation of TLR pathways in acute and chronic HIV-1 infection. *AIDS* **26,** 533–541 (2012).

102. Carrington, M. & Alter, G. Innate Immune Control of HIV. *Cold Spring …* 1–12 (2012).

103. Nuvor, S. V., Sande, M. Van Der, Whittle, H., Jaye, A. & Rowland-jones, S. Natural Killer Cell Function Is Well Preserved in Asymptomatic Human Immunodeficiency Virus Type 2 (HIV-2) Infection but Similar to That of HIV-1 Infection When CD4 T-Cell Counts Fall Natural Killer Cell Function Is Well Preserved in Asymptomatic Human. *J. Virol.* **2,** 2529–2538 (2006).

104. Huber, G., Bánki, Z., Lengauer, S. & Stoiber, H. Emerging role for complement in HIV infection. *Curr. Opin. HIV AIDS* **6,** 419–426 (2011).

105. Jallow, S. & Simpson, S. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–5 (Springer New York, 2014). doi:10.1007/978-1-4614-9610-6_47-1

106. Simpson, S. & Jallow, S. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–10 (Springer New York, 2016). doi:10.1007/978-1-4614-9610-6_41-2

107. Hauser, H. *et al.* HIV-1 Vpu and HIV-2 Env counteract BST-2/tetherin by sequestration in a perinuclear compartment. *Retrovirology* **7,** 1–16 (2010).

108. Evans, D. T., Serra-Moreno, R., Singh, R. K. & Guatelli, J. C. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. *Trends Microbiol.* **18,** 388–396 (2010).

109. Neil, S. J. D., Zang, T. & Bieniasz, P. D. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* **451,** 425–430 (2008).

110. Bour, S., Schubert, U., Peden, K. & Strebel, K. The envelope glycoprotein of human immunodeficiency virus type 2 enhances viral particle release: a Vpu-like factor? *J. Virol.* **70,** 820–829 (1996).

111. Bunnik, E. M., Pisas, L., van Nuenen, A. C. & Schuitemaker, H. Autologous Neutralizing Humoral Immunity and Evolution of the Viral Envelope in the Course of Subtype B Human Immunodeficiency Virus Type 1 Infection. *J. Virol.* **82,** 7932–7941 (2008).

112. McMichael, A. & Rowland-Jones, S. Cellular immune responses to HIV. *Nature* **410,** 980–987 (2001).

113. Andrews, S. M., Wong, G. & Rowland-Jones, S. L. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–10 (Springer New York, 2014). doi:10.1007/978-1-4614-9610-6_38-1

114. Davenport, M. P. & Petravic, J. CD8+ T cell control of HIV - A known unknown. *PLoS Pathog.* **6,** 1–3 (2010).

115. Jiang, D., Niwa, M., Koong, A. C. & Diego, S. Polyfunctional T cell responses are a hallmark of HIV-2 infection Melody. *Eur. J. Immunol.* **49,** 48–56 (2016).

116. Duvall, M. G. *et al.* Maintenance of HIV-specific CD4+ T cell help distinguishes HIV-2 from HIV-1 infection. *J. Immunol.* **176,** 6973–6981 (2006).

117. Makvandi-Nejad, S. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–5 (Springer New York, 2014). doi:10.1007/978-1-4614-9610-6_50-1

118. Abbas, A., Lichtman, A. H. & Pillai, S. *Basic Immunology*. (Elsevier, 2015).

119. Hoffman, W., Lakkis, F. G. & Chalasani, G. B cells, antibodies, and more. *Clin. J. Am. Soc. Nephrol.* **11,** 137–154 (2016).

120. Ma, C. S., Deenick, E. K., Batten, M. & Tangye, S. G. The origins, function, and regulation of T follicular helper cells. *J. Exp. Med.* **209,** 1241–1253 (2012).

121. Tomaras, G. D. *et al.* Initial B-Cell Responses to Transmitted Human Immunodeficiency Virus Type 1: Virion-Binding Immunoglobulin M (IgM) and IgG Antibodies Followed by Plasma Anti-gp41 Antibodies with Ineffective Control of Initial Viremia. *J. Virol.* **82,** 12449–12463 (2008).

122. Pensieroso, S. *et al.* B-cell subset alterations and correlated factors in HIV-1 infection. *Aids* **27,** 1209–1217 (2013).

123. Moir, S. & Fauci, A. S. B cell responses to HIV infection. *Immunol. Rev.* **275,** 33–48 (2017).

124. Tendeiro, R. *et al.* Memory B-cell depletion is a feature of HIV-2 infection even in the absence of detectable viremia. *Aids* **26,** 1607–1617 (2012).

125. Rocha, C. *et al.* Potency of HIV-2-specific antibodies increase in direct association with loss of memory B cells. *Aids* **31,** 2431–2433 (2017).

126. Moir, S. *et al.* HIV-1 induces phenotypic and functional perturbations of B cells in chronically infected individuals. *Proc. Natl. Acad. Sci.* **98,** 10362–10367 (2001).

127. Makvandi-Nejad, S. & Rowland-Jones, S. How does the humoral response to HIV-2 infection differ from HIV-1 and can this explain the distinct natural history of infection with these two human retroviruses? *Immunol. Lett.* **163,** 69–75 (2015).

128. Buckner, C. M. *et al.* Characterization of Plasmablasts in the Blood of HIV-Infected Viremic Individuals: Evidence for Nonspecific Immune Activation. *J. Virol.* **87,** 5800–5811 (2013).

129. Moir, S. *et al.* Evidence for HIV-associated B cell exhaustion in a dysfunctional memory B cell compartment in HIV-infected viremic individuals. *J. Exp. Med.* **205,** 1797–1805 (2008).

130. Mayr, L. M., Su, B. & Moog, C. Non-neutralizing antibodies directed against HIV and their functions. *Front. Immunol.* **8,** (2017).

131. Richman, D. D., Wrin, T., Little, S. J. & Petropoulos, C. J. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4144–9 (2003).

132. Overbaugh, J. & Morris, L. The Antibody Response against HIV-1 The Antibody Response against HIV-1. *Cold Spring Harb. Perspect. Med.* **1,** 1–17 (2012).

133. Wei, X. *et al.* Antibody neutralization and escape by HIV-1. *Nature* **422,** 307–312 (2003).

134. Mouquet, H. Antibody B cell responses in HIV-1 infection. *Trends Immunol.* **35,** 549–561 (2014).

135. Kong, R. *et al.* Broad and Potent Neutralizing Antibody Responses Elicited in Natural HIV-2 Infection. *J. Virol.* **86,** 947–960 (2011).

136. Rodriguez, S. K. *et al.* Comparison of Heterologous Neutralizing Antibody Responses of Human Immunodeficiency Virus Type 1 (HIV-1)- and HIV-2-Infected Senegalese Patients: Distinct Patterns of Breadth and Magnitude Distinguish HIV-1 and HIV-2 Infections. *J. Virol.* **81,** 5331–5338 (2007).

137. Ozkaya Sahin, G. *et al.* Potent Intratype Neutralizing Activity Distinguishes Human Immunodeficiency Virus Type 2 (HIV-2) from HIV-1. *J. Virol.* **86,** 961–971 (2012).

138. Rocha, C. *et al.* Evolution of the human immunodeficiency virus type 2 envelope in the first years of infection is associated with the dynamics of the neutralizing antibody response. *Retrovirology* **10,** 1–14 (2013).

139. Özkaya Şahin, G. *et al.* Frequent intratype neutralization by plasma immunoglobulin a identified in HIV type 2 infection. *AIDS Res. Hum. Retroviruses* **29,** 470–8 (2013).

140. Chen, C.-Y. *et al.* Antagonism of BST-2/Tetherin Is a Conserved Function of the Env Glycoprotein of Primary HIV-2 Isolates. *J. Virol.* **90,** 11062–11074 (2016).

141. Centers for Disease Control and Prevention (CDC). Terms, Definitions, and Calculations Used in CDC HIV Surveillance Publications. (2016). Available at: https://www.cdc.gov/hiv/statistics/surveillance/terms.html. (Accessed: 5th September 2018)

142. Clavel, F. *et al.* Molecular cloning and polymorphism of the Human Immune Deficiency Virus type 2. *Nature* **324,** 691–5. (1986).

143. Wei, X. *et al.* Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob Agents Chemother* **46,** 1896–1905 (2002).

144. Weber, K., Bartsch, U., Stocking, C. & Fehse, B. A multicolor panel of novel lentiviral ‘gene

ontology' (LeGO) vectors for functional gene analysis. *Mol. Ther.* **16,** 698–706 (2008).

145. Platt, E. J., Bilska, M., Kozak, S. L., Kabat, D. & Montefiori, D. C. Evidence that Ecotropic Murine Leukemia Virus Contamination in TZM-bl Cells Does Not Affect the Outcome of Neutralizing Antibody Assays with Human Immunodeficiency Virus Type 1. *J. Virol.* **83,** 8289–8292 (2009).

146. de Wet, J. R., Wood, K. V, DeLuca, M., Helsinki, D. R. & Subramani, S. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7,** 725–737 (1987).

147. Dahabieh, M. S., Ooms, M., Simon, V. & Sadowski, I. A Doubly Fluorescent HIV-1 Reporter Shows that the Majority of Integrated HIV-1 Is Latent Shortly after Infection. *J. Virol.* **87,** 4716–4727 (2013).

148. O'Doherty, U., Swiggard, W. J. & Malim, M. H. Human Immunodeficiency Virus Type 1 Spinoculation Enhances Infection through Virus Binding. *J. Virol.* **74,** 10074–10080 (2000).

149. Montefiori, D. C. *Protocol for Neutralizing Antibody Assay for HIV-1 in A3R5 Cells*. (2011).

150. Oliveira, V. *et al.* Genetic Diversity and Drug Resistance Profiles in HIV Type 1- and HIV Type 2-Infected Patients from Cape Verde Islands. *AIDS Res. Hum. Retroviruses* **28,** 110919072739002 (2011).

151. De Pina-Araujo, I. I. M., Guimarães, M. L., Bello, G., Vicente, A. C. P. & Morgado, M. G. Profile of the HIV epidemic in Cape Verde: Molecular epidemiology and drug resistance mutations among HIV-1 and HIV-2 infected patients from distinct islands of the archipelago. *PLoS One* **9,** (2014).

152. Marcelino, J. M. *et al.* Potent and Broadly Reactive HIV-2 Neutralizing Antibodies Elicited by a Vaccinia Virus Vector Prime-C2V3C3 Polypeptide Boost Immunization Strategy. *J. Virol.* **84,** 12429–12436 (2010).

153. Gray, E. S. *et al.* The Neutralization Breadth of HIV-1 Develops Incrementally over Four Years and Is Associated with CD4+ T Cell Decline and High Viral Load during Acute Infection. *J. Virol.* **85,** 4828–4840 (2011).

154. Durand, C. & Ambinder, R. in *Encyclopedia of AIDS* (eds. Hope, T. J., Stevenson, M. & Richman, D.) 1–9 (Springer New York, 2021). doi:10.1007/978-1-4614-9610-6_24-1

155. Kozak, M. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44,** 283–292 (1986).

156. Zhang, H. *et al.* Novel Single-Cell-Level Phenotypic Assay for Residual Drug Susceptibility and Reduced Replication Capacity of Drug-Resistant Human Immunodeficiency Virus Type 1. *J. Virol.* **78,** 1718–1729 (2004).

157. Bour, S., Akari, H., Miyagi, E. & Strebel, K. Naturally occurring amino acid substitutions in the HIV-2 ROD envelope glycoprotein regulate its ability to augment viral particle release. *Virology* **309,** 85–98 (2003).

158. Exline, C. M. *et al.* Determinants in HIV-2 Env and tetherin required for functional interaction. *Retrovirology* **12,** 1–13 (2015).

159. Urata, S. *et al.* Tetherin antagonism by Vpu protects HIV-infected cells from antibody-dependent cell-mediated cytotoxicity. *J. Virol.* **70,** 4828–4840 (2018).

160. Urata, S. *et al.* BST-2 controls T cell proliferation and exhaustion by shaping the early distribution of a persistent viral infection. *PLOS Pathog.* **14,** e1007172 (2018).

Supplementary data

Table A: Patient and env clones identification with respective size of Env proteins in amino-acids. Plus, two control clones (ROD10 and ROD 14 env genes).
Table adapted from supplementary data of Chen, C.-Y. et al. Antagonism of BST-2/Tetherin Is a Conserved Function of the Env Glycoprotein of Primary HIV-2 Isolates. J. Virol. 90, 11062–11074 (2016).

Patient	Coreceptor usage	Clone ID	Sample ID	Size (amino-acids)
na	na	ROD14	ROD14env_HA	858
na	na	ROD10	ROD10_HA	858
P1	CCR5	P1-1	HCC1-1-1HA	862
		P1-3	HCC1-1-3HA	862
		P1-5	HCC1-1-5HA	862
		P1-6	HCC1-1-6HA	862
P2	CCR5	P2-1	HCC6-2-1HA	871
		P2-2	HCC6-2-2HA	871
		P2-3	HCC6-2-3HA	869
		P2-6	HCC6-2-6HA	871
		P2-7	HCC6-2-7HA	871
P3	CXCR4	P3-1	HCC10-3-1HA	858
		P3-3	HCC10-3-3HA	858
		P3-5	HCC10-3-5HA	858
		P3-9	HCC10-3-9HA	856
		P3-10	HCC10-3-10HA	622
		P3-11	HCC10-3-11HA	753
		P3-12	HCC10-3-12HA	858
P4	CCR5	P4-1	HCC19-4-1HA	886
		P4-3	HCC19-4-3HA	886
		P4-6	HCC19-4-6HA	882
		P4-7	HCC19-4-7HA	886
		P4-8	HCC19-4-8HA	886
		P4-11	HCC19-4-11HA	882
		P4-7-m1	HCC19-4-7m1HA	885
		P4-7-m2	HCC19-4-7m2HA	883
		P4-7-m3	HCC19-4-7m3HA	886
		P4-7-m1+m2	HCC19-4-7m1+m2HA	882
		P4-7mA	HCC19-4-7m3HA	886
		P4-7mB	HCC19-4-7m4HA	886
		P4-7mC	HCC19-4-7m5HA	886
		P4-7mD	HCC19-4-7m6HA	886
P6	CXCR4	P6-1	HCC10-6-1HA (HSM10.04)	866
		P6-3	HCC10-6-3HA (HSM10.04)	861
P7	mixed population	P7-1	HSMAK-7-1HA	860
		P7-2	HSMAK-7-2HA	858
		P7-4	HSMAK-7-4HA	858
		P7-5	HSMAK-7-5HA	860

		P7-7	HSMAK-7-7HA	856
		P7-10	HSMAK-7-10HA	860
P8	CCR5	P8-2	HSMNC-8-2HA	865
		P8-3	HSMNC-8-3HA	731
		P8-4	HSMNC-8-4HA	753
		P8-6	HSMNC-8-6HA	865

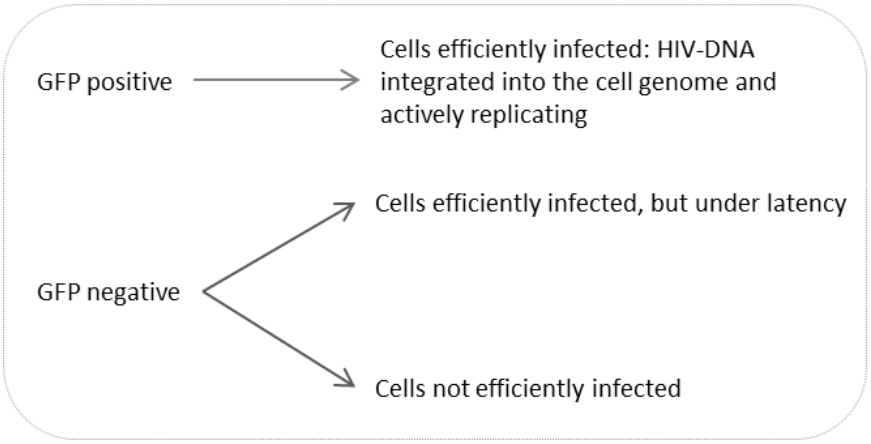


Figure A: Schematic representation of Jurkat cell infection assay.

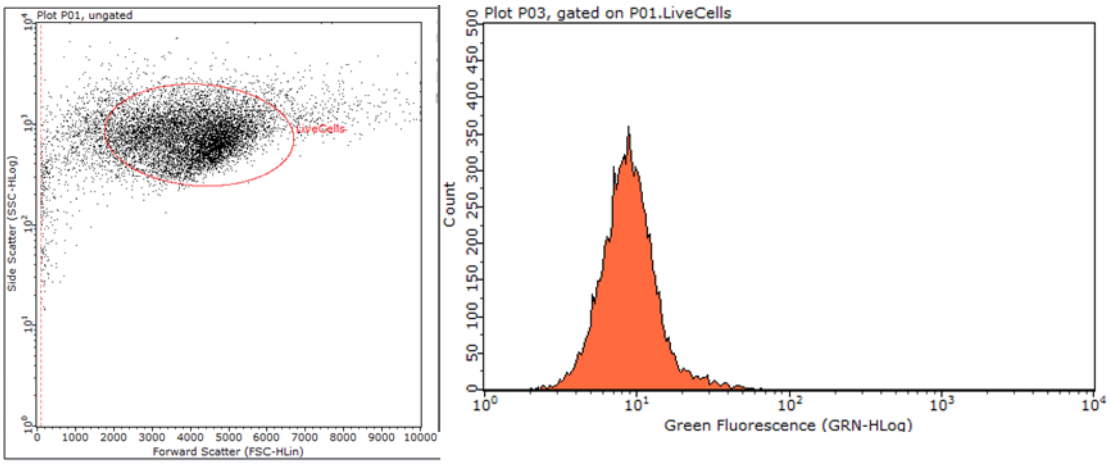


Figure B: Results (not statisticaly analized) from flow cytometry analysis of 5 000 to 10 000 live cell events of Jurkat E6.1 cells, after 48 hours (Negative Control – Mock). The peak observed in the graph corresponds to background flourescence.

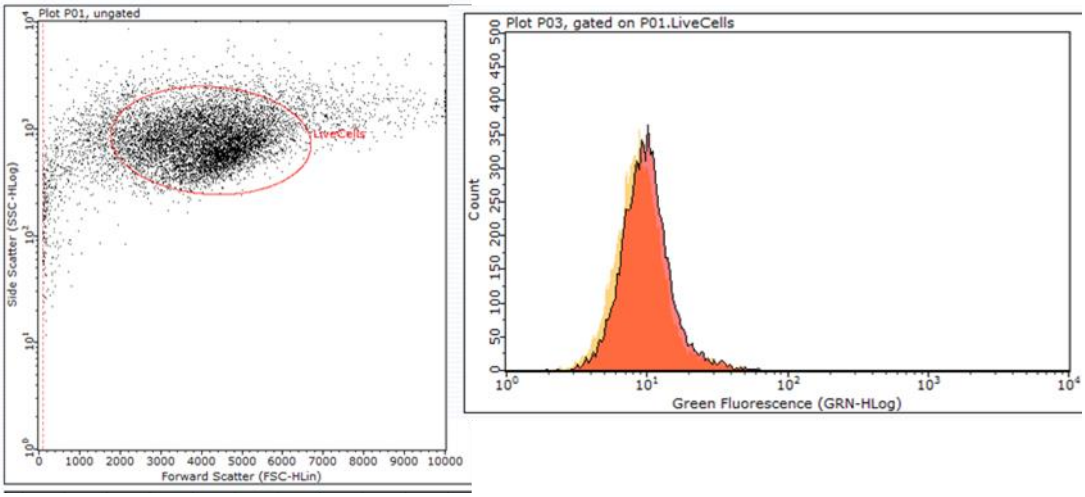


Figure C: Results (not statisticaly analized) from flow cytometry analysis of Jurkat E6.1 cells, 48 hours after infected with pseudovirus produced with HIV-2 recombinante clone pROD10-CMV-EGFP (positive control). The yellow peak observed in the graph corresponds to negative control (mock) and the orange peak to the result from infection.

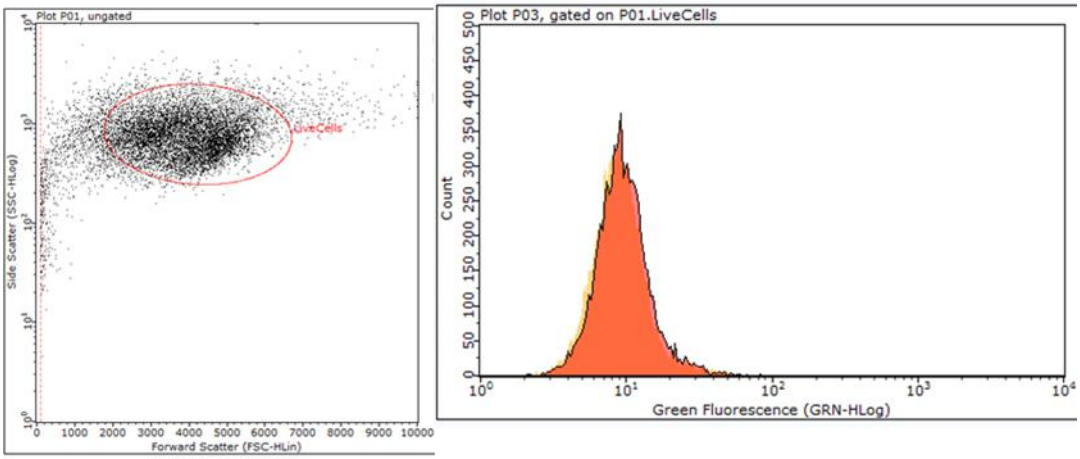


Figure D: Results (not statisticaly analized) from flow cytometry analysis of Jurkat E6.1 cells, 48 hours after infected with pseudovirus produced with HIV-2 recombinante clone pROD10-EGFP. The yellow peak observed in the graph corresponds to negative control (mock) and the orange peak to the result from infection.

Table B: Neutralization percentage of specificity assays with VSV and pSM119 enveloped pseudovirus and 1:4 dilutions of sera 2, 4, 20, 37 and 43.

Conditions	Neutralization %	Infection %
pSM119 + Serum 2	-60.36	161.38
pSM119 + Serum 4	-41.00	160.36
pSM119 + Serum 20	2.60	141.00
pSM119 + Serum 37	-59.45	97.40
pSM119 + Serum 43	-79.41	179.41
VSV + Serum 2	-64.71	159.45
VSV + Serum 4	18.76	81.24
VSV + Serum 43	-61.38	164.72

The infection ability of virus in presence of sera, exceeding 100% (compared to a positive control of virus + cells) is frequent, though it has not yet been explained

HCC1-1-5HA (P1-5)

Dilutions	1:320	1:1280	1:5120	1:20480	1:81920	1:327680	1:1310720	1:5242880	1:20971520
Serum 2	98.40	99.38	96.89	95.50	79.69	56.77	18.72	16.86	14.38
Serum 4	-12.63	19.11	10.42	13.71	14.75	na	na	na	Na
Serum 20	99.15	100.61	99.33	99.44	88.57	63.30	35.66	25.15	15.56
Serum 37	98.79	99.39	100.31	100.73	96.84	80.59	49.08	9.98	29.21
Serum 43	98.16	97.01	82.65	61.21	37.95	22.11	14.50	10.32	23.40

HCC6-2-7HA (P2-7)

Dilutions	1:320	1:1280	1:5120	1:20480	1:81920	1:327680	1:1310720	1:5242880	1:20971520
Serum 2	58.61	67.39	71.49	95.86	90.26	64.19	42.50	29.78	-17.08
Serum 4	-14.17	4.06	-9.06	7.78	5.62	na	na	na	na
Serum 20	74.92	79.00	70.55	100.24	88.58	49.37	27.57	9.82	-21.26
Serum 37	87.48	99.34	91.15	99.64	91.98	93.81	74.96	60.89	23.18
Serum 43	64.04	72.58	62.88	89.30	56.60	47.70	23.05	22.45	29.59

HCC10-3-11HA P(3-11)

Dilutions	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	1:40960
Serum 2	-27.13	-6.23	-22.54	-2.37	-13.79	-14.26	12.46	5.22	7.14	-5.41	1.58
Serum 4	-29.53	-15.10	-43.31	-16.12	-24.29	-11.55	-19.50	-8.55	-21.45	-29.61	-4.70
Serum 20	21.39	11.96	21.27	17.61	18.58	9.09	17.58	3.74	1.25	-14.76	17.02
Serum 43	-21.61	-25.78	-11.60	-26.13	-21.43	-0.02	18.42	-7.00	6.89	-0.44	17.51

HCC19-4-6HA (P4-6)

Dilutions	1:40	1:80	1:320	1:1280	1:5120	1:20480	1:81920	1:327680	1:1310720	1:5242880	1:20971520
Serum 2	89.71	87.71	82.24	84.30	76.70	74.18	41.92	52.03	47.98	24.48	43.12
Serum 4	10.07	-7.75	2.13	27.29	23.21	12.23	19.74	na	na	na	na
Serum 20	87.05	87.37	84.73	85.67	75.99	55.40	35.37	44.92	32.02	21.59	40.15
Serum 37	81.14	84.12	89.10	84.67	92.27	88.73	80.23	42.26	45.83	21.15	22.29
Serum 43	84.20	78.09	82.67	73.89	57.87	39.10	26.34	29.28	27.51	46.19	38.01

Figure E: Antibody neutralization of pseudovirus formed with patient Env-clones. Heat maps of several dilutions of five HIV sera against three *env*-clones pseudovirus. na – not applicable