HIV-2 Chronic Infection

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

Susana Tracana

Master of Science Degree in Microbiology 2017/2018
Instituto Superior Técnico – Universidade de Lisboa
Supervisors: Prof. Dr. Pedro Borrego and Prof. Dr. Arsénio Fialho

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

Introduction

The Human immunodeficiency virus type 1 and type 2 (HIV-1 and HIV-2) belongs to the *Retroviridae* family and *Lentivirus* genus [1]. The HIV-2 was isolated in 1986 from two West African patients with acquired immunodeficiency syndrome (AIDS) [2,3] . While HIV-1 infection is pandemic, infection by HIV-2 is predominant in West African countries and countries with historical and socio-economic ties with them such as Portugal. In Portugal, 3.3% of HIV/AIDS cases are caused by HIV-2 infection [4]. The HIV replication cycle is divided in seven general steps [5] : 1) binding of the envelope glycoproteins to cellular CD4 receptor and co-receptors (CCR5 and CXCR4) [6–8] ; 2) fusion of HIV to the host cell surface [6–8]; 3) reverse transcription of viral RNA [9]; 4) integration of the provirus into the cellular genome [9]; 5) virus replication [9]; 6) virion assembly; 7) budding and virion maturation [6,9]. After viral DNA integration 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. In latent infection, infected cells do not produce viral proteins and escape the viral cytopathic effects, are ignored by the immune system [10] and escape treatment. Therefore, HIV latency represents a major barrier towards eradication of HIV infection [11] . HIV-2 infection has higher tendency for latency than HIV-1 infection [11,12]. Compared to HIV-1, the transmission of HIV-2 occurs less frequently, and this may contribute to the declining prevalence of HIV-2 infection worldwide [13] and is associated with the lower viral loads observed in HIV-2 infections [12,14] . Although proviral DNA loads are very similar between the two [12,15]. The HIV infection can be divided in three stages: the acute phase, the chronic phase and AIDS [16]. The acute phase is characterized by intense HIV replication and massive loss of CD4 T cells in the absence of an immune response [17]. 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 [18,19]. Constant antigen stimulation cause

chronic immune activation, cellular exhaustion, senescence, and low renewal potential [18,19]. Generally, HIV-2 infection is characterized by a lower state of immune activation (since the majority have low to undetectable viremia), which might account for the slower disease progression [20]. When the human immune system detects a pathogenic microorganism activates defence mechanisms to eliminate the infection by innate immune responses, cellular responses and humoral responses. Tetherin (BST-2) is an antigen and its activity is an intrinsic interferon-induced antiviral cellular response that acts against enveloped viruses such as HIV [7,21–23]. 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 [7,21,24]. Neutralizing antibodies are antibodies that bind to envelope glycoproteins of cell-free HIV [25]. HIV-2 infected patients have a potent autologous and heterologous neutralizing antibody response [26–28] , in contrast to HIV-1 infection [29]. Compared with HIV-1, HIV-2 is a highly pathogenic immunodeficiency virus normally well controlled by the host immune response, being thus generally considered as an attenuated form of HIV. Although, there are few studies about HIV-2 and consequently the level of knowledge about its characteristics is lower. The general aim of this project is to provide a better description of the mechanism underlying HIV-2 chronic infection, namely viral 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 to contribute for a better understanding of the mechanisms governing latency of HIV-2, but also may offer an important model for the study of many aspects of HIV immunopathogenesis; and 2) Characterization of the susceptibility of the HIV-2 envelope to neutralizing antibodies, to contribute to a more detailed picture of the interaction of the HIV-2 envelope with neutralizing antibodies.

Materials and Methods

Env sequences

Chen et al, in 2016, produced and analysed 35 different *env* gene clones originated from 7 HIV-2 infected patients from Portugal [30]. 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 [30]. Chen et al [30] 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 [30]. 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 [30]. 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 [30].

A total of 35 plasmids with different *env* genes were studied. These plasmids derive from pCM10 and have a hemagglutinin (HA) tag, as described [30]. 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®).

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°C/30 minutes before neutralization.

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 [31]); *pSG3.1 Δenv* (plasmid expressing an infectious molecular clone of HIV-1 strain 3.1 with the *env* gene deleted [32]) 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).

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.

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°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.

Production of HIV-2ROD-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* [33] and then cloned into the plasmid *pcDNA^{TM}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 1 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.

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).

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.

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) [34]. Luciferase is an enzyme that emits light when luciferin is converted to oxyluciferin [35]. 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).

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.

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 [36–38] in guava® Flow Cytometry easyCyte™ Systems. Cell supernatants without virus were used as negative controls.

Neutralizing assay

The neutralizing activity of antibodies present in the each serum was analysed using a luciferase gene reporter assay in TZM-BL cells. 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 (Adapted from [39]). Luciferase activity was quantified 48 hours later on a luminometer (TECAN) using the One-Glow Luciferase Assay System (ThermoFisher).

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 [26]).

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

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 [40].

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

Sequence Primers (Forward/Reverse)	Sequence amplified (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)

Results and Discussion

In vitro model of HIV-2 latency

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 green fluorescent protein (GFP) under the control of the HIV promoter in the 5' long terminal repeat (LTR) [41] and other recombinant HIV-2 clone under the control of the Human cytomegalovirus (CMV) promoter to be used as a positive control since GFP is constitutively expressed.

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. 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. 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.

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 [30] (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 1 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*.

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 2 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.

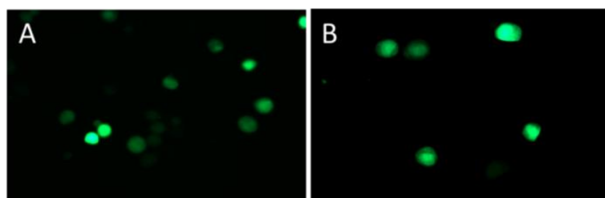

Figure 1: A: Image from fluorescence microscope of 293T cells transfected with the clone pROD10-EGFP 15. B: Image from fluorescence microscope of 293T cells transfected with the clone pROD10-CMV-EGFP 44.

Table 2: Luciferase assay results from TZM-bl cells infected with the infectious Env-pseudotyped viruses.

Conditions	Luminescence [b] (mean)
Mock 1 [a] (Negative Control)	5448
pROD10-CMV-EGFP 18 | RODenv8	26773
pROD10-CMV-EGFP 44 | RODenv8	37408
Mock 2 [a] (Negative Control)	5144
pROD10-CMV-EGFP 44 | HCC6-2-7HA	187606
Mock 3 [a] (Negative Control)	4119
pROD10-EGFP 5 | RODenv 8	10978
pROD10-EGFP 5 | HCC6-2-7HA	184549
pROD10-EGFP 15 | RODenv 8	12435
pROD10-EGFP 15 | HCC6-2-7HA	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.
[b] Relative Light Units

Infection of Jurkat cell

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 schematically represented in the Figure 2. 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 [37].

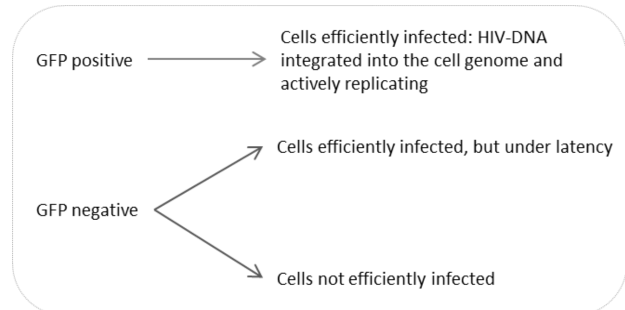

Figure 2: Schematic representation of the rational of Jurkat cell infection assay. Adapted from [41].

Jurkat cells infected with pROD10-EGFP and the positive control (pROD10-CMV-EGFP) showed fluorescence at the mock level, i.e. only background fluorescence, being considered GFP negative. According to the literature, the events leading to latency have low frequency (1% of the cells) [37], therefore cells were considered not infected. The results were not statistically analysed since they are negative. 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 3 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 3: Luciferase assay results of pROD10-EGFP transfection in 293T cells.

Conditions	Luminescence [b] (mean)
Mock [a] (Negative Control)	3047
pROD10-EGFP	103389

[a] Mock is negative control of infection
[b] Relative Light Units

Study of HIV-2-env characteristics

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 [30]. 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) were chosen due to their high level of infection in TZM-bl cells.

Table 4: Luciferase assay results for evaluation of infection capacity of pseudovirus produced with each *env*-gene plasmid.

Conditions	Luminescence [b] (mean)
Mock 1 [a] (Negative Control)	2650
HCC1-1-5HA (P1-5)	139684
HCC6-2-7HA (P2-7)	766084
HCC10-3-11HA (P3-11)	181157
HCC19-4-6HA (P4-6)	42397
Mock 2 [a] (Negative Control)	2443
HCC6-2-3HA (P2-3)	7009
HCC6-2-6HA (P2-6)	2026
HCC19-4-1HA (P4-1)	8352
HSMNC8-2HA (P8-2)	4142

[a] Mock is negative control (cells and medium) for each test
[b] Relative Light Units

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 [42,43]. 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 [26,44]. 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* [40]. An *env* with a lower molecular and phenotypic evolution and origin from an R5 tropic virus is commonly more susceptible to neutralization [40].

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.

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 3 and Table 5).

P1-5 pseudovirus was the most susceptible to antibody neutralization. A dilution of $1:2.048\times 10^4$ of serum samples 2, 20 and 37 neutralized 95%, 99% and 100%, respectively, of P1-5 replication. For serum 43, a dilution of $1:5.12\times10^3$ neutralized 82% of P1-5 (Figure 3). The ID50 calculated was $1:2.63018\times10^5$ for serum 2, $1:3.02786\times10^6$ for serum 20, $1:8.99909\times10^5$ for serum 37 and $1:2.3246\times10^4$ for serum 43 (Table 5). P1-5 viruses, a R5 tropic virus [30] 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 [30], 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 6) [30]; 2) has a lower Vpu-like activity [30] 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 [30].

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 3). In addition, the ID50 calculated corroborate this affirmation (Table 5). 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) [30] 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 [30] (Table 6), suggesting that he was infected before that patient 1. Potent neutralizing antibodies were associated with low CD4+ T cell count [45] and consequently to the virus evolution to escape [29].

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 3). ID50 was calculated for that four sera but they are inconclusive (Table 5). 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 3). 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 6) [26,40,46]. Thus, patient 3, who is the patient with the lowest CD4+ T cell count and diagnosed seven years before the time of sampling [30] (Table 6), is at an advanced stage of infection where virus neutralization resistance (X4 tropism) is common [26,40]. 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 [30].

Table 5: 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

Table 6: 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

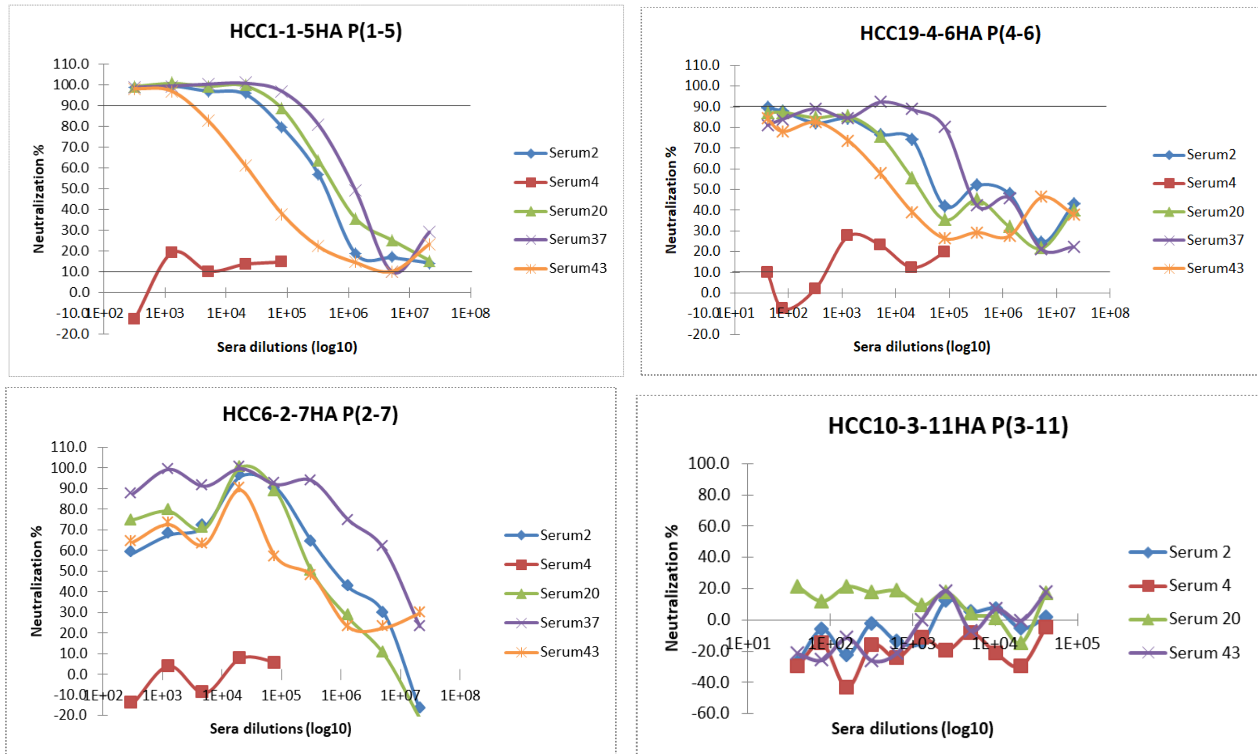

Figure 3: Relationship between HIV sera dilutions and neutralization percentage of the four HIV-2-env clones (P1-5, P2-7, P3-11 and P4-6) analysed. HCC1-1-5HA (P1-5): the most susceptible to neutralization; highlighted correlation of sera dilutions to 90% and 10% of neutralization. HCC19-4-6HA (P4-6): 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.

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 [47] 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 [48]. 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.

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 [49]). 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 [50]. As well as the T568I mutation between two env sequences of the patient 4 (P4-7 and P4-11) [30]. Both mutations involved in a conformational change of the gp36 to allow a decrease of affinity between BST-2/tetherin and the env glycoprotein [30]. However, binding is essential but is not enough to Vpu-like activity [30,51]. In HIV-1 specific mutations in Vpu increase the susceptibility of the virus to immune humoral responses [52]. 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* [53], it would be interesting to study this effect of BST-2 on HIV-2 infection, considering the imunopathogenesis and disease progression of this infection.

References

1. 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)
2. Clavel, F. *et al.* Isolation of a New Human Retrovirus African Patients with AIDS from West. **248,** (1986).
3. Clavel, F. *et al.* Human immunodeficiency virus type 2 infection associated with AIDS in West Africa. *N. Engl. J. Med.* **316,** 1180–1185 (1987).
4. Martins, H. C. *Infeção VIH e SIDA: a situação em Portugal a 31 de dezembro de 2016*. (2017).
5. 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)
6. Freed, E. & Martin, M. *Fields Virology. Fields Virology* (Wolters Kluwer, 2013). doi:9781451105636
7. 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
8. Azevedo-Pereira, J. M. in *Manual sobre SIDA* (ed. Antunes, F.) 13–30 (Permanyer Portugal, 2011).
9. Taveira, N., Borrego, P. & Bártolo, I. in *Manual sobre SIDA* (ed. Antunes, F.) 31–54 (Permanyer Portugal, 2011).
10. 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).
11. 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).
12. 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).
13. 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).
14. 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).
15. 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).
16. 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)
17. Daskalakis, D. HIV diagnostic testing: evolving technology and testing strategies. *Top. Antivir. Med.* **19,** 18–22 (2011).
18. Moir, S., Chun, T.-W. & Fauci, A. S. Pathogenic Mechanisms of HIV Disease. *Annu. Rev. Pathol. Mech. Dis.* **6,** 223–248 (2011).
19. Mogensen TH, Melchjorsen J, Larsen CS & Paludan SR. Innate immune recognition and activation during HIV infection. *Retrovirology* **7,** 54 (2010).
20. Leligdowicz, A. *et al.* Direct Relationship between Virus Load and Systemic Immune Activation in HIV- 2 Infection. *J. Infect. Dis.* **201,** 114–122 (2010).
21. 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).
22. 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).
23. 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).
24. 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).

25. 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).

26. 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).

27. 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).

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

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

30. 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).

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

32. 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).

33. 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).

34. 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).

35. 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).

36. 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).

37. 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).

38. 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).

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

40. 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).

41. Jordan, A. & Bisgrove, D. HIV reproducibly establishes a latent infection after acute infection of T cells in vitro. *EMBO J.* **22,** (2003).

42. 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).

43. 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).

44. 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).

45. 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).

46. 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

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

48. 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).

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

50. 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).

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

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

53. 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).