SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

HIV-2: insight into viral replication dynamics, evolutionary rate and role in dual HIV infection

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at 10:30; 15th of October, 2019.

Head of the Defense Committee: Prof. Dr. Szabó Gábor, MD, PhD, DSc

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The PhD defense will be organized online at 10:00 am 8th of December, 2020.

Participation requires registration. For registration and further information please email to szojka.zsofia@med.unideb.hu.

INTRODUCTION

Epidemiology and clinical manifestation of HIV-2

According to WHO, it is estimated that approximately 37.9 million persons are infected with HIV worldwide. Most of the infections are caused by HIV-1, and while recent statistical data about HIV-2 infection is lacking, early estimates put the total number of HIV-2 infected patients at around 1-2 million, which also included patients dually infected with HIV-1 and HIV-2 (HIV-D). HIV-2 infection and HIV-D are mostly prevalent in West Africa, and in regions sharing socio-ecomomic relations to that region; such as Portugal, France, and Spain. HIV-2 is mostly prevalent in West African countries; such as Sierra Leone, Gambia, Senegal, Ivory Coast, Guinea and Guinea-Bissau. Compared to HIV-1, infection with HIV-2 is less pathogenic, patients usually have undetectable or low plasma viral load (VL) (Avettand-Fenoel et al., 2014), slower CD4+ T-cell decline (Van der Loeff et al., 2002) and a slower rate of progression to AIDS, even in antiretroviral-therapy naive patients.

HIV-1 and HIV-2 dual infection

Where both HIV viruses circulate, patients can be dually infected. The estimated prevalence of HIV dual infection (HIV-D) is 0-3.2 % in West African countries, such as in Guinea-Bissau and Bissau (Da Silva et al., 2008). Moreover, 0.2% of French HIV infected individuals are dually infected, (Barin et al., 2007); and in Spain, 32% of registered HIV-2 infected are also HIV-1 positive (de Mendoza et al., 2017). Detection of CD4+ T-cell decline is one of the indicators of disease development. The percentages of CD4+ and CD8+ T-cells were significantly higher, moreover, a slower increase in the percentage of CD8+ T-cell was noticed in dually seropositive cases, in comparison to HIV-1 mono-infected ones (Esbjörnsson et al., 2012). VL is undetectable in roughly half of the HIV-D cases, similarly, in HIV-2 (Gottlieb et al., 2018). Patients dually infected with both viruses have lower HIV-1 plasma viral load compared to HIV-1 mono-infected individuals. Dually infected patients have slower progression to AIDS, compared to HIV-1 mono-infected individuals. The mechanism of dual infection with both HIV viruses remains poorly understood. While some studies reported approximately 70 % protection against re-infection with HIV-1 in HIV-D patients (Travers et al., 1995), in other studies this HIV-2 mediated protection was not noted (Greenberg, 2001).

Virus evolution and disease progression

Both HIV viruses evolve rapidly, due to high mutation and replication rates. A higher rate of spontaneous mutations may lead to faster evolution of the viruses, promotes eluding of neutralization, and indicates immune escape and the risk of disease progression. Rapid evolution of HIVs is attributed to the lack of proofreading activity of the reverse transcriptase, and the editing of viral cDNA by the cellular cytidine deaminases of the A3 family (Andrews and Rowland-Jones, 2017) (Cuevas et al., 2015). The mutation rate is extremely high for HIV-1, while there is no information about the mutation rate of HIV-2.

The association between HIV evolution and disease progression had been described in the literature (MacNeil et al., 2007) (Lemey et al., 2007). Although correlation between evolutionary rate and disease progression is well characterized for HIV-1 (Salemi, 2013), viral evolution of HIV-2; especially intra-patients evolution, is understudied.

Main differences of between IV-2 and HIV-1 genomes

HIV-1 and 2 share only a 55-48 % identity in the nucleotide level. HIV-2 genes can be classified into three main groups: main structural genes: *gag*, *pol*, *env*; regulatory genes: *tat* and *rev*, and accessory genes: *vif*, *nef*, *vpr* and *vpx*. The main structural genes also show comparable similarity: *gag*: 54 %, *pol*: 55 % and *env*: 35 %. Even though both viruses share a common ancestor, variability in their genomic sequences accounts for the presence of unique accessory proteins. For example, HIV-2 codes for the viral protein X (Vpx) accessory protein, while HIV-1 codes for the viral protein U (Vpu). Additionally, a difference can also be observed in the regulatory proteins; such as the transcriptional trans-activator protein (Tat).

Transcriptional trans-activator protein of HIV-2

Tat plays an important role in the initiation of viral transcription. Although Tat protein of both HIV viruses induces transcription, several differences can be observed. The identity in the amino acid sequence is less than 30 % between the two HIVs Tat proteins. The first three domains: the Pro-rich, Cys-rich and core region of Tat are involved in the transactivation function. Overall, only 10 % identitiy is observed between the Pro-rich regions of the two Tat proteins. While mutations in the Pro-rich domain of HIV-1 Tat have no effect on the function of the protein (Rossenkhan et al., 2012). It is well described that Tyr to Ala substitution in the Cys-rich domain of HIV-1 and SIV results in aberrant

folding, leading to the inactivation of Tat (Das et al., 2007), however, the effect of tyrosine to alanine subsitution in the Pro-rich region of HIV-2 has not been described.

Vpx

Viral protein X (vpx) is only present in HIV-2, and in its ancestor SIVsmm. HIV-2 Vpx is important in the nuclear transport of preintegration complex (PIC), wherein K68 and R70 residues of the protein mediate the function (Belshan, 2002). Another major function of Vpx is to counteract the antiviral effect of sterile α motif domain and HD domain-containing protein 1 (SAMHD1), a specific HIV-1 restriction factor, which is able to block HIV replication by restricting reverse transcription, through maintaining low cellular free dNTP level in the early stage of infection (Hollenbaugh et al., 2014). Vpx induces the proteosomal degradation of SAMHD1 by binding to the Cullin4-DDB1-DCAF1 E3 ligase complex (Ciftci et al., 2015). K68 residue of HIV-2 Vpx is critical for Vpx-E3 ligase complex interaction, and the subsequent ubiquitation of SAMHD1 (Mcculley et al., 2012).

AIMS

Even though HIV-2 was discovered more than 30 years ago, relatively limited information exist about its pathomechanism, viral evolutionary rate and infectivity, compared to HIV-1. Moreover, although dual infection had been described in the literature, studies about its pathomechanism are widely lacking. The two viruses share only a 40 % similarity in their genome. Each virus encodes for unique genes; such as *vpx* in HIV-2 and *vpu* in HIV-1; moreover, some of the viral proteins are highly polymorphic, and substantially differ in their amino acid sequence between the two viruses; such as the Tat protein (Kurnaeva et al., 2019).

Furthermore, HIV-2 infection is mostly associated with a slower rate to AIDS progression (Esbjörnsson et al., 2018), although it probably has a high mutation rate, similarly to its counterpart (Rambaut et al., 2004). There are limited number of publications about the contribution of HIV-2 evolutionary rate to disease progression.

Therefore, our aim was to carry out an in-depth study of HIV-D, in addition to characterize inactivating mutations in HIV-2 Tat, and to develop a sensitive and robust method for detection of HIV-2 DNA load, and to understand the correlation between intra-patient HIV-2 viral evolution and disease progression.

Our aims were achieved by carrying the following:

1. Characterization of HIV dual infection in cell culture

2. *In silico* and *in vitro* characterization of the effect of mutations in the Pro-rich domain of HIV-2 Tat

3. Development of a sensitive HIV-2 DNA quantification protocol

4. Studies on the differences in HIV-2 evolutionary rate between fast and slow HIV-2 progressors

MATERIALS AND METHODS

Plasmids and vectors

2nd generation lentiviral vectors were used for HIV-1 and HIV-2 virion production. For production of HIV-1 pseudovirions, the following plasmids were used: psPAX$_2$ as a packaging plasmid (a kindly gift from Dr. D. Trono at the University of Geneva Medical School), pWOX-CMV-GFP transfer vector, which was modified to code for mCherry instead of Green Fluorescent Protein (GFP), and pMD.G; encoding for the envelope protein of vesicular stomatitis virus. The following plasmids were used for HIV-2 pseudovirion production: HIV-2 CGP (a ROD based HIV-2 protein expression vector), a transfer plasmid CRU5SINCGW, which has a GFP expression cassette under CMV promoter, and pMD.G plasmid. HIV-2-CRU5SIN-WPRE vector contains U5 regions and HIV-2 *gag*, lacking of *pol* and *env*. (HIV-2 CGP and CRU5SINCGW plasmids were a kindly gift of Joseph P. Dougherty at the Robert Wood Johnson Medical School). pcDNA3.1-Vpx-NeGFP encodes for HIV-2 Vpx-GFP fusion protein, and the sequence of HIV-2 *vpx* gene is identical to that found in the HIV-2 CGP vector. pcDNA3.1-Vpx-NeGFP was obtained from Genscript services. As a mock plasmid, we created pcDNA3.1-NeGFP from pcDNA3.1-Vpx-NeGFP plasmid using KpnI and XbaI restriction enzymes, then ligation. pTY-EFeGFP was also used as a control vector in some experiments carried out in THP-1 cells.

Mutagenesis

We used QuikChange II and QuikChange Lightning Multi Site-Directed Mutagenesis Kits (Agilent Technologies) to generate the following mutations in the HIV-2 CGP plasmid. Protease: D25N, Tat: Y44A / Y55A, Rev: H73R, Vif: S144A, Vpr: R78A, Vpx: K68A and R70A. Inactivating mutations of Vpx (K68A and R70A) were also generated in the pcDNA3.1-Vpx-NeGFP vector. PCR sequencing was used to confirm the success of mutagenesis.

In silico predictions of Tat mutations

For *in silico* predictions, the sequence of HIV-2 Tat protein was downloaded from UniProt database (UniProt ID: P04605), and protein model structures were obtained from Swissmodel repository (Bienert et al. 2017). For predictions of disorder and point mutation-induced stability changes we used IUPred (Dosztanyi et al. 2005) and I-Mutant 2.0 servers (Capriotti, Fariselli, and Casaio 2005); JPred4 server was used for secondary structure predictions (Drozdetskiy et al. 2015). To estimate the effect of point mutations

on Tat protein stability, we used SDM server (Pandurangan et al. 2017) and FoldX algorithm (Guerois, Nielsen, and Serrano 2002) structure-based predictions.

Experiments on HEK293T cells to study HIV-1/2 dual infection

Production of HIV-1 and HIV-2 viral particles

293T human embryonic kidney (HEK293T) cells (Invitrogen) were used for pseudovirion productions. For the production of HIV-1 pseudovirions, we used psPAX$_2$, pWOX-CMV-mCherry, and pMD.G plasmids; for HIV-2 production, we used HIV-2 CGP, HIV-2 CRU5SINCGW, and pMD.G. For the production of Y44A and Y55A Tat mutant HIV-2 virions we used mutant Tat carrying CGP, HIV-2-CRU5SIN-CGW, and pMD.G plasmids. HEK293T cells were passaged in order to achieve around 70 % confluency on the next day. Transfection was carried out using polyethylenimine (PEI), then HEK293T cells were incubated at 37°C, 5 % CO_2 in antibiotics-free DMEM containing 1% FBS after adding the PEI-DNA solution for 5-6 hours. The media was then replaced by fresh culturing media, and the supernatant containing virions was collected on day 1, 2 and 3, concentrated by ultracentrifugation, and the viral pellet was dissolved in 200 µl phosphate-buffered saline (PBS). Reverse transcriptase (RT) activity and amount of HIV-2 capsid was then measured using ELISA-based colorimetric reverse transcriptase assay (Roche Applied Science), and ELISA-based colorimetric p24 (for HIV-1) or SIV p27 (HIV-2) assay (Express Biotech International).

HIV-1 and HIV-2 dual transduction assays

Simultaneous infection (transduction) model

The day before transduction, HEK293T cells were seeded in a 48-wells plate, then infected with 30 ng RT-equivalent of HIV-1 and HIV-2 pseudovirions simultaneously. The cells were complemented with serum- and antibiotic-free media containing 8 µg/ml polybrene. For HIV-1 and HIV-2 mono-infection controls, cells were infected with 30 ng RT-equivalent of HIV-1 or HIV-2 virions. On the next day, the cells were supplemented with double DMEM (containing 20 % FBS, 2 % glutamine, and 2 % penicillin-streptomycin), and were incubated for 4 days. FACS analysis was then performed to analyze infectivity.

"Superinfection" (over-transduction) models

Similarly to simultaneous transduction, HEK293T cells were plated in 48-well plate a day before superinfection. For HIV-1" superinfection" experiments, cells were first transduced with 30 ng RT-equivalent of HIV-2, after 24 hours incubation 30 ng RT-

equivalent of HIV-1 and double DMEM was added to the cells, followed by a 4 days incubation. HIV-2 "superinfection" was carried out similarly, with the exception that HIV-2 transduction was followed by transduction with HIV-1. After 4 days incubation, FACS analysis was performed to quantify HIV-1 (mCherry) and HIV-2 (GFP) positivity in 5,000 cells.

HIV-1 transduction of HEK293T cells pre-transfected with wild-type and mutant HIV-2 CGP vectors

Using the transfection protocol described previously, cells were transfected with 10 µg of HIV-2 CGP vectors carrying the wild-type or the mutant gene of interest using PEI, cells were incubated and media was changed after 5-6 hours. After 30 hours incubation, transfected cells were split and transferred into 48-well plate, supplemented with 50 µl antibiotics- and serum-free DMEM containing 8 µg/ml polybrene and transduced with 30 ng RT-equivalent of HIV-1. Thereafter incubated for 24 hours, and supplemented with double DMEM, followed by further incubation for 3 days. Cells were then scraped and fixed in PBS containing 1 % formaldehyde. FACS was used to detect the percentage of HIV-1 positivity (mCherry) in 5,000 cells.

HIV-1 transduction of HEK293T cells transfected with Vpx

Using the previously described transfection protocol, HEK293T cells were transfected with 10 µg of pcDNA3.1-Vpx-NeGFP plasmid. After 30 hours incubation, Vpx-transfected cells were seeded in 48-well plate and transduced with 20 ng RT-equivalent of HIV-1 pseudovirions. After 4 days incubation, cells were mechanically scraped off, and HIV-1 mCherry positivity was analyzed in 5,000 cells using flow cytometry. For control experiments, non-transfected, HIV-1 transduced cells, pcDNA3.1-NeGFP vector, and K68A-R70A functionally restricted mutant Vpx was used to transfect cells, followed by transduction with HIV-1.

Western blotting of HIV-2 Vpx expressed in HEK293T cells

Western blotting was used to verify the presence of HIV-2 Vpx. Using the above described method HEK293T cells were transfected with 10 µg of HIV-2 CGP, pcDNA3.1-Vpx-NeGFP or pcDNA3.1-NeGFP. After 30 hours of incubation the media was discarded, and cells were scraped in PBS, centrifuged, then dissolved in lysis buffer (50 mM Tris-HCl, 250 mM NaCl, 0.5 % NP-40, 5 mM EDTA, 50 mM NaF, pH 7.4), and disrupted by short sonication, the lysate was then centrifuged and the proteins were

separated on 14 % SDS polyacrylamide gel and detected by antibodies after transfer to a nitrocellulose membrane. HIV-2 Vpx monoclonal antibody (Kappes et al., 1993) was used as a primary antibody, followed by a secondary anti-mouse IgG (Sigma-Aldrich) to detect the presence of Vpx. β-actin antibody (Covalab) was used as a primary antibody, followed by anti-mouse IgG, used for normalization of total protein.

Quantitative real-time PCR analysis of vpx transfected, HIV-1 transduced cells

Viral DNA was extracted from HIV-1 transduced, vpx pretransfected HEK293T cells using Blood and Cell culture DNA Mini Kit (Qiagen). Real-time PCR was used for quantification of HIV-1 2-LTR circle junctions. qPCR was carried out with an initial denaturation step at 95°C for 10 min, followed by 45 cycles of 95°C for 1 min, and 60°C for 1 min. pWOX-CMV-mCherry plasmid was used as a standard, as it encodes for the long terminal repeat region. HIV-1 DNA was normalized to the expression of PBDG (porphobilinogen deaminase) housekeeping gene.

Detection of HIV-2 Vpx incorporation into HIV-1 pseudovirions

HEK293T cells were transfected with 10 μg of pcDNA3.1-Vpx-NeGFP vector, then after 30 hours of incubation, the transfected cells were also transfected with plasmids used for HIV-1 production. For control experiments, pTY-EFeGFP was used. RT ELISA was used to detect the RT activity from the harvested pseudovirions. Furthermore, western blot analysis was performed to detect the presence of Vpx from the virions.Virions were lysed in pH 7.8 lysis buffer (50 mM Tris, 80 mM KCl, 25mM DTT, 0.75 mM EDTA, 0.5 % Triton X-100), then lyzed virionwere separated on 14 % SDS polyacrylamide gel, proteins were transferred to a nitrocellulose membrane then after blocking the membranes anti-Vpx monoclonal antibody and anti-p24 monoclonal antibody (Chesebro et al., 1992) were used as primary antibodies, and anti-mouse IgG (Sigma-Aldrich), was used as a secondary antibody. The harvested pseudoviorions were also used to transduce HEK293T cells using the previously mentioned transduction protocol. Fluorescence microscopy was used to detect positively transduced cells.

Experiments on THP-1 cells

Simultaneous and "superinfection" assays in THP-1 cells

THP-1 cells were plated for transduction in 96-well plate in serum and antibiotic-free RPMI media containing 8 μg/ml polybrene. Cells were transduced with 10 ng RT-equivalent HIV pseudovirions. The day after the transduction, cells were complemented

with double RPMI and incubated for 3 days. For HIV-1 superinfection, cells were transduced with 10 ng of HIV-2 in the presence of polybrene, followed by incubation for 30 hours. Cells were then superinfected with 10 ng RT-equivalent HIV-1 virions. After 3 days incubation, FACS analysis was carried out.

Activation, transfection and transduction of THP-1 cells

THP-1 cells were plated into 48-well plate and 50 nM phorbol 12-myristate 13-acetate (PMA) was added to activate cells. Differentiation of monocytes to adherent macrophages was detected by optical microscopy. Activated cells were transfected with pcDNA3.1-Vpx-NeGFP or pcDNA3.1-NeGFP vectors using Lipofectamine LTX Reagent (Thermofisher Scientific). After 30 hours of incubation the supernatant was changed to a fresh medium, and cells were infected with 13 ng RT-equivalent of HIV-1. This was followed by 3 days of incubation, and fluorescent cells were detected using flourescent microscope and by flow cytometry.

Studying the effect of HIV-2 Tat Y44A mutation

Experiments in GHOST(3) cells

Transduction of GHOST(3) cells

GHOST(3) cells were seeded in a 12-well plate and were transduced with 5 ng (normalized for p27) HIV-2 virus (wild-type and Y44A / Y55A Tat mutant) in the presence of polybrene, after 2 hours of incubation media was completed and the cells were incubated for 3 days. FACS analysis was performed to quantify the percentage of LTR-induced GFP positive cells.

Dot Blotting

1 ng (normalized for HIV-2 capsid) pseudovirions carrying wild-type or mutant (Y44A or Y55A) Tat were lyzed using pH 7.8 lysis buffer, then 20 µl of the lysed pseudoviruses were loaded onto nitrocellulose membrane and detected using HIV-2 Tat anti-serum as a primary antibody, followed by the use of secondary anti-rabbit IgG. Dot-blotting was perfomred from GHOST(3) indicator transduced with 5 ng (normalized for p27) of wild-type or Y44A / Y55A mutant Tat HIV-2 pseudovirions, as well. Cells were trasnduced as described above, the media was discarded, then cells were scraped in PBS, centrifuged and dissolved in pH 7.4 lysis buffer, followeb by disruption using sonication. 20 µl of supernatant was loaded onto nitrocellulose membrane. HIV-2 Tat anti-serum (Echetebu and Rice, 1993), followed by secondary anti-rabbit IgG were used to detect HIV-2 Tat in the cell lysate. β-actin was used as a control.

Experiments in HEK293T cells

Detection of HIV-2 RT and Tat by Western-blot

To detect the presence of HIV-2 RT and Tat by Western-blot in pseudovirions, 1 ng of HIV-2 pseudovirions (normalized for p27 capsid) were suspended in pH 7.8 lysis buffer, then the proteins of the lysate were separated on 14% SDS polyacrylamide gel. After protein transfer to nitrocellulose membrane and blockint the free sites, HIV-2 Tat antiserum, anti-HIV-2 RT (Klutch et al., 1993), or anti-p24 monoclonal primary antibodies were used, followed by the use of secondary anti-rabbit IgG, for anti-Tat, and anti-RT antibodies and anti-mouse IgG; for anti-p24 monoclonal antibody. To detect intracellularly expressed RT and Tat, HEK293T cells were transfected with 10 µg of HIV-2 CGP plasmid coding for either the wild-type or Y44A mutant HIV-2 Tat. 24 and 72 hours after transfection, transfected cells were mechanically deattached, and were dissolved in PBS. After disruption by short sonication, 30 µl of the supernatant was then loaded onto 10 % SDS polyacrylamide gel. After electrophoresis, transter to nitrocellulose membrane and blocking, HIV-2 Tat antiserum or anti-HIV-2 RT primary antibodies were used, followed by the use of secondary anti-rabbit IgG. For normalization β-actin was used.

Quantification of HIV-2 DNA in whole blood

DNA extraction from PM1, U1 cells and Whole Blood

DNA was extracted from 5 x 10^6 PM1 cells and 5 x 10^6 U1 cells (subclones of U937 chronically HIV-1 infected cells) using Qiagen QIAamp DNA Mini Kit. U1 cells were used as control to detect the specificity to HIV-2. Viral DNA was extracted from 200 µl of whole blood in the presence of 5 µg carrier RNA using QIAamp Blood DNA Mini Kit (Qiagen).

Preparation of HIV-2 standard for Real-Time PCR

HIV-2 RNA was extracted from 1.44 x 10^{11} RNA-copies/ml of electron microscopy-counted HIV-2 particles using miRNeasy micro Kit (Qiagen). Then, cDNA synthesis was performed using SuperScript III One-Step RT-PCR System (Thermo Fisher Scientific) and Platinum Taq DNA polymerase (Thermo Fisher Scientific). Reactions were prepared in 25 µl solution containing: 2x reverse transcription buffer, 1 µl of eluted total RNA (5,000 copies/µl), 100 ng forward and reverse primers, 1 µl SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase (Thermo Fisher Scientific), and RNase/DNase–free sterile water up to the final volume. RT-PCR was carried out with an

initial cDNA synthesis step for 30 min at 50 °C, followed by initial denaturation for 2 min at 94 °C, 35 cycles of 15 s at 94 °C, 30 s at 50 °C, 45 s at 68 °C, and a final elongation step for 5 min at 68 °C. Product was visualized on 1% agarose gels stained with Gel Red, and was purified using QIAquick PCR purification Kit (Qiagen).

Preparation of PBDG standard for Real-Time PCR

PBDG fragment was amplified using two steps PCR (an outer and a nested PCR). For outer PCR, the reaction contained 10 ng eluted DNA of PM1 cells, 0.125 µl Dream Taq DNA polymerase (5 U/µl), 5 mM dNTP mix and 100 ng primers (PBGD-CF1 and PBGD-CR2). For nested PCR, the reaction contained 1 µl of outer PCR product, 0.125 µl Dream Taq DNA polymerase (5 U/µl), 5 mM dNTP mix and 100 ng primers (PBGD-CF1 and PBGD-CR1). Products were visualized on 1% agarose gels stained with Gel Red. Nested and outer PCRs were carried out with an initial denaturation of 2 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 45 s at 72 °C and a final elongation step for 5 min at 72 °C. PCR product was purified using QIAquick PCR purification Kit.

Quantitative real-time PCR analysis for detection of HIV-2 DNA

Reactions were prepared in 30 µl solution containing: 15 µl Thermo Fisher Maxima probe qPCR Master Mix (Thermo Fisher Scientific), 4 µM of probe, 100 ng/µl of each primer (Damond et al., 2002) and 8 µl of template DNA (concentration ranging between 800-1000 ng). qPCR was carried out with an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 30 seconds and extension at 72 °C for 30 seconds. For quantification, a series of ten-fold and two-fold dilution of the HIV-2 *gag* fragment corresponding to 10^6 to 1 copies DNA was used as a standard. HIV-2 DNA was normalized to the expression of PBDG housekeeping gene. HIV-2 DNA concentrations were firstly calculated as HIV-2 DNA copies/µl obtained from the qPCR reaction. Copy number of HIV-2 DNA was then normalized to PBDG. Finally, the results were reported as the number of copies/10^5 cells, using the following formula: (HIV-2 DNA copies per µl)/(PBDG copies per µl/2 chromosomes per cell) x 10^5 cells = HIV-2 DNA copies per 10^5 cells. Limit of detection (LOD) and limit of quantification (LOQ) were calculated using a formula described by *Schwarz et al.* (2004).

Analysis of the association between CD4$^+$ T-cell level and HIV-2 evolutionary rate

Population study of cohort in Guinea-Bissau

Samples were collected from a large cohort of police officers in Guinea-Bissau by members of the SWEGUB CORE group between 1990 and 2011 (Mansson et al., 2009) (Norrgren et al., 1995) (Esbjörnsson et al., 2012). The cohort included 438 seroincident HIV-2 infected individuals. 83 members of the cohort had an estimated date of infection. V1-C3 region of HIV-2 *env* was amplified from 53 samples collected from 16 individuals of a cohort of police officers in Guinea-Bissau (Mansson et al., 2009) (Norrgen et al., 1995).

Amplification and sequencing of V1-C3 region of HIV-2 *env*

HIV-2 RNA was extracted from plasma samples using Qiagen miRNeasy micro kit. cDNA synthesis was performed from extracted RNA using SuperScript III One-Step RT-PCR System and Platinum Taq DNA polymerase, KH2_OF, and TH2_OR primer pairs. This was followed by a semi-nested PCR using Platinum Taq High Fidelity, KH2_OF, and KH2_OR primer pairs (MacNeil et al., 2007) (Barroso and Taveira, 2005). One-step and nested-PCRs were carried out with an initial cDNA synthesis step for 30 min at 50 °C, followed by initial denaturation for 2 min at 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 50 °C, 60 s at 68 °C, and a final elongation step for 5 min at 68 °C. Amplified fragments were cloned into pCR2.1 TOPO cloning system, and then 12 individual clones were picked for subsequent sequencing. HIV-2 V1-C3 region of *env* sequences were edited using CodonCode Aligner v1.5.2 and MEGA5 using the Clustal algorithm (Tamura et al., 2011).

Survival and phylogenetic analysis

Kaplan-Meier analyses were performed for AIDS progression time, and log rank test was used for statistical comparisons. To determine the non-significant interactions between covariates, Cox proportional-hazards model was used. PHI test was used to identify intra-patient recombinants (Bruen et al., 2006), then Maximum-likelihood (ML) phylogenetic trees were used to reconstruct the inferred model using Garli v2.0 (Zwick). Internal branches were identified by Maximum-likelihood-based approximate likelihood ratio test (aLRT) Shimodaira-Hasegawa (SH)-like branch support, in PhyML 3.0, where SH-values of > 0.9 were significant (Guindon et al., 2010) (Anisimova et al., 2011).

Subtype analysis was performed using reference sequences from Los Alamos database and Clustal algorithm (Tamura et al., 2011), then phylogenetic analysis was performed.

Evolutionary rate analysis of HIV-2 envelope

BEAST v1.7.5 was used to reconstruct time-measured and Bayesian rooted phylogenetic trees. All analyses were performed by Markov Chain Monte Carlo (MCMC) $50x10^6$ generations with sampling every 2500-5000 generation, and effective sample sizes >100 and inspection of traces, as assessed in Tracer v1.6. To estimate the rate of substitutions, the Hasegawa, Kishino and Yano (HKY) substitution model was used (Hasegawa et al., 1985), and exploratory analyses were performed by single analysis of $50x10^6$ MCMC generations. Results of duplicate analyses were combined in LogCombiner v1.7.5 (Drummond and Rambaut, 2007). Nucleotide (HKY) and codon (GY94 codon model) substitution rates were estimated for V1-C3 regions of HIV-2 *env* (Hasegawa et al., 1985) (Goldman and Yang, 1994). BEAST v1.7.5: hierarchical phylogenetic model (HPM) was used to compare the evolutionary rate between slow and fast disease progressors (Drummond and Rambaut, 2007) (Edo-Matas et al., 2011), and Bayes Factors (BFs) >3 were considered as a significant association (Kass and Rafter, 1995).

Absolute rates and divergence plots

The indicator of selection and molecular adaption is the ratio of nonsynonymous and synonymous rates (dN/dS rate ratio), which cannot be used to detect the simultaneous increases and decreases (Seo et al., 2004), thus, every branch in the tree of the substitution rate needs to be differed into expected nonsynonymous (E[N]) and synonymous (E[S]) substitution rates (Lemey et al., 2007). For each individual, 200 random trees generated by HPM analysis were used to define the [N] and E[S] rates in HyPy 2.2.0 (Pond et al., 2005).

Analysis of selected sites of HIV-2 env

BEAST v1.8.1 (Drummond and Rambaut, 2007) was used to approximate the ratio of nonsynonymous (E[N]) and synonymous (E[S]) substitution at each codon, and identify the differences under positive and negative selection (Lemey et al., 2012). Analysis was carried out using strict clock model with HKY nucleotide substitution model (Hasegawa et al., 1985) and constant population size model. For conservation analysis, reference sequences of HIV-2 and SIVsm *env* were aligned by using Clustal algorithm.

Surface accessibility of envelope protein

To quantify solvent-accessibility surface area of the HIV-2 gp125 (PBD ID:5cay), DSSP running web server was used (Touw et al., 2015). Relative solvent accessibility (RSA) was calculated as described by Tien et al (Tien et al 2013). Residues were considered to be exposed on the surface when residues of RSA of ≥5% (Miller et al., 1987). HIV-2 envelope protein (PBD ID: 5cay) was used to visualize the surface exposed positively selected amino acids, by Chimera programe.

Statistical analysis

One-way ANOVA analysis was performed to compare RT activities, transduction efficiencies and LTR-induced GFP positivity of wild-type and Tat mutant HIV-2 transduced GHOST(3) cells, using GraphPad Prism 7.0. For analysis of dual infection experiments: statistical analysis using One-way ANOVA analysis of variance for independent samples were used to show the difference of HIV-1 infectivity when a wild-type or mutant HIV-2 CGP vector, and when wild-type or mutant Vpx coding pcDNA3.1-Vpx-NeGFP vector was used.

Regarding the phylogenetic analysis: IBM SPSS Statistics 21 was sued to perform statistical analyses for cohort study: two-tailed Mann-Whitney U test, two-tailed Fisher's Exact Test, Friedman's test for compare multiple groups and Spearman's rho for correlation analyses. Bonferroni corrected Wilcoxon signed rank test was performed after significant Friedman's tests**.**

RESULTS

Inhibitory effects of HIV-2 Vpx on the replication of HIV-1

HIV-1 and HIV-2 dual transduction assays

In the case of simulataneous transduction experiments, HIV-1 related fluorescence was significantly decreased by more than 60%, compared to that of HIV-1 mono-infected cells (P-value = 0.001), while HIV-2 related fluorescent signal did not change significantly (P-value = 0.7). When HEK293T cells were pre-transduced with HIV-2 virions followed by HIV-1 "superinfection", HIV-1 related fluorescent signal decreased by more than 80 % (P-value ≤ 0.01), indicating a protective nature of HIV-2 againts HIV-1 "superinfection".

HIV-2 Vpx protects cells against "superinfection" with HIV-1

To determine which HIV-2 protein plays a role in the inhibitory mechanism of HIV-2 against HIV-1 "superinfection", HEK293T cells were transfected with CGP plasmid carrying defective HIV-2 genes, and the cells were "superinfected" with HIV-1 pseudovirions. Following transfection of cells with HIV-2 CGP vector coding for a defective protease, Rev, Tat, Vpr and Vif, HIV-1 transduction efficiency decreased by 40 %, 30 %, 51 %, 36 % and 45 %. HIV-1 "infectivity" was not altered when cells were pre-transfected with the control HIV-2-CRU5SIN-WPRE vector. However, when cells were first transfected with HIV-2 CGP vector encoding a functionally restricted Vpx, HIV-1 "infectivity" was restored (P-value ≤ 0.05), reaching levels similar to those obtained in mock-transfected cells (P-value = 0.32).

HIV-1 transduction of GFP-Vpx expressing cells

Based on the previous findings, HEK293T cells were transfected with pcDNA3.1 vector coding for HIV-2 Vpx in fusion with GFP (pcDNA3.1-Vpx-NeGFP), followed by transduction with HIV-1. The sequence of the encoded *vpx* gene is identical to that found in the wild-type HIV-2 CGP vector. A mock vector was designed by restriction of the *vpx* coding sequence from the pcDNA3.1 plasmid using KpnI and XbaI restriction endonucleases. In the presence of HIV-2 Vpx, HIV-1 infectivity (transduction efficiency) was reduced by more than 80 % compared to the control transduction. Western blotting was used to detect the intracellular expression of Vpx following transfection with pcDNA3.1-Vpx-NeGFP and HIV-2 CGP vectors. These results indicate that HIV-2 Vpx plays a crucial role in the inhibition of HIV-1's replication.

Effect of Vpx on the replication of HIV-1

To characterize the inhibitory effect of HIV-2 Vpx on HIV-1, we performed qPCR of HIV-1 2-LTR junctions. When HEK293T cells were first pre-transfected with a Vpx-GFP coding plasmid followed by transduction with HIV-1 pseudovirions, the copy number of 2-LTR circle junctions were reduced by > 40 % (P-value < 0.01). Furthermore, p24 capsid production and RT-activity were hampered.

Vpx-GFP incorporation into HIV-1 pseudovirions

We studied whether or not Vpx-GFP is incorporated into HIV-1 pseudovirions. For this, HIV-1 pseudovirions were produced and collected from cells pre-transfected with a plasmid coding for Vpx-GFP. Western blotting was used to detect the presence of the 37 kDa Vpx-GFP fusion protein from lyzed pseudovirions, and p24 capsid was used for normalization. The harvested virions were used to transduce HEK293T cells, and the presence of Vpx-GFP was confirmed using a fluorescent microscope.

Experiments on THP-1 cells

When THP-1 cells were transduced with both HIV-1 and 2 pseudovirions concomitantly, or when HIV-1 "superinfection" followed pre-transduction with HIV-2, the transduction efficiency of HIV-1 did not change significantly. When THP-1 cells were activated and differentiated into macrophages using PMA, then transfected with the pcDNA3.1-Vpx-NeGFP plasmid followed by a transduction of HIV-1; HIV-1-related mCherry fluorescence was restored. And similarly to results obtained in HEK293T cells, pre-transfection with wild-type Vpx decreased the transduction efficiency of HIV-1 (P-value ≤ 0.05).

Effects of Y44A mutation in the Pro-rich domain of HIV-2 tat on the expression and activity of RT, and the transactivation of proviral genome

***In silico* characterization of HIV-2 Tat mutations**

Disorder predictions show that while the central region (39-78 residues) of the protein has a globular nature, both N- and C-termini are unstructured. SWISS-MODEL repository was used to map potentially destabilizing mutations, as no crystal or NMR structures are available for HIV-2 Tat. To map possible inactivating mutations, structure-based methods were performed. Alanine-scanning was performed using FoldX algorithm and SDM server. Mutations which showed destabilizing nature of alanine-substitution (G36, L40, Y44, L47, L72, and G80); mainly in the central region of the protein, may induce changes in the structure and stability of the protein, which was proven using

sequence-based prediction by I-Mutant server. IUPred2A and Jpred4 servers were used to predict the effect of Y44A mutation in the secondary structure of HIV-2. Y44A mutation in HIV-2 indicate disruption of the α-helix at the N-terminal part of the globular region.

In vitro characterization of HIV-2 Y44A mutant Tat

Experiments in GHOST(3) cells

GHOST(3) cells contain a *tat*-dependent HIV-2 LTR-GFP construct, and a GFP fluorescence is obtained in response to transduction with a functional Tat. To examine the effect of Tyr mutations on Tat-induced HIV LTR transactivation, HIV indicator cells were infected with 5 ng (normalized for p24 capsid) of virions. GFP fluorescence signal was significantly decreased by more than 93 % and 91 % in the presence of HIV-2 Tat Y44A and Y55A mutations, respectively (P value < 0.0001), compared to that of the wild-type. Furthermore, dot-blotting was used to confirm the presence of HIV-2 Tat from cell lysate, following transduction, and also in the virions.

Effects of Tat mutations on RT

RT activity was determined from viral supernatant using an ELISA-based colorimetric assay. RT activity was decreased by 97% for Y44A and by 96% for Y55A Tat mutations, compared to the wild-type HIV-2 (P-values < 0.0001), implying a detrimental effect of the mutations on the activity of RT. Western-blot was carried out to determine whether the Y44A Tat mutation had any effect on the quantity of RT packaged into the pseudovirions. RT was undetectable in the presence of Y44A mutant Tat from the lysate of pseudovirions. This finding may explain why RT activity was abolished, while the amount of capsid and Tat in the pseudovirions was not affected by the mutation. To explore the changes in RT as a result of the mutation, HEK293T cells were transfected with wild-type and Y44A mutant Tat coding HIV-2 CGP plasmids, and changes in RT quantity was then followed by Western-blotting of transfected cell lysate over a period of 3 days. After 24 hours of transfection, the amount of RT was lower in the presence of Y44A mutation compared to that found in the wild-type, and after 3 days, RT was undetectable.

Quantification of HIV-2 DNA

During qPCR reactions, several different controls have been used to establish the specificity: extracted DNA from U1 cells, DNA from HIV-1 infected patients, and DNA from negative participants. None of the control samples gave positive results, thus,

specificity was near 100%. Sensitivity of our assay was near 100% at 5 copies/reaction (detected in 14/14 runs, 0.69 $\log_{10}$ with SD 0.44), while 78% at 1 copy/reaction (11/14 runs). Limit of detection (LOD, respectively) was found to be 1 copies/reaction (11/14 runs), and the median correlation coefficient was 0.999 (range: 0.998-1.000), and limit of quantification (LOQ, respectively) was 5 copies/reaction at CT 33 (SD: 0.9). Overall, detection limit of proviral DNA was 0.5 copies/10^5 leukocytes. To exclude the possibility that human DNA contamination might have any effect on the sensitivity of our assay, qPCR was also performed in the presence of 1 µg PM-1 cell's DNA. And no effect was observed in the presence of background DNA.

Low postseroconversion $CD4^+$ T-cell level is associated with faster disease progression, and higher viral evolutionary rate in HIV-2 infection

Phylogenetic analysis of V1-V3 region of *env* sequences

Maximum likelihood analysis was performed on 409 *env* sequences collected from 16 study participants. For each participants, in each time-point, a median of 7 clones were sequenced, in total, 528 sequences, however, for evolutionary analyses, 119 recombinant sequences were removed, thus, only 409 sequences were used. Phylogenetic analyses also showed that all the sequences are subtype A.

Classification of HIV-2 infected participants into faster and slower progressors

Classification of study participants into different group of progressors was based on the dynamics of longitudinal CD4+ T-cells. All HIV-2 infected cohort members were classified into two groups: faster and slower disease progressors. To define faster and slower progressors, 3 stratifications were used: the decline rate of CD4 %, the level of CD4% at the midpoint between the first and last records, and the combination of the decline rate and the level of CD4 %. Based on decline rate of CD4 %: 111 patients were categorized as slower progressors, and 81 patients were classified as faster progressors. Mean CD4 % decline rate was 2.7% /year (SD: 2.6), and the median time to AIDS was 11.7 years (95% CI 7.3-16.1) for faster progressors. Mean CD4% decline was 0.5% / year (SD: 1.6) and the median time to AIDS was 16.8 years (CI 12.3-21.3) for slower progressors, from the first recorded CD4 % (p=0.008). The level of CD4%; at the midpoint between the first and last recorded CD4% levels, shows 87 patients as faster progressors with a mean CD4% level of 21.0% (SD 4.3), and the median time to AIDS was 9.4 years (CI 6.7-12.1). While 105 participants were classified as slower progressors with a mean CD4 % level of 35.0% (SD 5.7), and the median time to AIDS was 15.5

years (CI 14.3-16.6) (p<0.001). When the decline rate and the level of CD4 % was combined: 85 patients were faster progressors, where the median time to AIDS was 8.6 years (95% CI 6.5-10.8) and 107 participants were slower progressors with 18.7 years (CI 13.6-23.8) median time to AIDS (p<0.001).

HIV-2 evolutionary rate association with the level of CD4%

Evolutionary rate of HIV-2 was evaluated within hierarchical phylogenetic modelling (HPM) framework. Evolutionary rate of V1-C3 region was $23.5x10^{-3}$ codon substitutions/site/year. When classification was based on CD4% level or a combination of CD4 % decline rate and level, the mean evolutionary rate was significantly higher for faster disease progressors ($28.6x10^{-3}$ codon substitutions/site/year). Evolutionary rate on the other hand was only $14.9x10^{-3}$ codon substitutions/site/year for slower progressors. Also, strong association was found between disease progression and evolutionary rate in the first variable regions (V1V2) (BF=11.8), and the conserved domains of Env (C2 (BF=28.4); C3 (BF=6.1)). To find out the difference in evolutionary rate between different disease progressors, ratio of non-synonymous to synonymous substitution rates (the dN/dS rate ratio) were estimated. When dN/dS rate ratios were compared between the different groups for different regions, only the C2 regions was found to be strongly negatively selected in faster progressors, compared to slower progressors (BF=3.7).

Positive selection in conserved sites of HIV-2 envelope in slower disease progression

To estimate the number of residues that go under positive selection during disease progression, a renaissance counting procedure was used to estimate the dN/dS rate ratio at each codon site. The number of positively selected sites was similar in the V1/V2, V3 and C3 regions in both groups. C2 region of Env in slower progressors has a higher number of positively selected sites, compared to that in faster progressors (35 vs 20 sites; *P*=0.026, two-tailed Fisher's Exact Test [FET]). Some of the amino acids of Env, which are critical to viral fitness, are conserved in HIV-2. Among the 246 amino acids, 84 were conserved in HIV-2 V1-C3 regions, and 20 conserved amino acids showed positive selection in slower progressors, compared to only 5 in faster progressors (*P*=0.002, FET). After visualization of these positively selected amino acids, the majoritiy (15 of 22) were surface-exposed. Furthermore, surface accessibility of amino acids showed that the positively selected amino acids were associated with solvent-exposed surfaces in the C2 region (29 out of 68 AAs, P=0,04), FET).

DISSCUSSION

HIV-1 and HIV-2 share a common ancestral origin, and a similar replication cycle; however, HIV-2 differs from its counterpart in many aspects. Infection by HIV-2 is mostly characterized by lower pathophysiology and reduced transmissibility. While most HIV-2 infected patients were registered in West African countries (Sousa et al., 2016), the incidence of HIV-2 infection had been on the rise in many European countries (Soriano et al., 2000) (Barin et al., 2007) (Dougan et al., 2005) (de Mendoza et al., 2014) (Ruelle et al., 2008). Patients can be dually infected with both HIV viruses, where the presence of the two viruses overlaps. The mechanism of dual infection is poorly understood. While Travers and his colleagues suggested protection of HIV-2 against HIV-1 infection (Travers et al., 1995), other studies did not reach this conclusion (Norrgren et al., 1999) (Greenberg, 2001).

Our goal was to construct an *in vitro* model to study HIV dual infection, and determine whether HIV-2 regulatory and accessory proteins might have any interfering role in HIV-D. In our dual infection assays, simultaneous infection of HEK293T cells with both HIV virions resulted in a decrease in the infectivity of HIV-1 by more than 60 %, while that of HIV-2 had not changed, "superinfection" of HIV-2 pre-transduced cells by HIV-1 resulted in a 90 % decrease of HIV-1 related fluorescence signal. In order to determine the protein responsible for the decrease of HIV-1's infectivity, we carried out dual sequential transduction assays using functionally restricted accessory/regulatory HIV-2 proteins. Our results showed that in the presence of a functionally restricted Vpx, HIV-1 infectivity was restored. Furthermore, we carried out experiments using a vector coding only for a Vpx-GFP fusion protein, results of which showed that the infectivity of HIV-1 decreased by more than 80 %. In the presence of Vpx, the 2-LTR circle junctions amount of HIV-1 was significantly reduced by > 40 %. We also found that Vpx has an inhibitory effect on HIV-1 capsid production and RT activity. Additionally, Vpx incorporation into HIV-1 pseudovirions was also demonstrated using Western blotting. When we experimented the HIV-1 superinfection of Vpx pre-transfected differentiated THP-1 monocytes, HIV-1 related fluorescency was decreased by > 80 % in the presence of Vpx, which was similar to that observed in HEK293T cells.

Another aim was to study the effect of mutations in the Pro-rich (acidic domain) of HIV-2 Tat *in silico* and *in vitro*. Using sequence-based predictions and stability analysis, we found that Y44A substitution may inactivate the protein. Based on our *in silico* analyses, we assumed that Y44A mutation may inactivate HIV-2 Tat or negatively affect

its function. We carried out a series of *in vitro* experiments to study the inactivating nature of the Y44A mutation. To determine the effect of the Y44A Tat mutation on transactivation of the proviral genome, we carried out experiments in GHOST (3) HIV indicator cell line. When GHOST(3) cells were transduced with HIV-2 pseudovirions carrying mutant Tat (Y44A), LTR-induced GFP signal was significantly decreased by more than 90 %, compared to that observed for wild-type HIV-2 Tat. Our finding is in good agreement with the previous studies, which showed that the 38-48 region is crucial for transactivation of the LTRs in HIV-2. HIV-1 Tat interacts with both subunits of RT *in vitro*, and this interaction can stimulate the activity of RT (Apolloni et al., 2007). To examine whether HIV-2 Y44A mutation has any effect on the activity and expression of RT, we carried out a series of experiments *in vitro*. Our findings showed that RT was undetectable in pseudovirions carrying the Y44A mutant Tat. We carried out further experiments to detect the changes in RT in transfected cell lysate by Western-blot. While RT was still detectable in day 3 post-transfection in the case of wild type, it was undetectable in day 3 in the presence of Y44A Tat mutant. Our findings indicate that Tat plays an important role in the stabilization of RT, and perhaps, Y44A Tat mutation may have induced the degradation of HIV-2 RT in a ubiquitination-dependent, or -independent manner.

While plasma viral load of the two viruses differ, proviral DNA load was reported to be similar for both viruses (Popper et al., 1999) (Popper et al., 2000). We aimed to develop a sensitive cellular HIV-2 DNA quantification method using whole blood. HIV-2 DNA load was normalized to PBDG host gene, and both specificity and sensitivity of the assay was 100 %. Furthermore, we were able to detect viral DNA in participants with undetectable viral RNA.

Even though HIV-2 infection is mostly characterized by a long asymptomic phase, infection in some patients can rapidly progress, similarly to HIV-1 infection (Van der Loeff et al., 2010). To determine the association between evolutionary dynamics of HIV-2 and disease progression, firstly, we classified participants of a cohort into groups of faster and slower progressors based on longitudinal $CD4^+$ T-cell datas. We found that CD4% level or the combined CD4% level/decline rate stratifications were associated with the evolutionary rate of HIV-2 infection, and it was almost twice as high in the case of fast disease progressors, compared to slower progressors. We could also determine that slower progressors had more residues that underwent positive selection in the C2 region

of *env,* compared to faster disease progressors. These positively selected amino acids in the C2 region; which are conserved in the envelope of HIV-2/SIVsm, are surface exposed.

In conclusion, our analyses of dual infection with both of HIVs, and experiments on the association between viral evolutionary rate and disease progression of HIV-2, may help to understand the clinical manifestation of HIV-2 and dual infection with HIV-1 and HIV-2 viruses. Our findings may provide new insights into associations between pathogenesis and evolution of HIV-2 virus, and may provide a base to understandwhy HIV-1/2 dual infection and HIV-2 mono-infection is less pathogenic, compared to HIV-1 mono-infection.

SUMMARY

In summary, we presented a cell culture model to study and analyze HIV-1 and HIV-2 dual infection. In the presence of HIV-2, HIV-1 transducing capability was significantly decreased. After carrying out loss-of-function mutations in HIV-2 accessory and regulatory genes, we were able to identify viral protein X as the protein implicated in the diminishment of HIV-1`s infectivity (in both HEK293T and activated THP-1 cells). HIV-1 transduction of Vpx transfected cells resulted in decreased quantity of 2-LTR circle junctions, impaired capsid production, and hindered RT activity of HIV-1.

Also, we were able to show that even though HIV-2 Tat protein shares similarities in its structure and function with its HIV-1 counterpart, the N-terminal region; which differs the most between the two proteins, plays a crucial role in viral replication. Our results suggest that Tat Y44A mutation has a negative effect on the infectivity, expression and stability of HIV-2 RT. Our findings highlight the importance of the Pro-rich domain in the regulation and activity of HIV-2 RT, unlike in Tat protein of HIV-1.

To understand the mechanism of disease progression of HIV-2, we analysed HIV-2 envelope sequences to determine the connection between disease progression and viral evolutionary rate, with our collaboration partners. Our analysis shows that CD4% level, or the combined CD4% level and decline rate stratifications are associated with evolutionary rate of HIV-2. We classified the study participants into groups of slower and faster progressors, based on their longitudinal T helper cell datas. Our results show that the evolutionary rate of faster progressors is twice as high, compared to that of slower progressors, and this would appear to indicate that high evolutionary rate implies faster disease progression. Additionally, we could determine that more residues in the C2 region of envelope went under positive selection for slower progressors, compared to faster progressors; and these residues are surface exposed. Last but not least, we developed a sensitive HIV-2 DNA quantification protocol, which could be applied when viral plasma RNA level is undetectable. We are confident that our proviral DNA assay provides a sensitive approach to HIV-2 DNA viral quantification from whole blood, and will aid in the monitoring and diagnosis of HIV-2 infection in the future.

Altogether, we believe that our findings may help to understand the mechanism of dual-infection and disease progression of HIV-2; highlight the importance of the Pro-rich (acidic) domain of HIV-2 Tat, and help to monitor HIV-2 infection and the success of therapy, when viral RNA is under quantification level or undetectable.

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List of publications related to the dissertation

1. **Szojka, Z.**, Mótyán, J. A., Miczi, M., Mahdi, M., Tőzsér, J.: Y44A Mutation in the Acidic Domain of HIV-2 Tat Impairs Viral Reverse Transcription and LTR-Transactivation.
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   DOI: http://dx.doi.org/10.3390/ijms21165907
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List of other publications

5. Karlsson, I., Tingstedt, J. L., Şahin, G. Ö., Hansen, M., **Szojka, Z.**, Buggert, M., Biague, A., da Silva, Z., Månsson, F., Esbjörnsson, J., Norrgren, H., Medstrand, P., Fomsgaard, A., Jansson, M., Sweden-Guinea-Bissau Cohort Research Group: Cross-Reactive Antibodies With the Capacity to Mediate HIV-1 Envelope Glycoprotein-Targeted Antibody-Dependent Cellular Cytotoxicity Identified in HIV-2-Infected Individuals.
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LIST OF POSTERS

2020: V. Varga, *Zs. Szojka,*T. R. Linkner, M. Mahdi and J. Tőzsér. The role of HIV-2 Vpx in dual HIV infectionand analysis of its function in the virallife cycle, 13th Molecular Cell and Immune Biology Winter Symposium, Debrecen, Hungary

2020: *Zs. Szojka,*T. R. Linkner, V. Varga*,* M. Mahdi and J. Tőzsér. Elucidating the role of HIV-2 viral protein X. Viruses conference. Barcelona, Spain

2019: M. Mahdi, *Zs. Szojka,* J. A. Mótyán and J. Tőzsér. HIV-2 Vpx mediates inhibition of HIV-1 through interaction with the reverse transcriptase. GINOP, Debrecen, Hungary

2015: M. Mahdi, *Zs. Szojka,* and J. Tőzsér. Evaluating the efficacy of protease inhibitors against HIV-2, and the effect of the I54M-L90M double mutation. 27th International Workshop on Retroviral Pathogenesis. Mülheim an der Ruhr, Germany.

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2013: M. Mahdi, *Zs. Szojka,* K. Matuz and J. Tőzsér. HIV-2 protease as a chemotherapeutic target. Hungarian Molecular Life Sciences. Siofok, Hungary

2013. *Zs. Szojka*, M. Mahdi, J. Tőzsér. Biochemical and Kinetic characterization of HIV-2 Protease. 6th Molecular Cell and Immune Biology Winter Symposium, Galyatető, Hungary

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