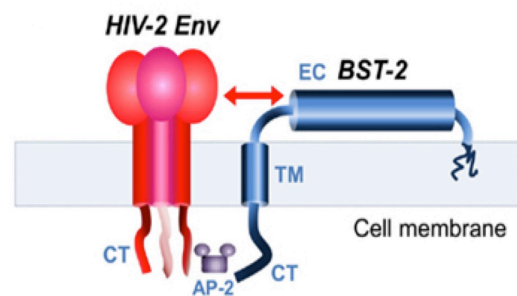


Multilayered and versatile functions of the HIV-2 envelope glycoprotein in the viral replication cycle



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Abstract

The Human Immunodeficiency Virus type 2 (HIV-2) is the second etiologic agent causing AIDS (acquired immune deficiency syndrome) in the human host. However, this retrovirus may be considered as an attenuated model of retroviral infection since the HIV-2-infected individuals show a longer asymptomatic phase, lower plasmatic viral loads without antiviral therapy, and do not experience AIDS-like symptoms in 60-70% of cases, in striking contrast with HIV-1-infected patients. HIV-2-positive individuals appear to control the virus replication and the main hypothesis that could explain this phenotype is a strong T cell-mediated immunity and a robust early innate immune response against HIV-2. In this thesis research project, we focus on a host restriction factor involved in the antiviral immune response: BST-2/Tetherin. This interferon (IFN)-inducible protein is able to retain the budding virions at the plasma membrane of the infected cells, and promotes the endocytosis and degradation of the “tethered” viral particle. HIVs have evolved means to counteract BST-2/Tetherin in order to ensure the viral dissemination in the human host. HIV-1 uses the accessory Vpu protein to bind and degrade BST-2/Tetherin, whereas HIV-2 - that lacks Vpu - relies on its envelope (Env) glycoprotein. It has been reported that the HIV-2 envelope glycoproteins bind and remove BST-2 from the cell membrane, but this countermeasure does not result in the degradation of BST-2. HIV-2 Env and BST-2 interact mainly through their ectodomain. However, little was known concerning the domain in the Env protein that can exert this function.

During this research project, we first revealed the precise motif within the ectodomain of HIV-2 Env protein involved in the anti-tetherin function. Indeed, we demonstrated that the N659 residue is clearly involved in the antagonism of BST-2/Tetherin. We also showed that the cytoplasmic tail (CT) of the HIV-2 Env protein cooperates in this anti-tetherin function.

BST-2/Tetherin can also prime the expression of numerous antiviral genes via the activation of the NF- κ B signaling pathway - a key regulator of the innate and adaptive immune response toward viral pathogens. The activation of the NF- κ B transcription factor results in the expression of numerous genes involved in the antiviral response. Interestingly, we demonstrated that the HIV-2 Env glycoprotein, like HIV-1 Env, acts as a potent activator of the NF- κ B signaling pathway and that the HIV-2 Env CT is involved in this activation. However, while HIV-1 Vpu completely abolishes the activation of NF- κ B at the late stages of infection mainly through the potent antagonism of BST-2, we

revealed that HIV-2 is unable to inhibit NF- κ B primarily due to the inefficient Env-mediated BST-2 antagonism. As a consequence, numerous antiviral genes are upregulated during the late stages of the HIV-2 viral cycle, as compared with HIV-1. Thus, we may conclude that the differential regulation of the NF- κ B-mediated antiviral response - through the viral countermeasure of the BST-2 restriction - is a major determinant of the differences between HIV-1 and HIV-2 pathogenesis, and may explain at least partly the stronger immunological control of the HIV-2 infection commonly seen in the majority of HIV-2-infected individuals.

List of abbreviations

aa: amino acid
ab: antibody
ADAR1: double-stranded RNA-specific adenosine deaminase
ADCC: antibody-dependent cellular cytotoxicity
agm: African green monkey
AIDS: acquired immune deficiency syndrome
ALIX: ALG2-interacting protein X
AP: adaptor protein or activator protein
APC: antigen-presenting cell
APOBEC: apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like protein
ART: antiretroviral therapy
ARV: AIDS-associated retrovirus
BCL10: B cell lymphoma/leukemia 10
BST-2: bone marrow stromal antigen 2
 β -TrCP: beta-transducin repeats-containing protein
CA: capsid protein
CARD11: caspase recruitment domain-containing protein 11
CCL: chemokine ligand
CCR5: C-C chemokine receptor type 5
CD4: cluster of differentiation 4
CDK: cyclin-dependent kinase
cDNA: complementary deoxyribonucleic acid
C/EBP: CCAAT enhancer binding protein
cGAMP: cyclic GMP-AMP dinucleotides
cGAS: cyclic GMP-AMP synthase
CpG: cytosine-phosphate-guanine dinucleotides
CPSF6: cleavage and polyadenylation specificity factor 6
cpz: chimpanzee
CRF: circulating recombinant form
CT: cytoplasmic tail
CTL: cytotoxic T lymphocyte
CXCR4: C-X-C chemokine receptor type 4
CycT1: cyclin-T1
CypA: cyclophilin A
DC: dendritic cell
DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
den: Dent's mona monkeys
DNA: deoxyribonucleic acid
dNTP: deoxyribonucleotide triphosphate
drl: drill monkeys
dsDNA: double-stranded DNA
EC: elite controller
EIAV: equine infectious anemia virus
Env: envelope glycoprotein
ER: endoplasmic reticulum
ESCRT: endosomal sorting complex required for transport
FIV: feline immunodeficiency virus
FP: fusion peptide
gag: group-specific antigen
GALT: gut-associated lymphoid tissue
gor: gorilla
gp: glycoprotein
GPI: glycosyl-phosphatidylinositol
GPR1: G-protein coupled receptor 1
gsn: greater spot-nosed monkey
GTPase: guanosine triphosphatase
HIV: human immunodeficiency virus

HLA: human leukocyte antigen
 HLA-DR: human leukocyte antigen-DR isotype
 HR: heptad-repeat region
 HTLV: human T lymphotropic virus
 IFI16: IFN γ -inducible protein 16
 IFITM: IFN-inducible transmembrane protein
 IFN: interferon
 IFNAR: IFN receptor
 I κ B: inhibitor of κ B
 IKK: inhibitor of κ B kinase
 IL: interleukin
 ILT-7: immunoglobulin-like transcript 7
 IN: integrase
 Indel: insertion/deletion
 IMC: infectious molecular clone
 IRES: internal ribosome entry signal
 IRF: interferon regulatory factor
 ISG: interferon-stimulated gene
 ITAM: immunoreceptor tyrosine-based activation motif
 JAK: Janus kinase
 kb: kilobase
 kDa: kilodalton
 KE: Kennedy epitope
 LAV: lymphadenopathy-associated virus
 LCK: lymphocyte-specific protein tyrosine kinase
 LLP: lentiviral lytic peptide
 LTNP: long-term nonprogressor
 LTR: long terminal repeat
 MA: matrix protein
 mac: rhesus macaque
 MALT1: mucosa-associated lymphoid tissue lymphoma translocation protein 1
 MEKK3: MAP3K3 ; Mitogen-activated protein kinase kinase kinase 3
 MHC: major histocompatibility complex
 MIP: macrophage inflammatory *protein*
 mnd2: mandrill monkey
 mon: mona monkey
 MPER: membrane-proximal external region
 mRNA: messenger RNA
 MSD: membrane spanning domain
 mus: mustached monkey
 Mx: myxovirus resistance protein
 MyD88: myeloid differentiation primary response 88
 nAb: neutralizing antibody
 NC: nucleocapsid protein
 Nef: negative factor
 NFAT: nuclear factor of activated T cells
 NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
 NHP: non-human primate
 NK: natural killer
 NLS: nuclear localization signal
 nm: nanometer
 NPC: nuclear pore complex
 Nup: nucleoporin
 OASIS: cyclic AMP-responsive element-binding protein 3-like protein 1
 PAMP: pathogen-associated molecular pattern
 PBS: primer binding site
 PD-1: programmed cell death protein 1
 PI3K: phosphoinositide 3-kinase
 PIC: pre-integration complex

PKC θ : protein kinase C theta
 PLC γ 1: phospholipase C γ 1
 PM: plasma membrane
 Pol: polymerase
 PPT: polypurine tract
 PR: protease
 Pr: precursor
 PRA1: prenylated Rab acceptor 1
 PRR: pattern recognition receptors
 P-TEFb: positive acting elongation factor b
 PTL: parthenolide
 rcm: red-capped mangabey
 Rev: regulation of viral protein expression
 RER: rough endoplasmic reticulum
 RhoGEF: guanine nucleotide exchange factor for Rho GTPases
 RIG-I: retinoic acid-inducible gene I
 RM: rhesus macaque
 RNA: ribonucleic acid
 RNase H: ribonuclease H
 RRE: Rev-responsive element
 RT: reverse transcriptase
 RTC: reverse transcription complex
 SAMHD1: sterile alpha motif and histidine aspartate domain containing protein 1
 SERINC: serine incorporator protein
 shRNA: short hairpin RNA
 siRNA: small interfering RNA
 SIV: simian immunodeficiency virus
 Skp1: S-phase kinase-associated protein 1
 SLFN11: schlafen protein 11
 SM: sooty mangabey
 smm: sooty mangabey monkey
 SNP: single nucleotide polymorphism
 SP: signal peptide
 Sp: spacer peptide
 ssDNA: single-stranded DNA
 ssRNA: single-stranded RNA
 STAT: signal transducer and activator of transcription
 STC: strand transfer complex
 STING: stimulator of interferon genes
 TAK1 (or MAP3K7): transforming growth factor β (TGF- β)-activated kinase 1 or mitogen-activated protein kinase kinase kinase 7
 TASK1 or KCNK3: potassium channel subfamily K member 3
 TAR: trans-activation responsive element
 Tat: trans-activation of transcription
 T-bet: T-box transcription factor
 TBK1: TANK-binding kinase 1
 TCC: target capture complex
 TCR: T cell receptor
 TFII: transcription factor II
 Tfh: follicular T cell
 TGN: trans-Golgi network
 Th17: interleukin 17-producing T helper cell
 TIP47: tail-interacting protein of 47 kDa
 TLR: Toll-like receptor
 TMD: transmembrane domain
 TNF: tumor necrosis factor
 TNPO3: transportin 3
 TRAF: TNF receptor-associated factor
 Treg: regulatory T cell

TREX-1: three prime repair exonuclease 1
TRIM5 α : tripartite motif protein 5 alpha
tRNA: transfer RNA
Vif: viral infectivity factor
Vpr: viral protein R
Vpu: viral protein U
Vpx: viral protein X
wol: Wolf's mona monkey
ZAP70: zeta-chain-associated protein kinase 70

Chapter I: Introduction

1.1 Discovery and origins of the Human Immunodeficiency Viruses 1 and 2

More than 40 species of wild-living African non-human primates (NHPs) are naturally infected with lentiviruses with a high prevalence in several sub-Saharan countries of the African continent. These lentiviruses, belonging to the *Retroviridae* family, are called Simian Immunodeficiency Viruses (SIVs). One of the first species to be reported to be infected was the African green monkey (*Chlorocebus* species) and the prevalence of infection was estimated to be 50% in adults. The SIV strain infecting the African green monkeys was termed SIVagm (agm for African green monkey) and, in the same way, all SIVs were christened with the name of the corresponding NHP species. The great majority of NHP species harbors a single strain of SIV, thus suggesting that the virus transmissions are essentially species-specific. Although multiple strains of SIV can be found in particular monkey species, each virus is closely associated to a monophyletic clade [1-4]. Chimpanzees were also reported to be infected with SIVcpz, but phylogenetic analysis revealed that this SIV arises from past recombination events between two (potentially three [5, 6]) different SIVs infecting distinct monkey species: SIVgsn/mus/mon and SIVrcm, isolated from greater spot-nosed monkeys (*Cercopithecus nictitans*; gsn), mustached monkeys (*Cercopithecus cephus*; mus) and mona monkeys (*Cercopithecus mona*; mon), and from red-capped mangabeys (*Cercocebus torquatus*; rcm) respectively [6-8]. Chimpanzees could have acquired both ancestral viruses by hunting and eating smaller monkeys since this behavior has been reported throughout Africa [9-11]. Thus, chimpanzees acquired contemporaneous SIVcpz from interspecies transmissions and it is now well known and largely accepted that the SIVcpz is the immediate precursor of Human Immunodeficiency Virus type 1 (HIV-1), which is responsible for the pandemic AIDS (acquired immune deficiency syndrome) formally recognized in several young homosexual men in the USA in 1981 [12, 13]. In 1983, two research groups announced that a novel virus, closely related to retrovirus family, may be the etiological agent of AIDS [14, 15]. The first group, an American team in which Robert Gallo was involved, revealed that this virus was highly similar in morphology to other Human T Lymphotropic Viruses (HTLV-I and -II). So, the researchers termed this newly isolated virus HTLV-III. Concurrently, a French group isolated a virus from a patient with pre-AIDS symptoms, but contrary to the Gallo's group, Luc Montagnier and Françoise Barré-Sinoussi showed that several proteins encoded by this virus were

different from those of HTLV-I or -II. Therefore, this team designated this virus Lymphadenopathy-Associated Virus (LAV), which was the syndrome affecting the patient. In fact, a third group, led by Dr. Jay Levy at the UCSF, was also working on the isolation of this new pathogen. This research group discovered the AIDS-associated virus and named it the AIDS Associated Retrovirus (ARV) in 1983 [16]. Finally, it was in 1986 that these (similar) viruses - LAV, HTLV-III or ARV - were christened HIV-1 [17, 18].

The first indication that SIVcpz was the ancestor of HIV-1 came from the finding of a wild-born chimpanzee from Gabon infected with a SIVcpz (SIVcpzGAB1) that was genetically related to HIV-1 [19]. However, several studies indicated that among 50 wild-caught chimpanzees tested for SIVcpz infection, only two chimpanzees (GAB1 and GAB2) were positive for SIVcpz infection or had HIV-1 cross-reactive antibodies [20, 21]. Furthermore, a third wild-caught chimpanzee (Noah) imported in Belgium was reported to be infected with a highly genetic divergent virus compared with the two chimpanzees GAB1 and GAB2 [22, 23]. Unexpectedly, it seemed that SIVs were very rare in this species, and it appeared possible that chimpanzees and humans had been infected from some other sources, probably other unidentified monkey species in central or west Africa [24, 25]. Resolution of this apparent paradox arose from analyses of mitochondrial gene sequences of chimpanzee that allowed to divide this species into four distinct subspecies: western (*Pan troglodytes verus*), Nigerian (*Pan troglodytes ellioti*), central (*Pan troglodytes troglodytes*) and eastern (*Pan troglodytes schweinfurthii*) chimpanzees [26]. These subspecies present distinct geographical distributions (Figure 1) and it has been reported that GAB1 and 2 chimpanzees belonged to the *Pan troglodytes troglodytes* subspecies, whereas Noah belonged to the *Pan troglodytes schweinfurthii* subspecies. Importantly, all other chimpanzees tested negative for SIVcpz infection were related to the *Pan troglodytes verus* subspecies, the latter exhibiting a very low prevalence of SIVs [27-29]. These initial observations showed that all HIV-1 strains were more closely related to SIVcpz from chimpanzees *Pan troglodytes troglodytes* in west central Africa than to SIVcpz *Pan troglodytes schweinfurthii* from eastern chimpanzees.

All HIV-1 viruses fall into four different groups, with distinct origins and independent monkey-to-human jumps: group M, N, O and P [2, 8, 24, 25, 30]. Phylogeographic and phylogenetic analyses demonstrated clusters within the lineages of SIVcpz from *Pan troglodytes troglodytes* and SIVcpz from *Pan troglodytes schweinfurthii* and allowed to describe the immediate precursors of the pandemic

HIV-1 group M in southeast Cameroon and/or in the northwest of the Democratic Republic of Congo (Figure 1), and the ancestors of HIV-1 group N in chimpanzee populations in south and central Cameroon [31-33].

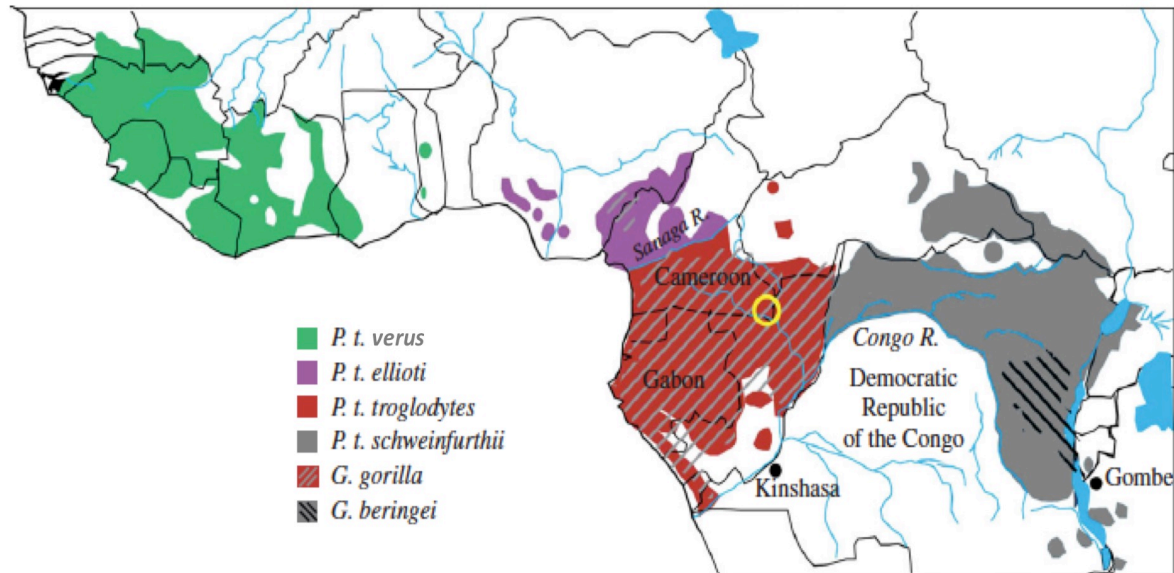


Fig.1: Geographical distributions of the four subspecies of chimpanzees and the two subspecies of gorillas that gave rise to the HIV-1 groups (subspecies ranges are color coded). The yellow circle depicts the probable site of HIV-1 origin (adapted from [24]).

HIV-1 group O and probably the recently discovered group P are related to another simian virus, SIVgor, identified in wild-living Western lowland gorillas (*Gorilla gorilla gorilla*) in Cameroon. Whereas the HIV-1 group M is widespread, the group O is restricted to Cameroon, Gabon and Equatorial Guinea with a low prevalence (~ 100,000 cases) and the HIV-1 group P prevalence is anecdotal (Figure 2) [2, 34-39].

Interestingly, the existence of a second human immunodeficiency virus, HIV-2, was suspected in 1985 with the observation of Senegalese patients who were infected with a virus more closely related to SIV than HIV-1 [40]. One year later, the virus responsible was isolated from blood sample from two AIDS patients (from Guinea-Bissau and Cape Verde, respectively) at the Paris Pasteur Institute by François Clavel and collaborators [30, 41-43]. The genomic structure and organization of this human virus were determined and, remarkably, it shared high similarities with HIV-1 virus [44, 45]. Several other cases of AIDS caused by this new human retrovirus (initially called HTLV-IV [46]) were described in different countries of West Africa [47].

Actually, the simian origin of human HIV-2 retrovirus was resolved before that of HIV-1, despite a later discovery. In 1984, SIVmac was identified in captive rhesus macaques (*Macaca mulatta*) in primate centers in the United States of America [48, 49]. However, it had been reported that free-ranging macaque monkeys were not naturally infected with SIVmac [50]. Shortly afterwards, another SIV was identified in captive monkeys, sooty mangabeys (*Cercocebus atys*) [51, 52]. This SIVsmm, very similar to SIVmac, were soon found in captive and in free-ranging sooty mangabeys living in West Africa [53-56] and reported to be at the origin of both SIVmac in macaques and HIV-2 in humans (Figure 2) [57, 58]. SIVsmm entered the human population through cross-species transmission on at least nine independent events, giving rise to nine HIV-2 groups (A to I). Phylogenetic and biogeographical analyses allowed to reveal that groups A, B, C, G, H and I are related to SIVsmm identified in sooty mangabeys from the Tai forest (Ivory Coast), group D is related to SIVsmm from Liberia, and groups E and F are related to SIVsmm from Sierra Leone. Among them, only the groups A and B are endemic and continue to spread in the human population, while the seven other lineages were only found in single individuals [59-63]. The exact route of cross-species transmission of the SIVsmm from sooty mangabeys to humans remains unknown but the most privileged routes are the bush-meat trade and the recreative use of sooty mangabey as pets [2, 24, 25, 64].

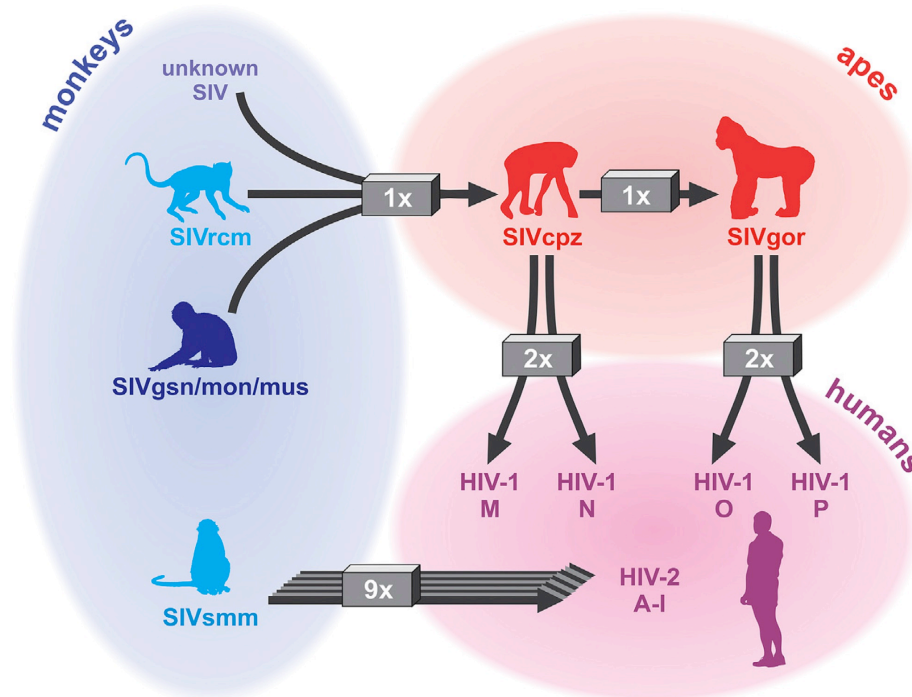


Fig.2: SIV cross-species transmissions. Several transmissions have occurred from monkeys to apes, and then from apes to humans arising the emergence of HIV-1 (groups M, N, O and P). HIV-2 (groups A to I) originated from direct transmission(s) of SIVsmm from sooty mangabey monkeys to humans (adapted from [6]).

Several studies have attempted to estimate the period when the ancestor of HIV-2 entered the human population. Viral gene sequences (*env* and *gag*) have been analyzed and the estimated time of most recent common ancestor for the group A was 1940 (± 16), and for the group B it was 1945 (± 14), approximatively one decade after the introduction of HIV-1 [65]. A more recent study conducted by Wertheim and Worobey in 2009 obtained nearly the same period for group A and B [66]. The introduction of the other groups of HIV-2 cannot be estimated by phylogenetic analyses because only single sequences are available without precise date of infection. In contrast to HIV-1, only two circulating recombinant forms (CRF, HIV strain arising from the recombination between two different subtypes or groups that circulates in the human population) of HIV-2 have been described so far. In 2010, CRF01_AB was detected in three subjects showing some evidence of disease progression and symptoms similar to AIDS caused by HIV-1 [67] and genetic sequences of this CRF were closely related to a HIV-2 recombinant form initially identified in 1994 from a patient in Abidjan [68]. The second new form of HIV-2 - which is rather an unique recombinant form (URF) - has been reported in Sierra Leone and is a particular form of the HIV-2 group F [69].

In contrast to HIV-1, HIV-2 is mainly restricted to West Africa where it infected ~ 2 million people. This number strongly needs to be re-evaluated across all African countries, especially in the light of data available in Guinea-Bissau showing a decreasing prevalence during the last years [70-72]. Several countries reported a prevalence of $>1\%$ in the population in the late 80's: Guinea-Bissau, Cap Verde, Ivory Coast, Gambia, Senegal, and Sierra Leone [73, 74]. The highest prevalence was reported in Guinea-Bissau. In this country, prevalence of HIV-2 infection was evaluated at 8% in adults and up to 21% in individuals over 40 years of age [75].

The groups A and B show two distinct distributions in West African countries. Group A is widespread in this area, while group B is only observed in Ghana, Ivory Coast, Burkina Faso and Mali [76]. It is surprising that the highest prevalence of HIV-2 group A is found in Guinea-Bissau, because this country is far from the presumed origin of this group in Ivory Coast. However, an identical observation has also been done for the HIV-1 group M strain: the chimpanzee reservoir has been observed in southeast Cameroon, far away from the source of the human epidemic in Kinshasa, the capital of the Democratic Republic of Congo [77, 78].

A phylogenetic study defined the geographical evolution and distribution of HIV-2 group A infection in Guinea-Bissau, by determining the effective number of infections. This study showed a strong increase of new HIV-2 infections in the middle of the 60's [65], which is concurrent with the Guinea-Bissau independence war (1963 to 1974). Therefore, Guinea-Bissau seems to be at the origin of the early dissemination of HIV-2 group A in West Africa and other former Portuguese colonies such as Angola, Cape Verde, Mozambique or several areas in India [79].

In the Northern countries, the two countries that are most seriously hit by HIV-2 infection are Portugal and France. In Portugal, HIV-2 represented up to 4.5% of AIDS diagnoses [80] and ~ 1,800 HIV-2-infected patients have been reported in 2008 [81]. In France, HIV-2 represents 1 to 2% of new HIV diagnosis with 1,000 patients included in the French HIV-2 ANRS cohort. Interestingly, these two countries present very different origins of HIV-2 infections, explained by their historical connections in Africa. Portugal is principally connected to Guinea-Bissau and Cape Verde, while France is mainly connected to Senegal and Ivory Coast. A few numbers of cases have been reported in USA, where 240 patients have been identified until 2009 [82]. Several surveys reported a decreasing HIV-2 prevalence in Senegal, Gambia and Guinea-Bissau [70, 83], highlighting that HIV-2 prevalence almost halved between 1987 and 2008 in Guinea-Bissau. It has been suggested that a decline of HIV-2 prevalence will occur in the next years, with an expected incidence under 0.5% of new infections by 2050 and a potential complete extinction of this virus in 2070. As shown in different studies, competition with HIV-1 seems to contribute only slightly to HIV-2 decline [72, 84, 85]. In conclusion, data allowing the prediction of a HIV-2 epidemic extinction are mainly based on the evolution of prevalence in Guinea-Bissau, even if some data from France and Portugal may also suggest a general decrease. Data available for HIV epidemiology in Africa are often based on less specific tests that may misclassify HIV infections (HIV-1 versus HIV-2 infections, or HIV-1/2 co-infections) and confuse the HIV-2 prevalence [86, 87]. In Belgium, although the distinction between HIV-1 and HIV-2 can be made since 2002, the number of effective HIV-2 infection cases reported by our laboratory (the AIDS Reference Laboratory of UCLouvain, Brussels) is 130 cases, which represents a very low prevalence and less than 1% of HIV infections. In Belgium, the number of persons living with HIV has been estimated around 19,000 at the end of 2017, corresponding to 1.7 persons per 1000 residents [88].

1.2 Viral particle and genomic organization of HIVs

The human immunodeficiency viruses are animal viruses from the *Retroviridae* family and belong to the lentivirus genus. Retroviruses form a family of enveloped viruses enclosing RNA molecule(s) as genome. This family is further divided into 7 distinct genera: *alpharetrovirus* (e.g. Avian Leukosis Virus), *betaretrovirus* (e.g. Mouse Mammary Tumor Virus), *gammaretrovirus* (e.g. Murine Leukemia Virus), *deltaretrovirus* (e.g. Human T lymphotropic Viruses; HTLVs), *epsilon*retrovirus (e.g. Walleye Dermal Sarcoma Virus), *lentivirus* (e.g. HIVs, Visna Virus, Equine Infectious Anemia Virus; EAIV) and *spumavirus* (e.g. Simian Foamy Virus and Bovine Foamy Virus). Recent bioinformatic research has revealed that retroviruses emerged during the Palaeozoic Era ($\pm$ 550 million years ago). Retroviruses genera share the common feature to replicate in vertebrate hosts through the unusual process of reverse transcription, i.e. the generation of a DNA genome from an initial viral RNA molecule [89, 90], also shared by *Hepadnaviridae* (as the Hepatitis B Virus) [91]. Based on these characteristics, HIV-1 and HIV-2 belong to the group VI following the Baltimore classification, which groups viruses with RNA genome (+ strand) with DNA intermediate in their replication cycle [92].

HIV viral particles are membrane-enveloped, present a spherical morphology and display a diameter of 100-120 nm on average (Figure 3 A). All retroviruses are obligate parasites and can enter into host cells, reverse-transcribe their positive-sensed single-stranded RNA molecules in double-stranded cDNA (complementary DNA) using their reverse transcriptase (RT), integrate this double-stranded DNA into the host genome using integrase (IN) enzymes, and ensure the production of viral mRNAs encoding all HIV proteins to generate new viral progeny that can bud from the infected cell in order to allow infection of surrounding cells [93, 94].

The viral particle consists of an envelope, i.e. a lipid bilayer which is actually the cell membrane of the infected cell that gave rise to the viral particle. Indeed, the budding virion carries with it a part of the infected cell membrane. An important viral protein is inserted into this external membrane: the envelope glycoprotein (Env) which is constituted of two subunits: gp120 or gp105 and gp41 or gp36 for HIV-1 and HIV-2 respectively. Just underneath the envelope, a lattice of matrix proteins (MA) allows Env proteins to be stably inserted in the membrane and allows also the viral particle to keep its spherical shape.

The two identical copies of viral single-stranded RNA are intimately covered by nucleocapsid

proteins (NC) which are themselves surrounded with a lattice of capsid (CA) hexamers that is closed by the insertion of 12 pentamers and forms a protective cone shaped core (Figure 3 A).

Structurally, the retrovirus RNA genome presents a length of ~ 9.7 kilobases and displays nine overlapping genes including several open reading frames that encode 16 viral proteins, which play essential functions in HIV replication. Like in all retroviruses, the HIV genome encompasses three major genes coding structural and enzymatic proteins: *gag* (for group-specific antigen), *pol* (for polymerase) and *env* (for envelope glycoprotein). The *gag* gene produces the Gag polyprotein precursor (Pr55^{Gag}) which is post-translationally cleaved by the viral protease (PR) to form matrix, capsid, nucleocapsid proteins and a small nucleic acid-stabilising protein, p6. Two spacer peptides (Sp1 and Sp2) are also included in the Pr55^{Gag}.

Downstream of the *gag* open reading frame, the HIV genome contains the *pol* gene coding a Pr160^{GagPol} processed to several highly conserved enzymes: protease, essentially involved in viral protein maturation in the budding virion, reverse transcriptase, and integrase. The *env* reading frame produces a glycosylated precursor protein (gp160 in HIV-1, gp140 in HIV-2), then cleaved by a cellular furin to form the extracellular glycoprotein and the transmembrane protein (see section 1.4 for more details on Env protein) [93, 95, 96].

The remaining genes in the HIV genome consist of two regulatory genes (*tat* and *rev*) and four accessory genes: HIV-1 and -2 both possess *vif*, *vpr*, and *nef* accessory genes, but only HIV-1 has a *vpu* gene, whereas HIV-2 encodes a *vpx* accessory gene [93, 96-99] (Figure 3 B and C).

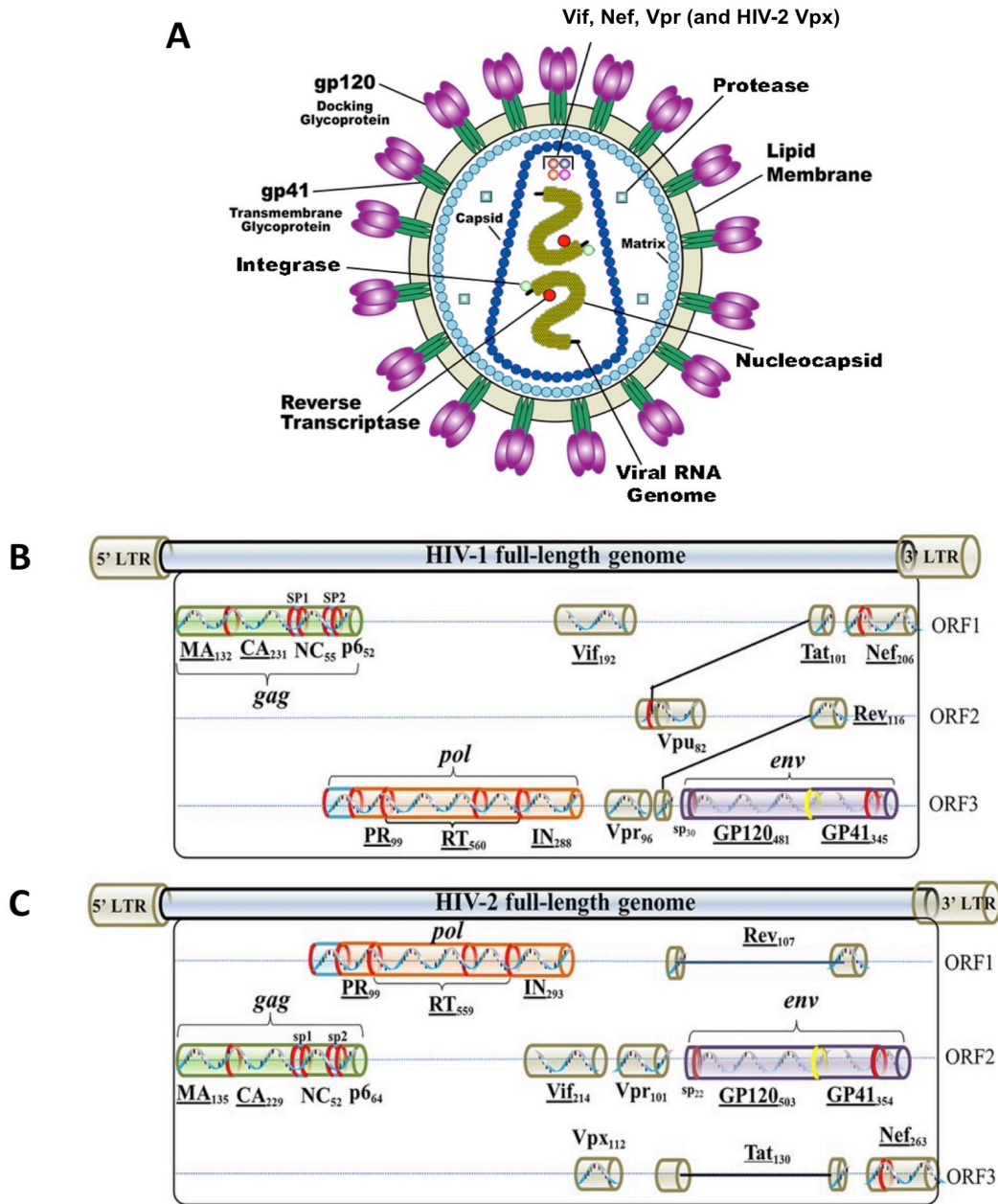


Fig.3: Viral particle and genomic organization of HIVs. **A.** Schematic representation of the HIV particle and the viral proteins that constitute the virion. Schematic models of the HIV-1 (**B**) and HIV-2 (**C**) full-length genome. The names and the numbers of amino acids of each HIV protein are indicated underneath the colored shape of the protein coding region in three open reading frames (red dashes indicate the sites of viral protease cleavage; yellow dash depicts the site of furin cleavage in the envelope precursor) (adapted from [99]).

The *vpu* gene is only found in SIVs infecting *Cercopithecus* species as SIVgsn/mus/mon, in SIVcpz and therefore in all groups of HIV-1. Evolutionarily, this accessory gene was probably acquired by the SIV infecting greater spot-nosed, mustached and mona monkeys (SIVgsn/mus/mon) [100-104]. It is interesting to note that Vpu protein shares some sequence and structural homologies with a mammalian K^+ ions channel, TASK1. This analogy suggests past infections of mammals with

retroviruses and highlights their potential ability to acquire and import cellular genomic sequences [103, 105-107].

Concerning *vpx* gene, it was acquired by SIVrcm, SIVdrl, SIVmnd2 and by SIVsmm, the ancestor of HIV-2, by recombination or duplication of the *vpr* gene [108]. This accessory gene was lost by SIVcpz after recombination events in the chimpanzee host, so HIV-1 does not encode *vpx* gene neither (Figure 4) [6, 108-110].

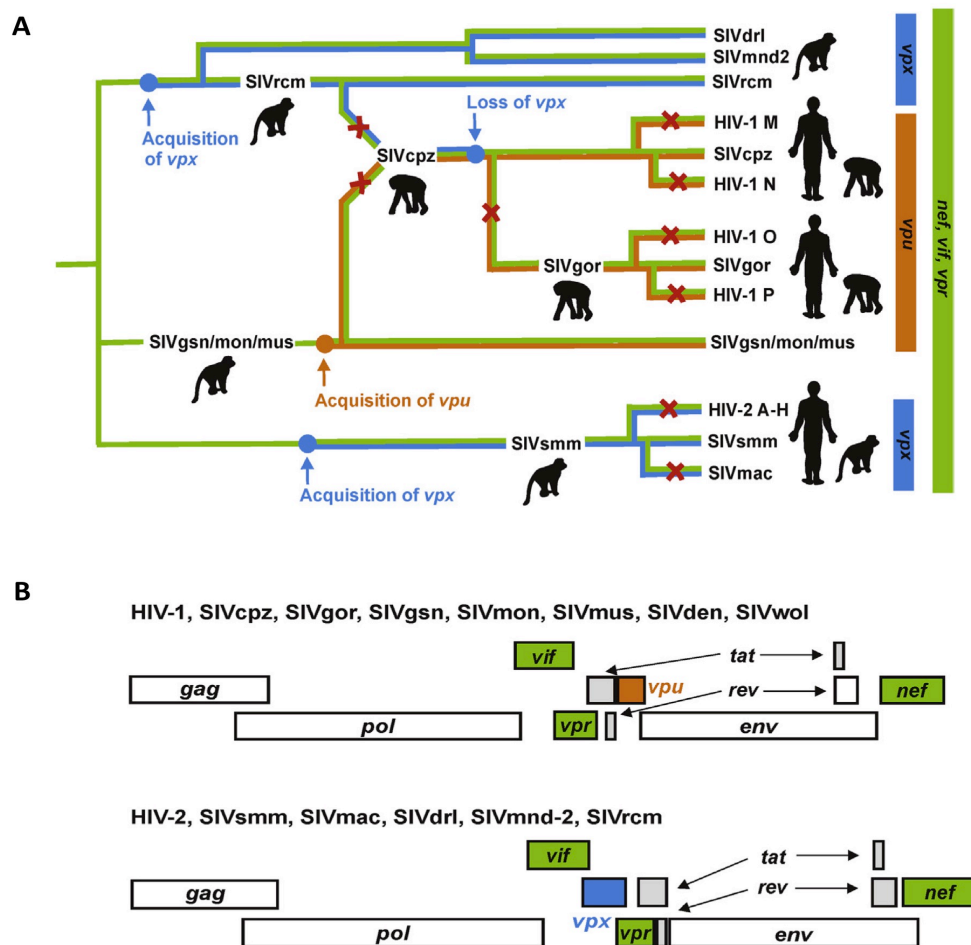


Fig.4: Acquisition and loss of *vpu* and *vpx* genes during the primate lentiviral evolution. **A**. The lines and rectangles colored in orange or blue show the presence of a *vpu* or *vpx* gene, respectively. Green rectangle indicates the presence of the accessory genes *vif*, *nef*, and *vpr*. Red crosses stand for cross-species transmissions. **B**. Genomic maps of the different SIVs and HIVs. mnd2: mandrill monkeys; drl: drill monkeys; den: Dent's mona monkeys; wol: Wolf's mona monkeys (adapted from [104]).

Molecular functions and features of the accessory proteins are detailed in chapter II of the present thesis. The different roles of each other viral protein will be described in the next section (i.e. « The viral replication cycle »).

The RNA genome of retroviruses containing the nine genes is flanked at its both edges by long terminal repeat sequences (LTRs), the 5' LTR and 3' LTR. These sequences contribute to the reverse transcription of the RNA molecules into DNA in the cytoplasm of newly infected cells, support viral DNA integration and act as promoter of viral gene transcription once the viral genome is integrated in the host genome, called provirus or proviral DNA [93, 96, 111]. LTRs are divided into three regions: U3, R and U5. The U3 region includes three functional domains, i.e. modulatory, enhancer and promoter domains (Figure 5).

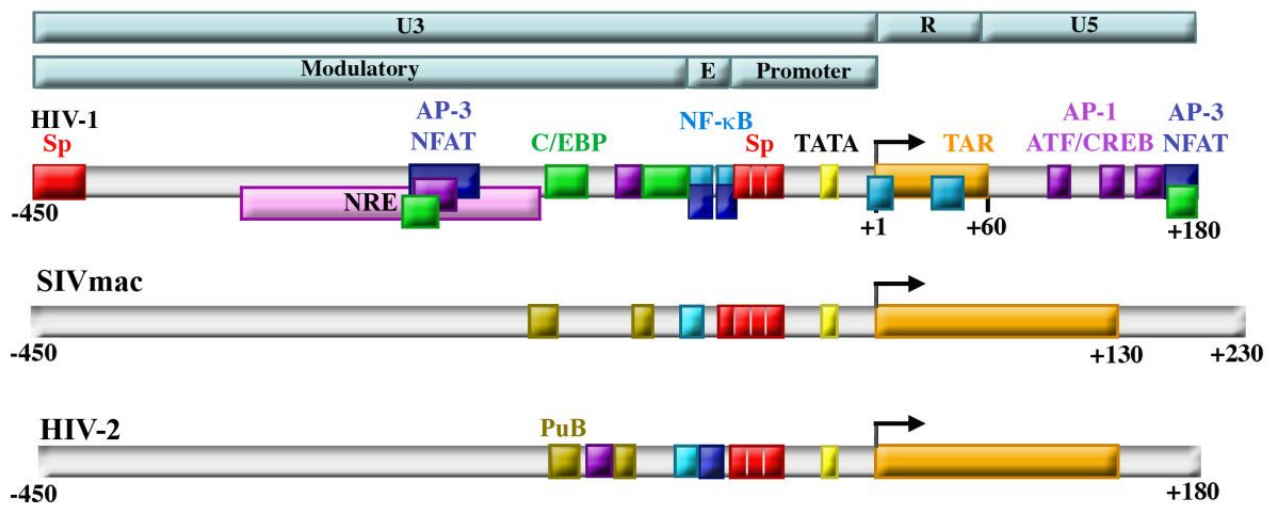


Fig.5: HIV 5'-LTR structure and binding sites of host transcription factors. LTRs are divided into the U3, R, and U5 regions. U3 is further divided into the modulatory, enhancer (E) and promoter domains, shown in the upper panel. TATA boxes (yellow) are highly conserved in all LTRs of HIV-1, SIV and HIV-2 (shown in the three bottom panels). Sp factor binding sites are depicted in red and the enhancer domain contains NF- κ B binding sites (shown in light blue). The R region displays the TAR element (trans-acting responsive element) in orange. NRE (negative regulatory element, in pink) serves as activator and repressor by binding NFAT proteins (blue), AP-1 proteins (purple), and C/EBP factors (green). The modulatory regions of SIV and HIV-2 also contain purine box bundles (PuB, gold) (adapted from [112]).

Prior to the expression of Tat, retroviral gene transcription relies on the host transcription factors to ensure the LTR-dependent gene expression. These host transcription factors mainly include the Sp family, activator protein 1 (AP-1) proteins, nuclear factor kappa B (NF- κ B) family, nuclear factor of activated T cells (NFAT/AP-3), and CCAAT enhancer binding protein (C/EBP) family members that exert key roles in the regulation of retroviral transcription by binding sites in the HIVs 5' LTR [112,

113]. Many of these host and viral proteins may form protein complexes to guarantee transcriptional regulation of viral genes. The LTRs of HIV-1, HIV-2, and SIV show a divergent number of binding sites for these host transcription factors, HIV-1 displaying the highest number and diversity of binding sites in its LTR sequences [93, 111, 112, 114].

The promoter region of LTRs includes a highly conserved and efficient TATA box to initiate the binding of the initiation factor TFIID, its associated transcription cofactors (TFIIA, B, E, F and H) and the mediator complex, thereby allowing RNA polymerases II to achieve viral gene transcription. In absence of Tat, recruitment of cellular transcription factors (TFs) to the HIVs promoter is sufficient to promote initiation of viral gene transcription. Nevertheless, only very low levels of mRNAs are produced because elongation is prematurely blocked and RNA polymerase II stops in a region nearby the transcription start site (Figure 6 A and B).

The LTR R region contains the trans-activation response element (TAR) implicated in Tat-mediated trans-activation. TAR actually forms an RNA stem-loop structure upon transcription which can bind to the viral protein Tat. Indeed, immediately after integration of viral DNA in the host genome, completely spliced mRNAs coding the regulatory viral proteins Tat and Rev are synthesized via recruitment of host transcription factors. Once expressed, Tat proteins enter the cell nucleus and interact with CDK9 kinase, CycT1 of the P-TEFb complex (positive acting elongation factor b). This complex shows high affinity for the TAR RNA stem structure in the R region of LTR. When Tat interacts with P-TEFb complex, it triggers the trans-activation mechanism that modifies positive and negative cellular elongation factors (Figure 6 C and D) ([113, 115] and references therein).

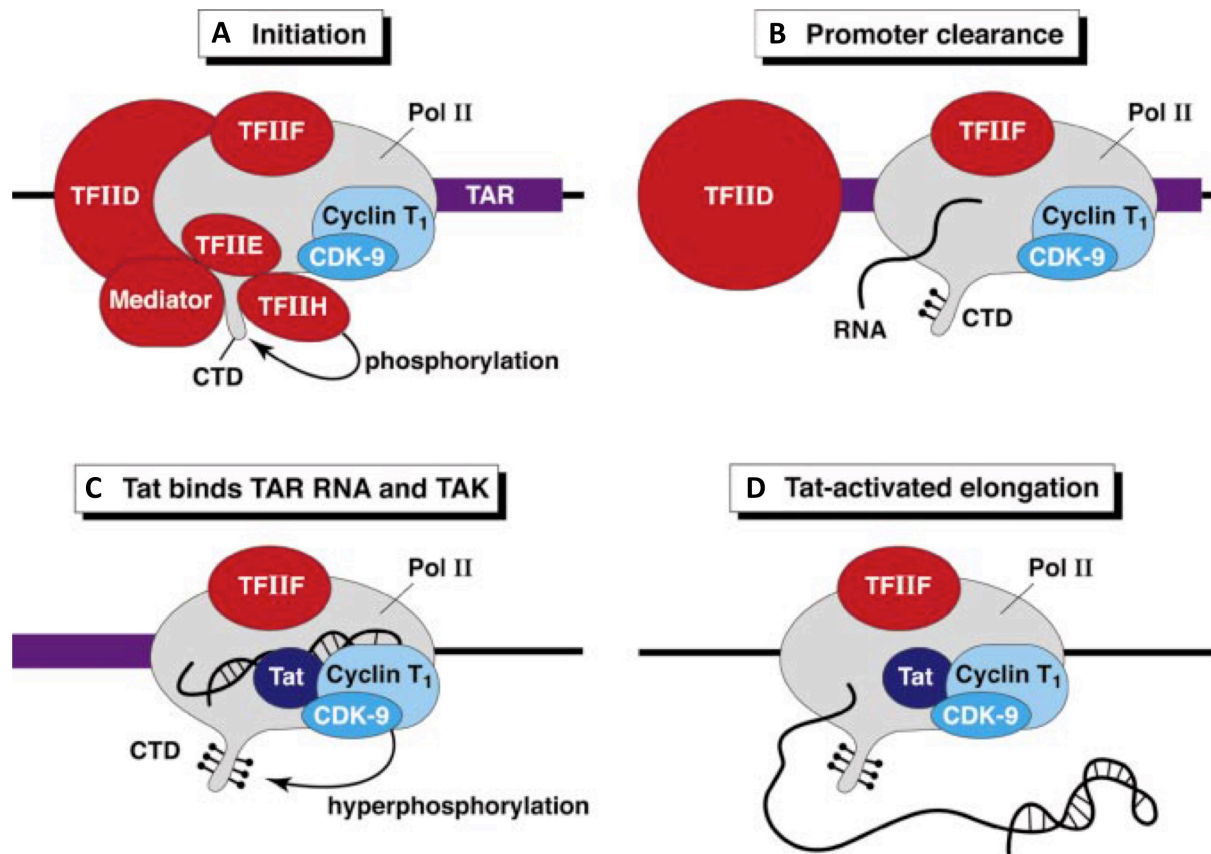


Fig.6: Tat and cellular cofactors involved in the activation of RNA polymerase II. **A.** The RNA Pol II is recruited to the LTR promoter, mainly via its interactions with TFIID. The C-terminal domain (CTD) of the RNA Pol II is phosphorylated by TFIIE. The RNA Pol II starts the transcription of TAR. **B.** The TAR RNA transcript forms a stem-loop structure and can bind the RNA Pol II. **C.** Tat associates with TAK (Tat-associated kinase) and is recruited in the transcription complex. Tat-TAK hyperphosphorylates the CTD of the RNA Pol II. **D.** The activated transcription complex transcribes the entire genome of HIV (adapted from [116]).

1.3 The viral replication cycle

HIVs recognize host immune cells (essentially CD4⁺ T lymphocytes), enter into these cells, replicate and express viral proteins to produce new infectious virions. From the entry to the budding virions, the replication of HIVs can be divided in several complex steps: binding and entry in the cell, uncoating and reverse transcription, nuclear entry and integration in the host genome, viral gene transcription, translation of viral mRNAs and expression of all viral proteins, assembly and budding of virions from the infected cell, and maturation of viral particle. The different steps of the viral replication cycle are usually subdivided into two groups: the pre-integration steps (entry, uncoating and reverse transcription, nuclear entry and integration), and the post-integration steps (viral gene transcription, translation of mRNA transcripts, expression of viral proteins, assembly, release and maturation).

In this section, we aim to review these sophisticated steps of the replication cycle in order to highlight, thereafter, the innate immune response mounted by the cell to counter or impede each of these viral replication steps. An overview of the viral replication steps is provided in Figure 7.

HIV entry

The HIV entry process begins with the attachment of a viral particle to the host cell and is followed by the fusion of the viral and target cell membranes with, subsequently, delivery of the viral capsid containing the viral genomic RNA into the cell cytoplasm. The envelope glycoprotein plays an essential role in the virus entry. Indeed, the extracellular domain of the Env protein specifically recognizes and binds the CD4 cellular receptor (cluster of differentiation 4), invariably expressed at the cell surface of all immune cells [117-119].

Nevertheless, attachment can be relatively non-specific : Env may interact with negatively charged cell surface molecules such as heparan sulfate proteoglycans [120], $\alpha 4\beta 7$ integrin [121, 122] or dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a C-type lectin receptor [123, 124]. After recognition and binding of the CD4 receptor, the Env gp120 subunit

undergoes conformational changes exposing the V3 loop, and thereby mediates subsequent binding of a cellular coreceptor, mainly C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) [125-127].

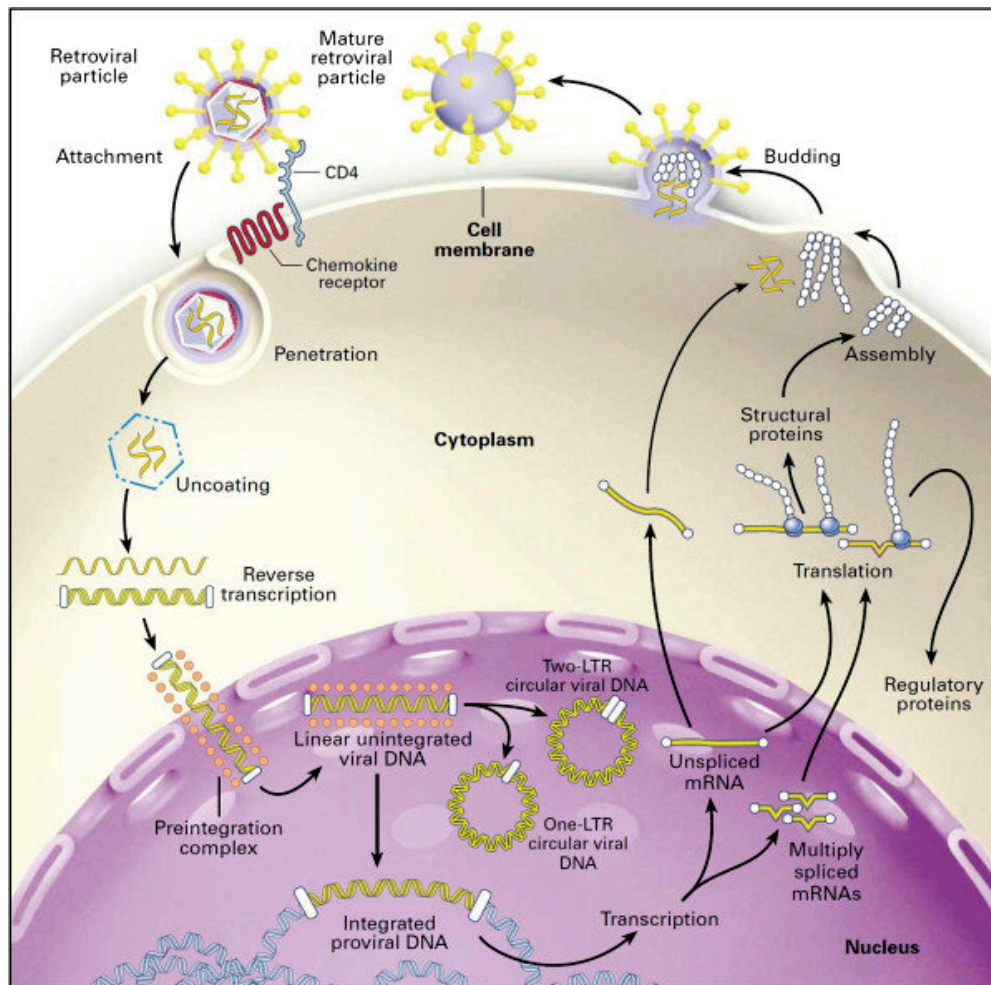


Fig.7: The HIV replication cycle. Each step of the viral life cycle is schematically depicted, from attachment (binding) of viral particle to budding and maturation of neoformed virions (adapted from [128]).

Binding to the CD4 and coreceptor triggers conformational changes in the Env trimers that mediate a pH-independent fusion of the viral and cell membranes [129]. In fact, the N-terminal ectodomain of the gp41 (or gp36 in HIV-2) displays a hydrophobic fusion peptide that is brought close to the cell membrane and is afterwards inserted in the cell membrane. Furthermore, two regions in the gp41 ectodomain (the heptad repeat 1 and 2, two leucine zipper-like motifs) form a stable antiparallel six-

helix bundle structure, favouring viral and cell membranes to get closer and ultimately allowing membrane fusion (Figure 8) [124, 129-133].

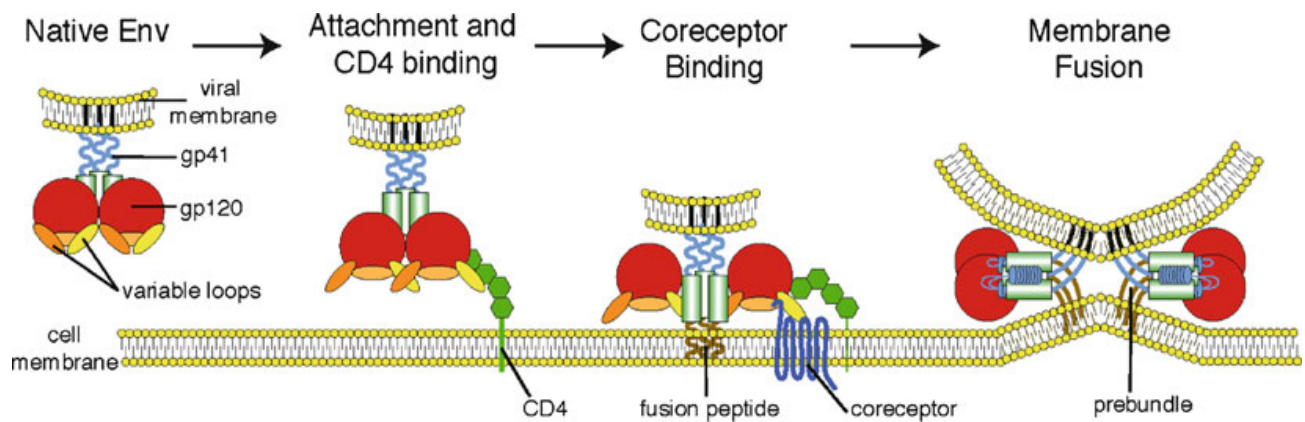


Fig.8: A schematic representation of the HIV entry process. First, the native Env protein binds to the host CD4 receptor. Second, CD4 attachment induces conformational changes in Env, enabling binding of coreceptor (CCR5 or CXCR4). Third, the hydrophobic fusion peptide of gp41 subunit is inserted in the cell membrane and allows fusion of the viral and host membranes (adapted from [124]).

Uncoating and reverse transcription

The Env-induced membrane fusion provides the opportunity for the viral core - the capsid shell containing the viral RNA genome - to reach the cell cytoplasm. The protective capsid polymer dissociates progressively, in a multistep process named uncoating, while allowing the RNA molecule to be reverse-transcribed and protected from the cellular immune sensors. In this transient reverse transcription complex (RTC), the viral double-stranded DNA synthesis occurs, mediated by the virus-packaged reverse transcriptase (RT) enzyme. Thus, initiation of reverse transcription is coupled to the uncoating process of the capsid core. Some other viral proteins have been reported to be present in this RTC: MA, NC, IN and Vpr. As the viral DNA synthesis takes place, the RTC transmutes to a pre-integration complex (PIC) which can attain the nuclear pore complex (NPC) and deliver the double-stranded DNA into the nucleus [134-137]. The precise timing and cellular location of the capsid core uncoating process and of the formation of PIC are still under debate. Several studies suggested that CA proteins remain associated with the RTC/PIC complex, even after its translocation into the nucleus

[138, 139]. However, subsequent studies showed that the upper size limit of the NPC (39 nm [140]) does not support the transport of an intact - non-dissociated - capsid core (50-60 nm [141]), although some CA proteins were identified in the nucleus [138, 139]. These results suggest that the disassembly of the capsid core occurs progressively during the journey of RTC from the membrane to the NPC (Figure 9) [142-144].

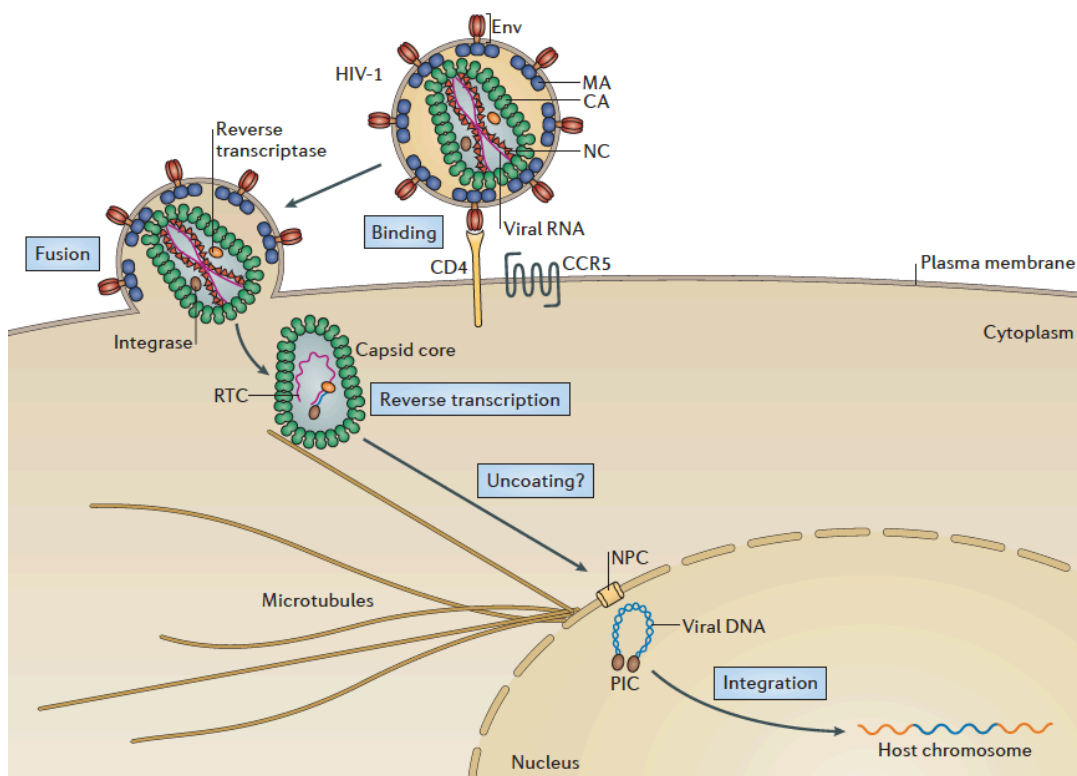


Fig.9: Schematic representation of the first steps of the viral replication cycle. Binding to the CD4 receptor via the Env protein and membrane fusion are followed by the delivery of the reverse transcription complex (RTC) in the cytoplasm. The reverse transcription and uncoating processes occur while the RTC reaches the nuclear pore complex (NPC). Finally, the pre-integration complex (PIC) is delivered in the cell nucleus to achieve the integration into the host chromosome (adapted from [142]).

It has been reported that several cellular factors interact with CA proteins and RTC/PIC complexes during this early phase of the HIV replication cycle. Thus, the uncoating process may be influenced by interactions between CA proteins, but also by interactions with specific host factors in a temporal manner. For example, Luban and collaborators revealed, 25 years ago, that host cyclophilin A (CypA),

a peptidyl-prolyl isomerase found in the cell cytosol, may interact with CA proteins [145]. Soon later, an interaction between CypA and CA was observed in the viral particle, demonstrating that this host factor is specifically incorporated into virions [146]. CypA interacts with a proline-rich domain of the HIV CA protein and catalyses the *cis-trans* isomerization of the Gly89-Pro90, resulting in conformational modifications in the CA residues lattice [147]. So, it is probable that CypA-induced conformational changes in CA may mediate disassembly of the capsid core, but contradictory results exist: a stabilization or a destabilization effect have been reported [148, 149], and moreover, these effects seem to be cell-specific [143, 150].

During transport of the RTC from the cytoplasm to the nucleus, the genomic single-stranded RNA (ssRNA) molecules are reverse-transcribed into double-stranded DNA (dsDNA) by the viral RT enzyme. This polymerase was discovered in 1970 by Temin and Baltimore, and at that time challenged the dogma of molecular biology. Indeed, this enzyme functions as an RNA-dependent DNA polymerase which can synthesize cDNA from ssRNA [151-153]. HIV RTs are heterodimers (p66/p51 in HIV-1 and p68/p55 in HIV-2) and have a ribonuclease H (RNase H) activity that promotes cleavage of template RNA strand of the RNA/DNA heteroduplexes [154]. The reverse transcription process is outlined in Figure 10. Reverse transcription is initiated by the binding of a cellular transfer RNA ($\text{tRNA}^{\text{Lys3}}$) to the primer binding site (PBS) [155]. After this binding step, RT synthesizes a ssDNA intermediate by extending the tRNA to the 5' end of the genome. RNase H activity of RT allows to remove the 5' end of the viral RNA (Figure 10 A and B).

The newly synthesized ssDNA intermediate is transferred to the R region (repeat region) in the 3' LTR where polymerization may continue to ultimately form a minus-strand DNA. Simultaneously to the elongation of the minus-strand DNA, the RT's RNase H activity degrades the template RNA (Figure 10 C and D). However, a purine-rich sequence, the polypurine tract (PPT), in the genomic RNA molecule transiently resists cleavage by the RNase H. PPT acts as primer for synthesis of a plus-strand DNA intermediate. The elongation continues until it reaches the tRNA (Figure 10 E). The tRNA sequence is then removed and a second transfer of the DNA intermediate allows annealing of the plus-strand DNA to the 3' end of the minus-strand DNA at the PBS sequence in the 5' side of the viral genome (Figure 10 F). Finally, the RT process is completed to form a full-length, double-stranded DNA with two identical LTRs at its both ends [154, 156, 157].

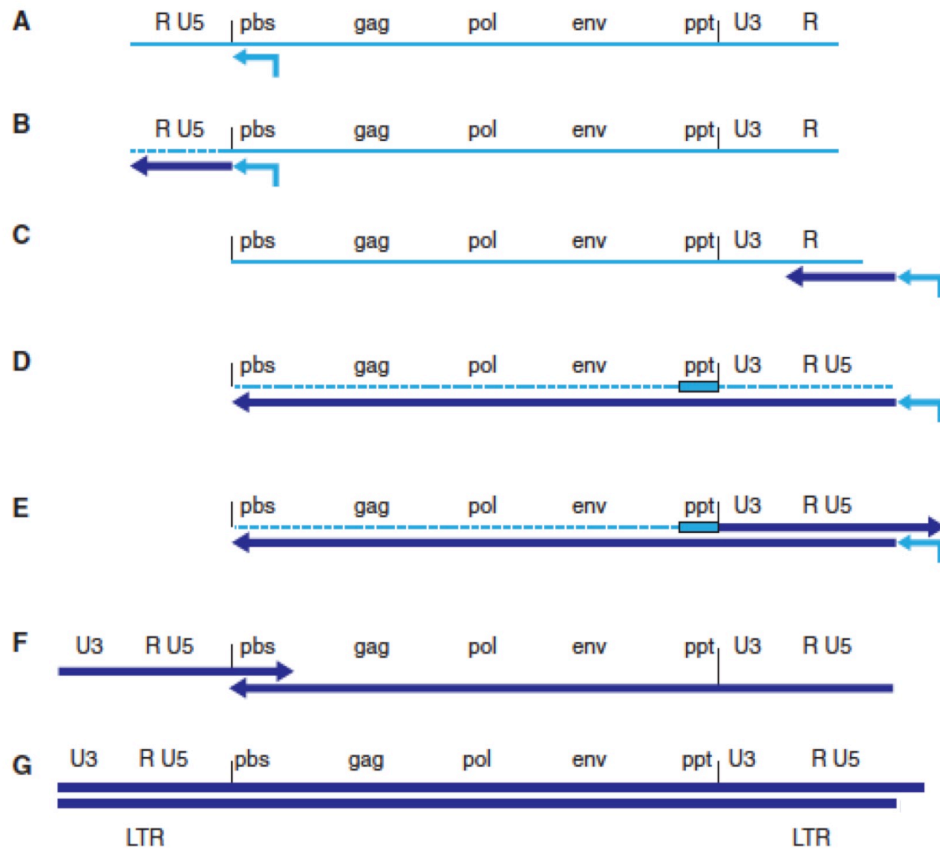


Fig.10 : The viral reverse transcription process. All steps of this process are depicted and are explained in detail in the main text. The R and U5 regions of the 5' LTR in addition of the U3 and R regions of the 3'LTR are shown in the RNA molecule (light blue line). DNA strands (single or double) are represented in dark blue lines. pbs: primer binding site; ppt: polypurine tract (adapted from [156]).

DNA integration

The PIC containing the viral double-stranded DNA enters the nuclear compartment through the nuclear pore complexes. The DNA integration process is catalyzed by the virus-packaged integrase (IN). Like the RT protein, IN is encoded by the *pol* gene and its dual function consists in the 3'-processing of both ends of the viral DNA and the strand transfer into the cell genome [158]. This integration is not reversible, and the cell becomes permanently infected. The integrated DNA is referred to as the provirus. Functionally, the 3'-processing of viral DNA ends occurs in a multimeric structure composed of assembled IN, known as the intasome [159]. The intasome reaches chromosomal DNA, forming the target capture complex (TCC).

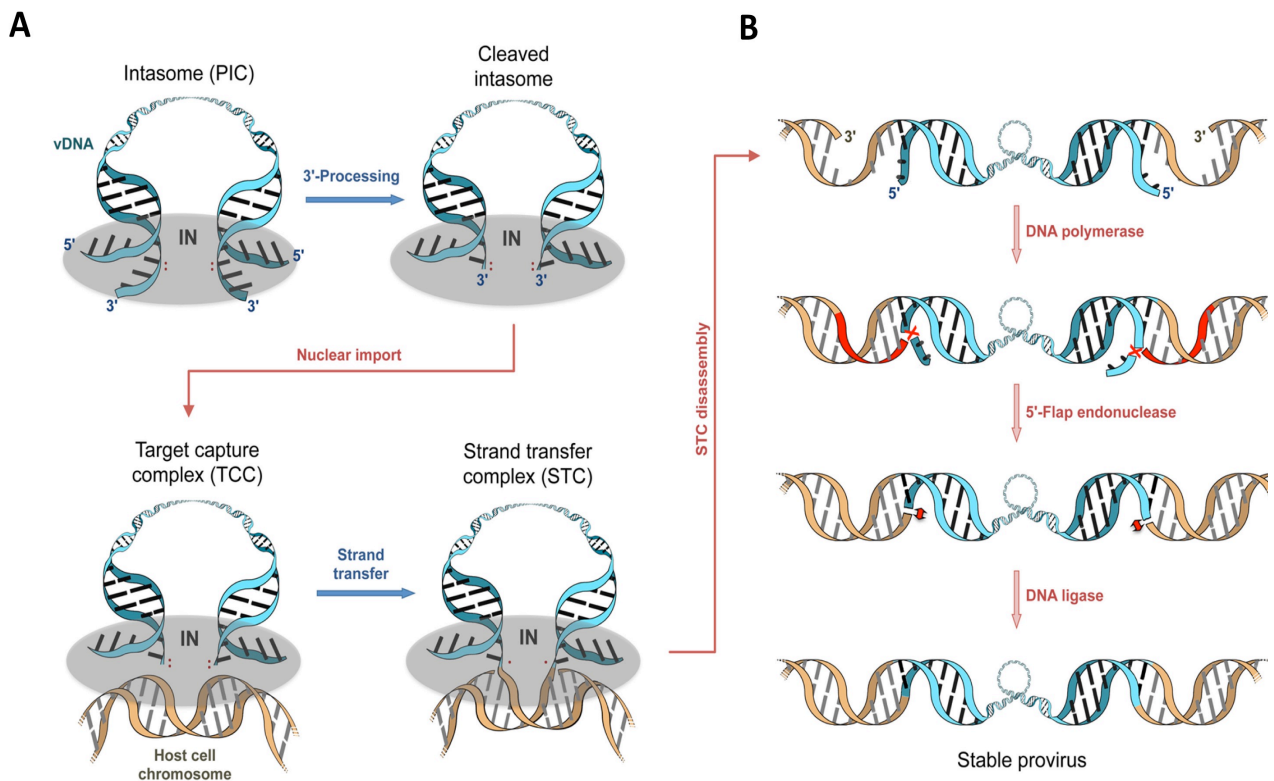


Fig.11: Intasomes and mechanism of viral DNA integration into the host genome. **A.** Viral and human DNA ends processing. **B.** Mechanism of viral DNA integration. vDNA: viral DNA; IN: integrase (adapted from [160]).

In the strand transfer complex (STC), IN uses 3'-hydroxyl groups as nucleophiles to cleave host DNA and simultaneously joins both 3' viral DNA ends to the apposing strands of chromosomal DNA (Figure 11 A). Then, a cellular DNA polymerase fills the gap between viral DNA 5' ends and 3' ends of host DNA (Figure 11 B) [156, 160-162].

Interaction between IN and viral DNA is sequence- and structure-specific, while IN does not display a high affinity for host chromosomal DNA. Nevertheless, the integration site selection is not random. Deep-sequencing assays have demonstrated that for primate lentiviruses, the integration of viral DNA mainly occurs in genomic sequences showing a high expression and active transcription profile ("gene-rich regions") in order to maintain an efficient viral replication (i.e. viral gene transcription) [162-165]. It has also been shown that the integration of viral DNA takes place near the outer shell of the nucleus (nucleus periphery), in close proximity to the nuclear pore [166].

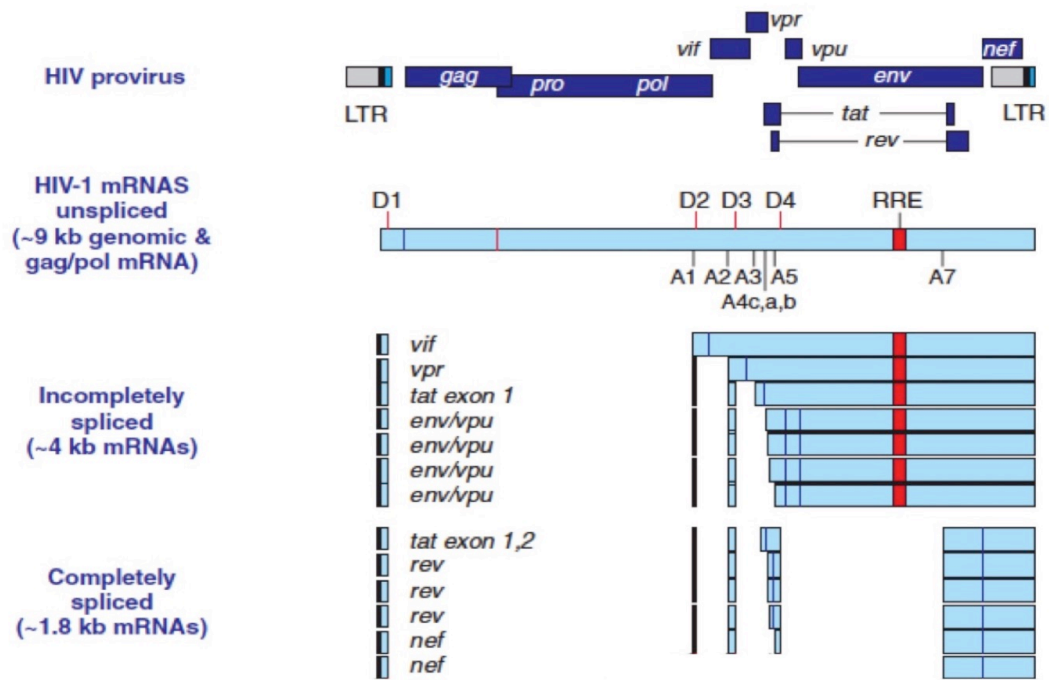
Viral gene transcription and protein expression

Once the provirus is permanently integrated into the host cell DNA, the HIV genome is expressed by the cell transcription machinery and viral mRNAs are produced. Retroviral mRNAs derive from alternative splicing of a pre-mRNA. These mRNAs encompass features and characteristics of cellular mRNAs. Indeed, they contain a 5' cap structure and a 3' poly(A) tail.

In HIVs, more than 30 different mRNAs are generated by alternative splicing, which are then exported to the cell cytoplasm. In regard to their incomplete or complete splicing, the distinct mRNAs could be classified in 3 categories: 1) the full-length unspliced mRNAs (~ 9-kb) from which are generated Gag and GagPol polyproteins. This unspliced mRNA also serves as genomic RNA, that will be packaged into the new viral particles; 2) the incompletely spliced mRNAs (~ 4-kb), and 3) the completely spliced mRNAs (~ 2-kb) [115].

The presence of several splice donor and acceptor sites in the full-length mRNA allows the generation of viral mRNAs containing the different open reading frames to ensure the expression of Vif, Vpr, Env and Vpu (in HIV-1) or Vpx (in HIV-2) proteins, and also Tat, Rev and Nef proteins [115, 167, 168]. While the completely spliced transcripts encoding Tat, Rev or Nef proteins are classically exported to the cell cytoplasm by the cell nuclear export machinery and are translated early, the incompletely spliced mRNAs must be exported toward the cytoplasm through the binding of the virally encoded protein Rev to the cis-acting RNA element termed the Rev-responsive element (RRE) present in the 3' side of the unspliced and incompletely spliced mRNAs (Figure 12 A) [115, 169, 170]. The incompletely spliced transcripts generate Env and Vpu (from a single bicistronic mRNA in HIV-1), Vif and Vpr proteins. In HIV-2, these transcripts also generate Env and Vif proteins, while Vpr and Vpx are expressed from a putative bicistronic mRNA [99, 171, 172]. The completely spliced transcripts rapidly express the regulatory Tat and Rev proteins, and the accessory Nef proteins, while the polyprotein precursors (Gag precursor and GagPol precursor) are expressed from the unspliced transcripts (Figure 12 B) [115, 167, 173].

A



B

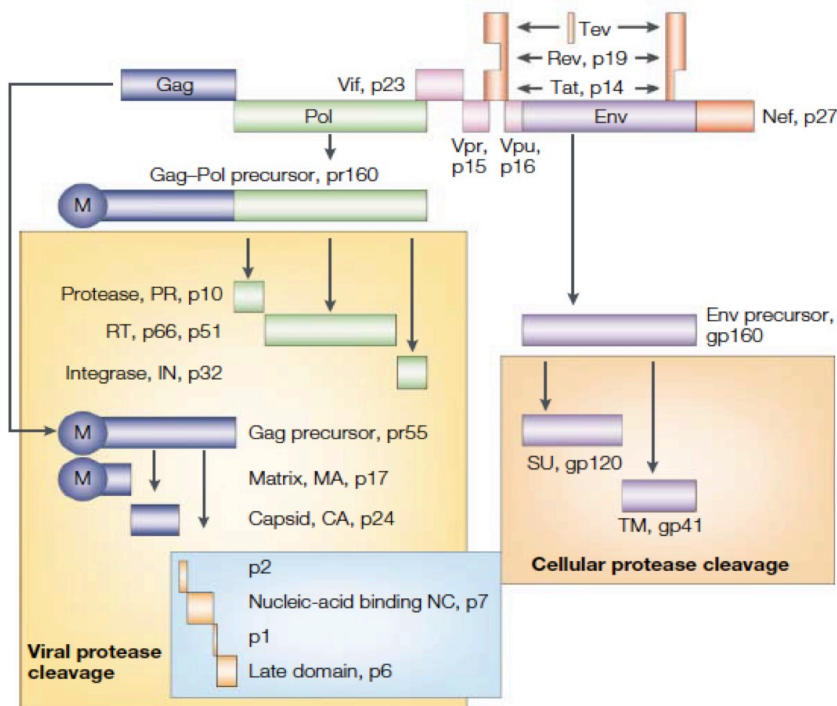


Fig.12: Schematic representation of the HIV-1 genome, the production of RNAs by the alternative splicing, and the generated polyprotein precursors. **A.** Genomic structure of HIV-1 genome and shematic representation of the alternative splicing. Top: Structure of the HIV-1 genome. Middle: the full-length, unspliced mRNAs. Localization of splice donor (D; red bars) and acceptor (A; black bars) sites are shown in the HIV genome. Bottom: representation of the different incompletely or completely spliced RNA transcripts generated by the alternative splicing. The locations of the AUG codons in the different RNA transcripts are shown as purple bars within the exons. RRE is represented by the red rectangle. **B.** The polyprotein precursors Gag, GagPol, Env gp160 and their respective domains (adapted from [115] for panel A and from [173] for panel B).

HIV assembly, release and maturation

The late stages of the viral replication cycle of HIVs allow formation and budding of infectious virions from the infected cells. The Gag precursor polyprotein (Pr55^{Gag}) plays an essential role in the assembly and release processes. Once expressed from the unspliced transcripts in the cytosol, the Gag precursors, containing the MA, CA, NC and p6 domains, begin to multimerize and reach the cell plasma membrane (Figure 13). The anchoring to the plasma membrane occurs through the MA domain of the Gag precursor. It has been reported that the lipid raft microdomains of the membrane are involved in this interaction. Besides this binding of the Gag precursor, the GagPol precursors which are also expressed from unspliced viral RNAs, can reach the multimerization site of Gag precursors at the inner leaflet of the cell membrane. The Env proteins traffic from the Golgi apparatus to the plasma membrane via the cellular secretory pathway. A few molecules of Env (15-20 trimers) are incorporated in the nascent virion through an interaction of the cytoplasmic domain of Env and MA proteins [95, 174, 175]. Furthermore, the NC domains of the Gag precursors interact with genomic RNA molecules. The viral RNA molecules contain a packaging signal (referred as the ψ -element) in the 5' UTR region. The NC domains can bind to this ψ -element and allow the packaging of two RNA molecules into the virion [95, 168, 174]. During the assembly process, the p6 domain of the Gag polyprotein precursor recruits the endosomal sorting complex required for transport (ESCRT-I) and the ALG2-interacting protein X (ALIX) proteins to initiate the membrane fission step, that leads to the immature virion budding. ESCRT-III is also recruited by the p6 domain to complete the membrane fission step [176]. The p6 domain of the Gag precursor also interacts with two other viral proteins: HIV-1 and -2 Vpr, and HIV-2 Vpx in order to incorporate them into the newly synthesized virions [177, 178]. Vif protein incorporation depends on interaction with both the NC domain and the viral genomic RNA [179]. Finally, Nef incorporation seems to be the result of a passive incorporation resulting from its association with the cellular membrane [180].

The last process occurring in the neoformed virion is the maturation that renders mature the viral proteins brought by the Gag and GagPol precursors into the assembling virions. This final step, driven by the proteolytic activity of viral protease, is involved in the formation of the conical capsid core and ensures the viral particle to be fully mature and infectious [95, 174, 181].

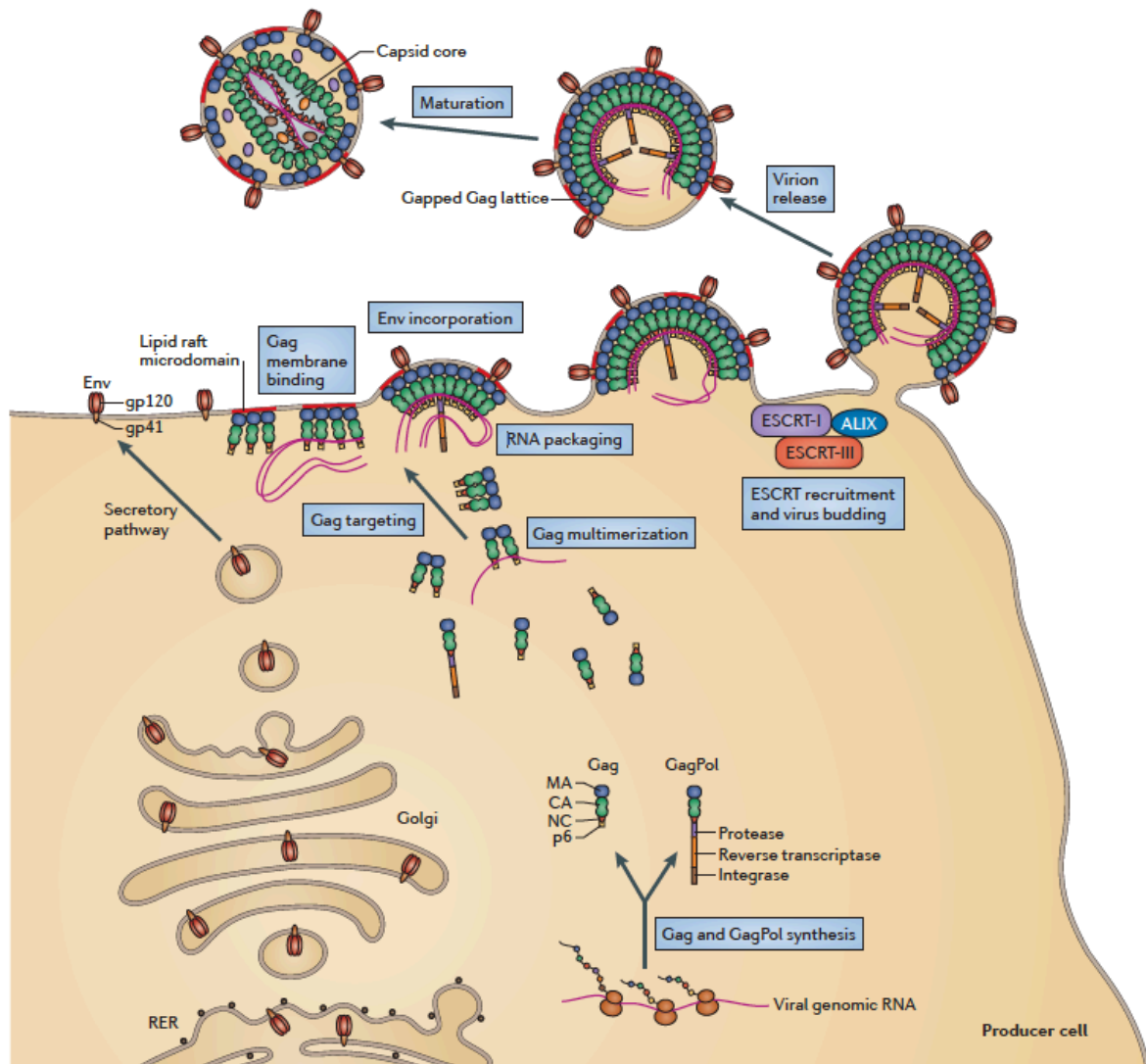


Fig.13: An overview of the HIV assembly, release and maturation. The Env proteins are addressed to the PM via the secretory pathway. Gag and GagPol precursors are synthesized in the cytoplasm and are targeted to the lipid raft microdomains of the PM. While the Gag precursors multimerize at the inner leaflet of the PM, two genomic RNA molecules (the “red filaments” in this image) are recruited by the NC domains. Release of the assembling virions is mediated by cellular proteins (ESCRTs and ALIX) (adapted from [95]).

1.4 The HIV envelope glycoprotein

Expression and structure of the HIV envelope glycoprotein

The HIV envelope glycoprotein (Env) is synthesized in the rough endoplasmic reticulum (RER) as a 160-kDa (in HIV-1; 140-kDa in HIV-2) polyprotein precursor from an incompletely spliced RNA transcript. This unprocessed precursor contains a N-terminal hydrophobic signal peptide (SP) targeting it to the ER membrane [182]. This signal peptide is cleaved by cellular peptidases and gp160 is extensively glycosylated with several N-linked (20-25) and O-linked (3-5) oligosaccharide side chains. The glycosylation accounts for ~ 50% of the molecular weight of lentiviral envelope glycoproteins. The glycosylated monomers of HIV-1 gp160 (or HIV-2 gp140) oligomerize predominantly into a trimeric structure [133, 183, 184]. The oligomerization facilitates the trafficking of gp160 from the RER to the Golgi apparatus by using the secretory pathway. In the Golgi or near the trans-Golgi network (TGN), unprocessed gp160 is cleaved by a cellular furin to produce mature Env glycoprotein, composed of the surface glycoprotein (SU or gp120 in HIV-1, gp105 in HIV-2) and the transmembrane glycoprotein (TM or gp41 in HIV-1, gp36 in HIV-2) (Figure 14 A). Thus, the whole Env glycoprotein is a trimeric structure of heterodimers (Figure 14 C). Subsequently to the furin-mediated proteolytic cleavage, these two subunits remain non-covalently associated. The proteolytic cleavage ensures the fusogenic activity and infectivity of the mature Env glycoproteins.

Throughout the gp120 and gp41, a series of cysteine residues (typically 18 residues) are found [185]. These conserved residues are involved in the formation of intramolecular disulfide bonds and in the folding process to maintain Env's tertiary structure (Figure 14 B). When Env is incorporated into the plasma membrane or into virions, the gp120 domain is entirely extracellular, while the transmembrane glycoprotein gp41 displays an extracellular domain, a transmembrane domain (TMD) and a cytoplasmic domain. The latter domain is entirely intracellular and is also called the cytoplasmic tail (CT) (Figure 14 A and C). The extracellular gp120 glycoprotein harbors five conserved domains (C1-C5), interspersed in the primary sequence with five hypervariable (V1-V5) amino acid sequences. Indeed, conserved regions are highly conserved, whereas the variable regions show a strong sequence variability among HIV-1 or -2 groups and isolates [186-190]. Env glycoprotein is the most variable region of the HIV genome, and sequence diversity among the distinct clades is up to 30-35%. The

gp120/105 ectodomain displays the highest variability, the gp41/36 ectodomain is the least variable, while the cytoplasmic domain shows an intermediate level of sequence diversity [191, 192].

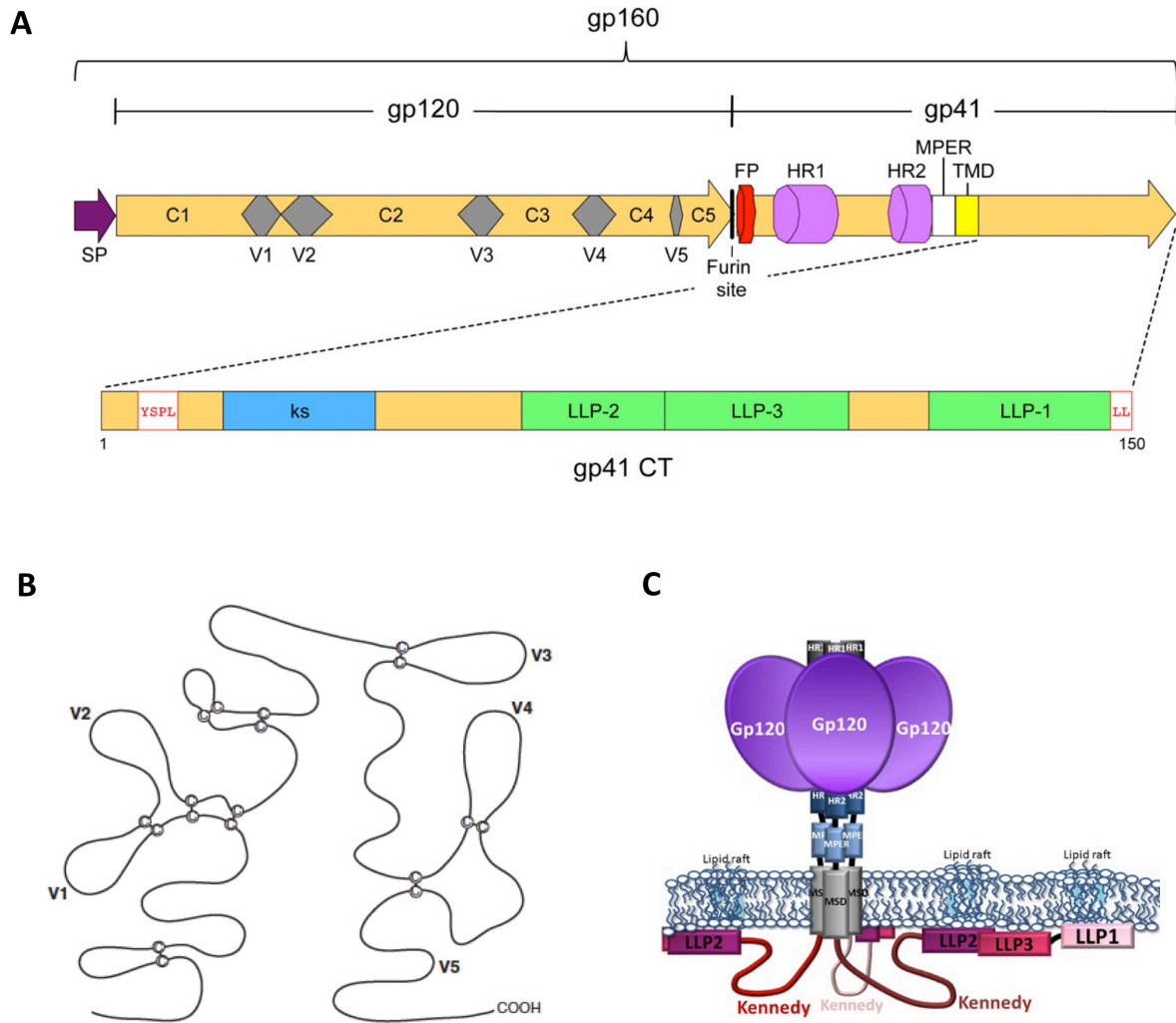


Fig.14: Structure and topology of the HIV Env glycoproteins. **A.** Structure of the Env precursor. Several domains within the Env protein are depicted: SP: signal peptide; C1-C5: conserved regions 1 to 5; V1-V5: variable regions 1 to 5; FP: fusion peptide in the gp41/36 ectodomain. In the CT of gp41/36, the endocytic motif (YxxΦ) and the Kennedy sequence (ks) are shown, as well as the lentiviral lytic peptides (LLP) 1 to 3. The dileucine motif (LL) at the end of the CT is also depicted. **B.** Schematic representation of the disulfide bounds that form the variable loops (V1-V5) in the Env ectodomain. **C.** Putative topology of the mature Env glycoprotein in the PM (adapted from [186] for panel A and B, and adapted from [175] for panel C).

The Env glycoprotein reaches the plasma membrane (PM) from the TGN through the secretory pathway. Upon its arrival at the PM, Env is either incorporated into nascent virions or rapidly recycled by endocytosis triggered by the interaction of an endocytosis motif in the Env CT and cellular clathrin

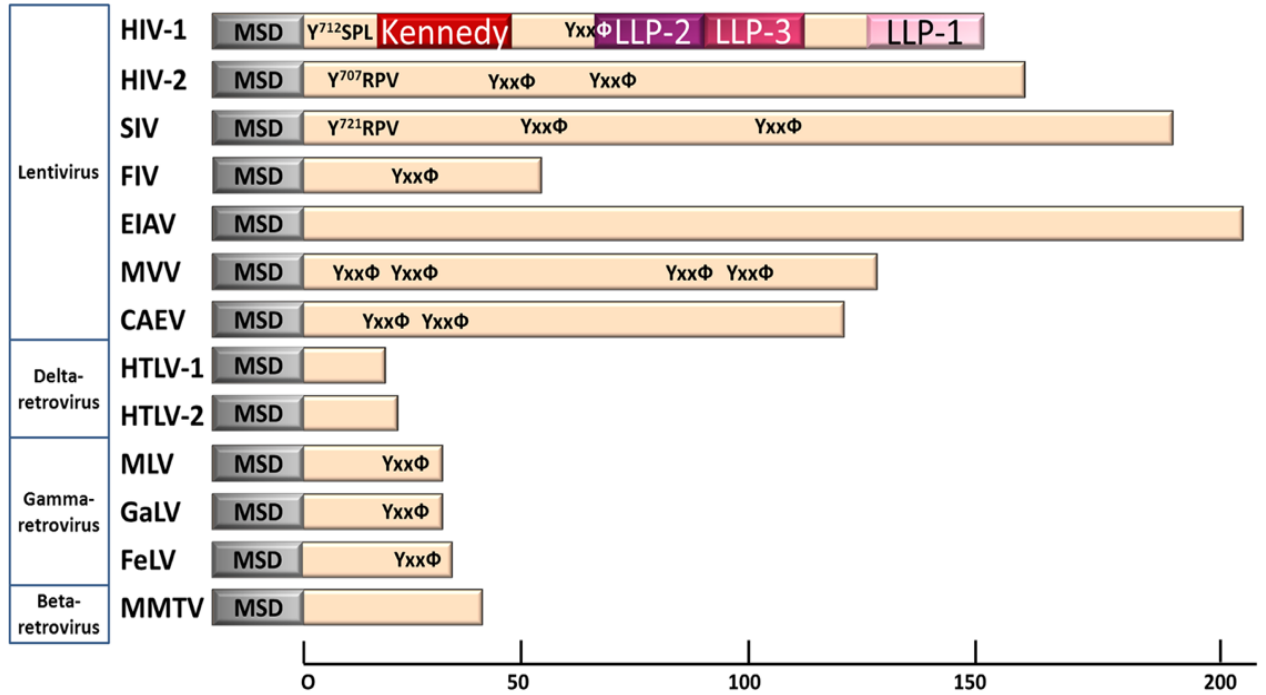
adaptor proteins (AP). Active recycling and the resultant low steady-state levels of Env surface expression are thought to be beneficial for HIV to evade immune recognition of exposed Env epitopes [193, 194]. Furthermore, the highly glycosylated state also contributes to protect Env proteins from the humoral immune recognition [194, 195]. In contrast to the HIV-1 Env, the HIV-2 envelope glycoprotein displays lower glycosylation levels, leading to a more “open” conformation of Env that may elicit production of human neutralizing antibodies (nAbs) [196].

The HIV gp41/36 transmembrane glycoprotein is composed of ~ 345 aa (amino acids) and harbors in its extracellular part the fusion peptide implicated in the viral and cell membrane fusion during entry process. Downstream of this hydrophobic region, two domains form two α -helical structures, termed the heptad-repeat regions (HR1 and HR2). Upstream of the transmembrane domain (TMD), a tryptophan-rich region – the membrane-proximal external region MPER – is located in the gp41/36 (Figure 14 A and C) [130, 131]. This region is essential for fusogenicity of the Env protein and virus infectivity, and is strongly recognized by neutralizing antibodies, such as 2F5 and 4E10 [197-199]. The TMD consists of 20-25 highly conserved hydrophobic aa and contains a consensus GXXXG helix-helix interaction motif. The main function of the TMD is the anchoring of Env protein into cell or viral lipid bilayer [186, 190]. The CT of gp41/36 is the intracellular (or “intravirionic”) domain of the HIV Env protein (Figure 14 A and C) and is likely the most variable in size and functionality among the primate lentiviruses.

Among the retroviruses, the HIVs Env glycoproteins harbor an unusually long CT domain, comprising approximately 150-157 residues. Only SIVs and EIAV lentiviruses display a longer CT domain (Figure 15 A) [175, 200].

Although the sequence identity of the different CT domains is not totally conserved among the retroviruses, numerous important motifs are highly preserved, especially in HIV-1, HIV-2 and SIVs, highlighting the evolutionary link between these primate lentiviruses as well as the essential roles of the CT domain in the viral replication (Figure 15 A and B) [192, 201].

A



B

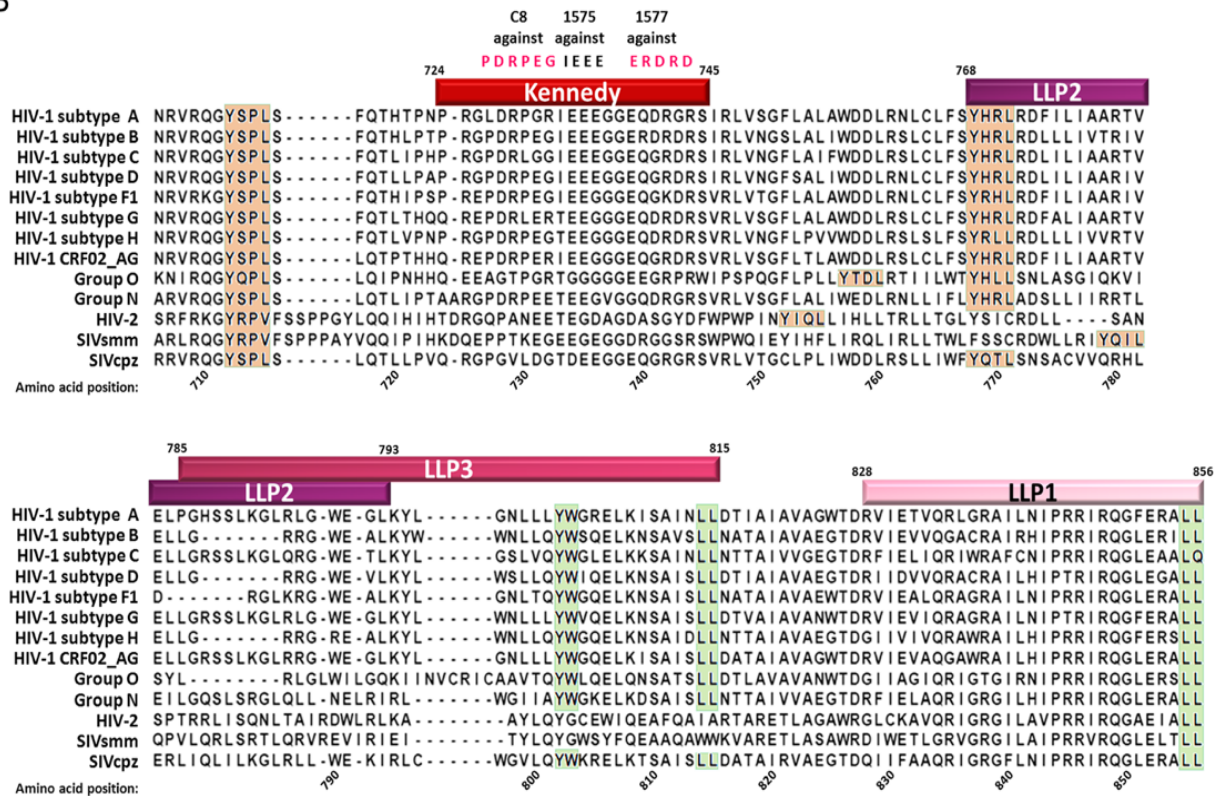


Fig.15: Structure and lengths of the Env CT of retroviruses. **A.** Comparison of the lengths of the Env CT of various retroviruses. The conserved YxxΦ motifs are highlighted, as well as the Kennedy sequence and LLP domains. **B.** Sequence alignment of Env CT from various retroviral (HIV and SIV) types and subtypes. Conserved YxxΦ motifs are color-coded in orange, the dileucine motifs (LL) and the important aromatic domains (YW) involved in Env trafficking are represented in green (adapted from [175]).

Immediately downstream the TMD, gp41/36 CT exhibits a consensus endocytic motif (YxxΦ) that can recruit the clathrin adaptor proteins (AP) complex and can trigger the internalization of Env from the plasma membrane. Interaction of Env CT and AP-2 proteins mediates the internalization of Env spikes from the plasma membrane, while interaction with AP-1 regulates the Env trafficking through the trans-Golgi network [202, 203]. A conserved region is found downstream this endocytic motif: the Kennedy epitope (KE) that elicits production of potent neutralizing antibodies, inhibiting the Env-mediated fusion (Figure 15 A and B) [204]. Existence of such humoral neutralizing antibodies binding to a specific intracellular region of HIV Env has led to the hypothesis that the CT is exposed to the extracellular compartment, at least partially and/or transiently. Indeed, several models of Env's topology have been proposed so far: in the first model, Env is a type I transmembrane protein with one MSD, placing its N-terminus in the extracellular compartment and its C-terminus in the intracellular compartment [205]. The alternative model suggests that the CT crosses the PM three times. This model is consistent with the potential exposure of the Kennedy epitope outside the cell or virus [206-208]. However, several lines of evidence do not support this alternative model but rather suggest that the CT may adopt different topologies and conformations when it lies in the cell membrane or in the virus membrane [209, 210] (reviewed in [175, 201, 211]). This issue is still under debate and further studies are needed to elucidate the CT (and Env protein as a whole) topology and conformation.

The Kennedy epitope is followed by three α -helical amphipathic structures named the lentiviral lytic peptides (LLP-2, LLP-3 and LLP-1) which are thought to be associated to the inner leaflet of the lipid bilayer (Figure 14 C and Figure 15). LLPs demonstrated a high level of conservation of structural properties among HIV and SIV strains and are characterized by arginine-rich domains. LLPs interact with the plasma membrane [212] and were reported to disturb the cell membrane integrity, thereby potentially inducing subsequent cytolysis and/or apoptosis of the producer cells [201, 211, 213, 214]. However, it has been reported that LLP-2 stabilizes the whole Env glycoprotein in its prefusion conformation [215].

At the C-terminal side of the CT, a dileucine (LL) motif is highly conserved among retroviral Env glycoproteins and is involved, together with the membrane-proximal endocytic motif, in the Env internalization [216, 217] (Figure 14 A and Figure 15 B).

Functions of the HIV envelope glycoprotein

Despite being a typical structural protein, the HIV mature Env glycoproteins have numerous functions in viral replication. The major role of Env is the CD4 receptor recognition and binding in order to mediate the fusion of cell and virion membranes. The gp120/105 ectodomain, which is exposed on the exterior of the virion, contains the determinants for the CD4 and coreceptor(s) binding. The conserved amino acid sequences C1, C3 and C4 are the principal Env determinants that bind the cellular CD4 receptor [218]. The HIV-1 Env V3 loop domain is involved in the binding of coreceptor(s) (CCR5 or CXCR4), while this activity is likely attributed to V1-V2 loops in HIV-2 [219-221]. HIV-2 Envs from different strains can also bind CCR5 and CXCR4, but interestingly and unlike HIV-1 Envs, are also able to recognize some other cell coreceptors such as CCR1, CCR4, CCR8, CXCR2, CXCR5 and GPR1. In addition to this broader recognition of receptors, it has been observed that some HIV-2 strains may infect human cells without binding the CD4 receptor. In this case, HIV-2 envelope glycoproteins may directly engage coreceptor binding, without previously recognizing the CD4 receptor at the PM. Such a broader range of coreceptor binding, unlike HIV-1, could be seen as an advantage for HIV-2 replication and dissemination in the human host. Nevertheless, these insights have been obtained by performing *in vitro* experiments. Thus, physiological relevance *in vivo* is currently unknown and warrants to be further investigated [219-223].

The Env cytoplasmic tail (CT) has received relatively little attention, whereas the Env ectodomain has been extensively investigated in the search toward an efficient HIV/AIDS vaccine, since the HIV Env glycoprotein is the only protein exposed at the surface of the virus. As mentioned above, the main function of Env is to drive the virus-cell membrane interaction and fusion, but a wide variety of regulatory functions mediated by the Env CT have been reported. As the intracytoplasmic localization of CT is the predominant topological model, this domain could interact with numerous cellular partners to achieve Env addressing/trafficking, recycling, stabilization and incorporation into newly formed virions, among other functions (reviewed in [175, 200]). All these interactions between the HIV gp41/36 domain and viral or cellular partners are summarized in Table 1 (see page 45).

Retroviral Env glycoproteins are addressed to the PM through the secretory pathway and colocalize with Gag. It has been reported that Env is preferentially targeted to the PM lipid rafts of polarized cells in a CT- and MA-dependent manner [224-227]. CT-MA interaction is the major regulator of Env

incorporation into virions when Env and Gag polyprotein precursors are localized at the PM [228]. Whether CT and MA interact directly or indirectly remains elusive and further studies are needed to elucidate this issue. However, two studies conducted by the same research group have reported that the intracellular TIP47 protein (Tail-interacting protein of 47 kDa, also known as perilipin 3) is an essential inter-connector between Env CT and MA proteins in CD4⁺ T cells [229] and in macrophages [230]. In addition, TIP47 may interact with diaromatic motifs (YW) within the CT and this association is thought to be important for the retrograde trafficking of Env glycoproteins [231]. PRA1 (prenylated Rab acceptor 1), involved in vesicular trafficking, is thought to associate with Env CT but no impact on viral release, Env incorporation or infectivity has been observed [232]. Another Rab protein, Rab7A, regulates the gp160/140 processing and incorporation of mature Env glycoproteins into virions. siRNA-based Rab7A knockdown experiments revealed an accumulation of Env (and other viral components) at the PM and an interference with viral release. Interestingly, authors speculated that the Rab7A depletion induces a BST-2/Tetherin-like phenotype on HIV release (see chapter II, section 2.3 “BST-2/Tetherin”) [233].

HIV Env CTs were also reported to interact with cellular calmodulin (CaM) through alanine and isoleucine residues within the LLP-1 sequence. Calmodulin acts as a calcium-induced mediator of FAS-mediated apoptosis and therefore, by binding calmodulin, Env CT may induce Ca²⁺ flux in cells. However, rapid and active Env recycling through the endocytic pathway may prevent the Env CT-calmodulin association, and hence may decrease the apoptotic potential of Env [234, 235] (see Table 1).

HIVs, like all vertebrate viruses, can manipulate the actin cytoskeleton of producer cells to promote viral replication and egress. SIV and HIV Env CTs were shown to interact with or hijack two human proteins implicated in regulation of the actin cytoskeleton. CT specifically interacts with p115-RhoGEF, a guanine nucleotide exchange factor and activator of the RhoA GTPase, to block the activation of RhoA [236]. Indeed, activated RhoA was shown to inhibit HIV replication in some cell types [237]. Interaction of CT and p115-RhoGEF is mediated by the LLP-3 domain, but the presence of LLP-2 and LLP-1 seems to be important for this association [236].

In addition to these functions, several other important activities of Env CT have been described, as the ability to overcome certain aspects of cellular restriction.

Firstly, the Env CT of HIV-1, HIV-2 and SIVs can interact with Luman protein (also called CREB3 or LZIP), a member of the OASIS family of transcription factors, that resides in the ER and is associated with the ER stress or unfolded protein responses [238]. Furthermore, Luman is also implicated in the repression of proviral transcription [239]. Blot and collaborators revealed that Env CT interacts with Luman in the ER through the N-terminal region of LLP-2, which leads to the rapid degradation of Luman. By so doing, Env CT impedes the ER-associated degradation of the transiently unfolded Env in the ER and counteracts the repression of proviral expression [239].

Secondly, Env CT also binds a cellular heterodimeric complex of the proteins prohibitin 1 and 2. Emerson *et al.* observed that abrogation of interaction between Env CT and prohibitin 1/2 hinders viral replication, suggesting a role of prohibitin 1/2 in the viral replication of HIV [240]. However, the exact mechanism underpinning this function of prohibitin 1/2 is not elucidated, mainly because prohibitin proteins are involved in a multitude of cellular signaling processes [241] (see Table 1).

Thirdly, and more importantly, HIV-2 envelope glycoprotein, unlike HIV-1 Env, is able to antagonize the host restriction factor BST-2/Tetherin through an interaction of their ectodomain [242]. This crucial function of HIV-2 Env - and the potential role of the cytoplasmic tail - in the BST-2 antagonism has been investigated in the first part of our thesis research project. Therefore, we dedicate a complete section to explain in detail this anti-tetherin function of the HIV-2 envelope glycoprotein (see Chapter II, section 2.3 “BST-2/Tetherin”) and we also present the research objectives that led to the publication of our first article (see Chapter III, section 3.1).

Finally, Postler and Desrosiers have reported the first description of a role for Env CT in the regulation of cellular activation. They observed that the Env CT of HIV-1 and SIVs can activate the transcription factor NF- κ B (nuclear factor κ B) [243]. In HIV-1, a consensus motif Yxx Φ in the N terminal side of LLP-2 can activate the serine/threonine kinase transforming growth factor β (TGF- β)-activated kinase 1 (TAK1) (see Table 1). Upon activation, TAK1 phosphorylates the inhibitor of κ B kinase (IKK) complex, resulting in the phosphorylation of inhibitors of κ B (I κ Bs). Consequently, I κ Bs are ubiquitinated and marked for proteosomal degradation. Subsequently, NF- κ B may translocate into the nucleus and bind to the promoter of various genes implicated in cell proliferation, cell survival, expression of proinflammatory cytokines and chemokines, interferons (IFNs) and IFN-stimulated genes (ISGs) [244-247]. However, activation of NF- κ B may also be beneficial for HIVs since the

LTRs harbor binding sequence(s) for NF- κ B that can induce expression of proviral genes (reviewed in [248]).

Moreover, it is worth noting that BST-2/Tetherin is also able to activate NF- κ B upon sensing of viral assembly and release processes [249, 250]. HIV-1 activates NF- κ B at the early stages of infection in order to ensure proviral transcription. Interestingly, however, HIV-1 completely inhibits NF- κ B activation at the late stages of infection, mainly by antagonizing BST-2, to restrain the NF- κ B-mediated antiviral responses [248, 251].

In the second part of the thesis research project, we sought to determine whether the HIV-2 envelope glycoprotein, the viral antagonist of BST-2, may also activate NF- κ B and we investigated the potential differences that could exist in the ability of HIV-1 and -2 to modulate or regulate the NF- κ B signaling pathway during their replication cycle. Thus, we dedicate a complete section to explain the interplay between retroviruses and NF- κ B in the pathogenesis of HIV infections (see Chapter II). In respect with this, we present the research objectives that led to the publications of our articles 2 and ‘in preparation’ (see Chapter III, section 3.2).

Table 1. Interactions between the HIV-1 gp41 (and HIV-2 gp36) and viral or cellular partners.

Interaction partner	Function(s)	aa of gp41/36 required for binding
AP-1	Intracellular trafficking	709-713 ; 853-854 (HIV-1) 706-710 ; 857-858 (HIV-2)
AP-2	Clathrin-mediated endocytosis	709-713 ; 853-854 (HIV-1) 706-710 ; 857-858 (HIV-2)
MA	Env incorporation	788-854
Luman	Overcoming the antiviral activity of Luman (?)	749-766
Calmodulin	Apoptosis (?)	769-788 ; 826-854
Perilipin 3 (TIP47)	Intracellular trafficking ; MA interaction (?)	784-822
Plasma membrane	Stabilization of Env in prefusion conformation (?)	769-788 ; 787-813 ; 826-854
PRA1	Intracellular trafficking (?)	?
Rab7A	Env gp160/140 processing and incorporation	?
Prohibitin 1/2	Overcoming the block in nonpermissive cells (?)	788-798
P115-RhoGEF	Impairing of RhoA signaling (?)	796-854
TAK1	NF- κ B activation	762-773 (Y ₇₆₆ HRL) for HIV-1 759-778 (Y ₇₇₀ SIC motif) for HIV-2
BST-2/Tetherin	Overcoming the BST-2 restriction	N659 for HIV-2 gp36 Cytoplasmic domain (?)

aa numbering is based on the sequence of the full-length HIV-1 NL4-3 envelope (GenBank accession no. AF324493.2) and on the sequence of the full-length HIV-2 ROD envelope (Genbank accession no. M15390.1).

The (?) means that the interaction and/or function are not fully defined. The motif of the HIV-2 gp36 domain required for binding the viral or cellular partner is indicated when it has been established (extensively reviewed in [175, 191]).

1.5 Cellular aspects and immunopathogenesis of HIV and SIV infections

HIV-1 infection

Most observations have been made in the case of HIV-1 infection. We will specify HIV-2 facts afterwards. HIV-1 is a blood-borne virus and can establish itself in human semen, pre-seminal, vaginal and rectal fluids, and in breast milk. Virus transmission occurs essentially through insertive or receptive sexual intercourse, vertical transmission (mother-to-child) and also by exposure to contaminated blood. This human retrovirus presents a tropism for immune cells expressing the CD4 receptor, such as CD4⁺ T lymphocytes, dendritic cells (DC) and macrophages. In the acute phase of infection, mucosal DCs are thought to be the first immune cells exposed to HIVs. Despite the fact that these cells are poorly infected by HIV-1, dendritic cells may carry viral particles from the primary site of infection to regional tissues, where a cell-to-cell transmission (*trans*-infection) of HIV particles occurs to efficiently infect CD4⁺ T cell, through an infectious synapse [111, 252-255]. Furthermore, DCs are professional antigen-presenting cells (APCs). After the internalization and sensing of the HIV particle, these cells express cytokines and interferons (IFNs) that regulate the antiviral immune responses, and present HIV antigens to immune cells [255].

In the absence of a pre-existing immune response, HIV disseminates rapidly and leads to a burst of viremia in the majority of infected individuals during the acute phase of infection [256, 257]. Low-level dissemination occurs early in partially activated CD4⁺ T cells. This first phase of infection is followed by substantial viral propagation in activated CD4⁺ T cells in the gut-associated lymphoid tissues (GALT) in the lamina propria. Dissemination of HIV to secondary regional lymphoid tissues occurs several days post-infection and an establishment of tissue reservoirs emerges (Figure 16 A). The immune response drops back the peak of viremia but provides only incomplete control of HIV-1 viral replication [98, 258, 259]. Typically, HIV-1 replicates at high levels, which is reflected in high concentrations of virus in plasma (high viral load) after 3-6 weeks post-infection, while viral RNA can be detected after only 10-12 days of infection.

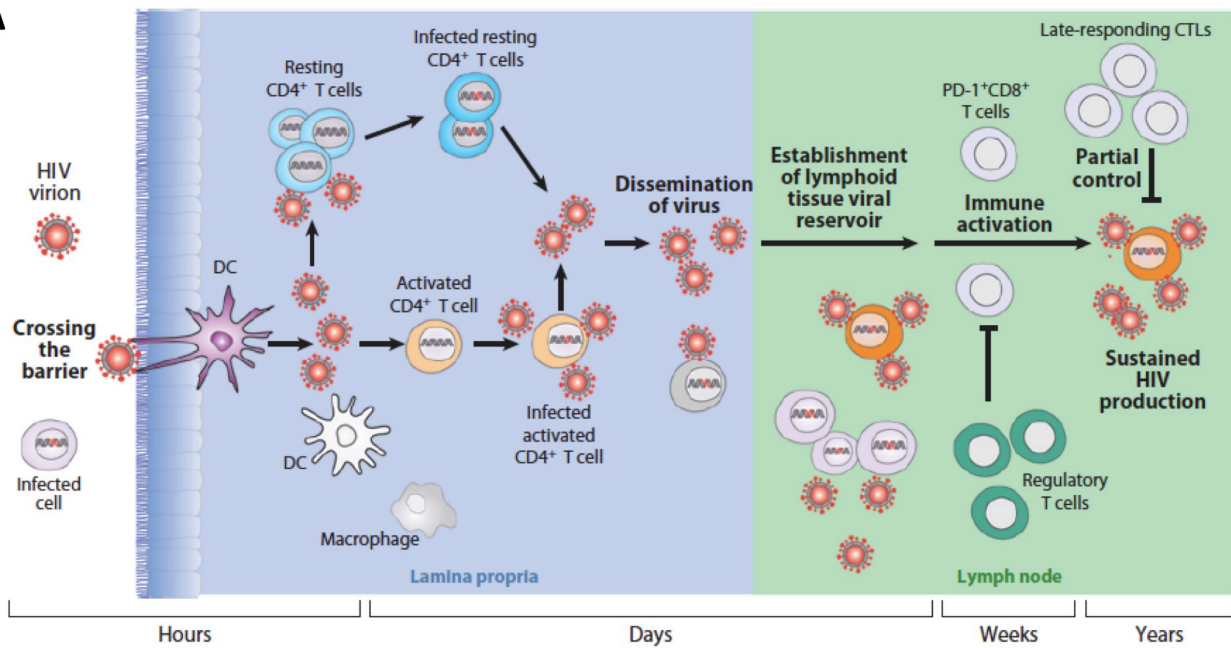
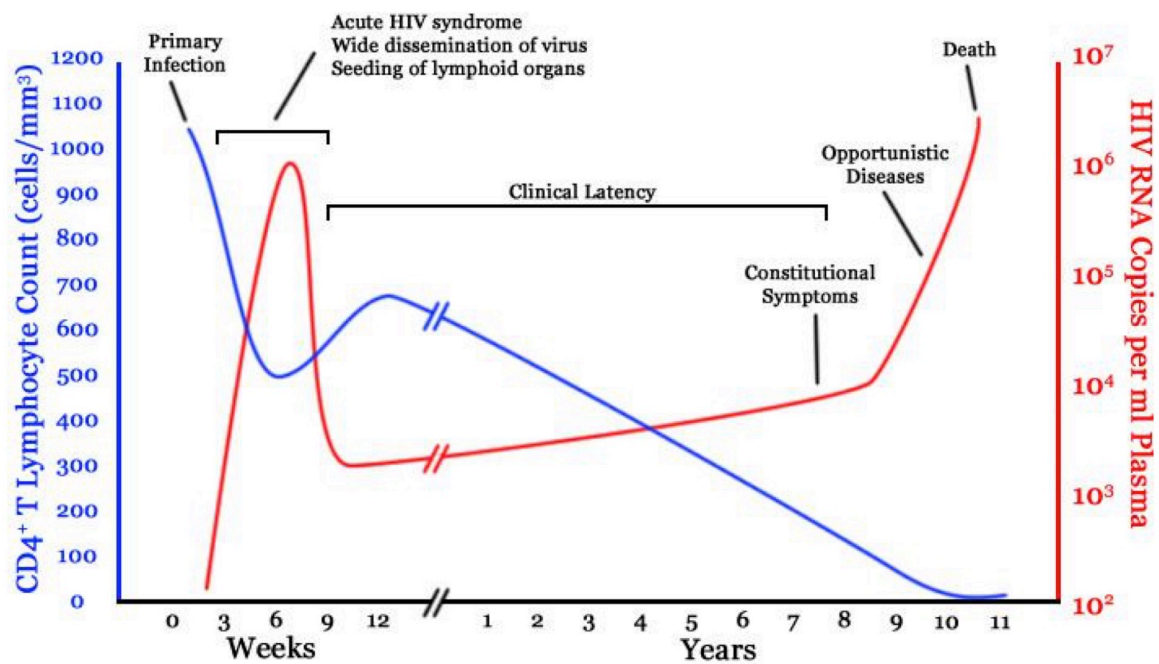
A**B**

Fig.16: The different phases of infection after exposure to HIV. **A.** The infection takes place with transmission across an epithelial barrier by a cell-free virus or virion carried by DC. The propagation of HIV occurs in partially activated CD4⁺ T cells and is followed by strong propagation in activated CD4⁺ T cells in the GALT (lamina propria). The dissemination of HIV particles to secondary lymphoid tissues (lymph nodes) and formation of reservoirs occurs after several days of infection. **B.** Evolution of both CD4⁺ T cell counts (blue line) and HIV RNA copies per mL of plasma (red line) during the acute and the chronic phase of HIV infection (adapted from [257] for panel A and [260] for panel B).

HIV-1 infects and destroys CD4⁺ T cells, thereby inducing dysfunction of T cell subsets and a considerable decline of the CD4⁺ T cell counts. During HIV-1 infection, loss of CD4⁺ T cells surpasses the production of new cells from the thymus, resulting in a progressive decrease of CD4⁺ T cells in infected individuals (Figure 16 B) [256, 261-264].

Soon after reaching the peak ($>10^6$ viral RNA molecules per mL of blood), viral replication generally drops by several orders of magnitude to establish a set-point. This reduction of viremia is concurrent with the appearance of virus-specific CD8⁺ cytotoxic T lymphocytes (CTLs) and the strong decline of HIV target cells, CD4⁺ T cells (Figure 16 A and B). However, early HIV-specific CD8⁺ T cell responses are narrowly directed to a few viral epitopes and are not able to completely inhibit viral replication, since immune driven viral escape occurs rapidly in the virus population [265]. Consequently viral replication is maintained and the CD4⁺ T cell count continues to decline, despite the extent of the HIV-specific CD8⁺ T cell response (Figure 16 B) [253, 257, 262, 266, 267]. Without an efficient antiretroviral therapy (ART), the progressive decline and dysfunction of CD4⁺ T cells during the clinical latency phase of the disease lead to the appearance of AIDS-associated symptoms and opportunistic infections in infected individuals, and ultimately cause death within 10-15 years (Figure 16 B) [268-270].

It is worth noting the existence of a small group of HIV-1-infected individuals (1-3%) who maintain low levels of viremia, stable and healthy CD4⁺ T cell counts (>500 cells/ μ l) for over 10 years and do not progress to AIDS during the HIV infection without clinical or pharmacological interventions. These individuals are called long-term nonprogressors (LTNPs) [271-273]. They have been subdivided into individuals who control viremia below the 400 copies/mL in blood for more than 5 years (viremic controllers) or below 50 copies/mL for at least one year (elite controllers, ECs) [269, 274, 275]. Understanding the immune mechanisms that underpin this strong immunological control of HIV infections is one of the major research fields towards a functional cure for HIV/AIDS. Accumulating evidence suggests a predominant role of the CD8⁺ T cell response in the long-term nonprogressive phenotype and containment of HIV replication [262, 263, 276-278]. Several studies demonstrated that the quality of CD8⁺ T cells from HIV controllers rather than the number is the major determinant contributing in the immune control of HIV infection, especially during the acute phase of infection [279, 280] (reviewed in [267, 281]). The CD8⁺ T cells in HIV controllers were shown to have a wider

diversity of effector functions and demonstrated greater cytotoxic abilities with high expression of granzyme B and perforin, resulting in a more efficient destruction of early infected CD4⁺ T cells at the site of infection [282-285].

Furthermore, CD8⁺ T cells from HIV controllers demonstrate superior capacities to secrete cytokines and chemokines, such as interleukin (IL)-2 [283, 286, 287]. Another evidence that the CD8⁺ T cell response is the main determinant in the control of HIV, is the association of specific human leukocyte antigen (HLA) class I alleles, like B*27 and B*57, with the elite controller phenotype [288, 289]. In fact, HLA-I alleles select epitopes of HIV that are presented to the host immune system, and these particular HLA-I alleles promote strong and effective CD8⁺ T cell responses [290]. Taken together, these features of CD8⁺ T cell responses in HIV controllers may contribute to immunological control during the acute phase of HIV-1 infection by governing an effective T cell immunity, in comparison to non-controllers individuals (see Figure 17 A) [265, 267, 281].

However, it has been observed that the CD8⁺ T cell response efficiency is also supported by the HIV-specific CD4⁺ T cell response. Indeed, CD4⁺ T cells play a crucial and central role in defense against HIV infection, by helping the aforementioned CD8⁺ T cell responses to control the disease in the acute phase of infection. Early expansion of CD4⁺ T helper (Th) cells with cytolytic abilities (high expression of perforin and granzyme B) is associated with control of HIV replication in the acute phase of infection and governs the disease progression (Figure 17 A) [258, 274, 291-296].

Interestingly, IL-21 is known to be essential in the cooperation between CD4⁺ and CD8⁺ T cell responses, since HIV-specific CD4⁺ T cells expressing IL-21 are enriched in the acute phase of infection in HIV controllers. Moreover, this cytokine contributes to durable control of HIV replication through both enhancing specific CD8⁺ T cell response and managing the regulatory CD4⁺ T cell (Treg) functions [297-301]. Several recent studies described that the frequency of Tregs, among the total CD4⁺ T cells may determine disease progression. In fact, low Tregs numbers and a balanced Tregs:Th17 ratio (see below) in peripheral blood and other tissues have been observed in HIV-infected individuals who control the viral replication and disease progression [302-304]. Tregs tightly regulate the CTL response of CD8⁺ T cells by a selective suppression of CTLs restricted by non-protective HLA-B*27 and B*57. Thus, Tregs select and promote effective and protective CD8⁺ T cell responses (Figure 17 A) [305-307].

Th17 (interleukin 17-producing T helper) cells are a subset of CD4⁺ T cells that mainly reside in mucosal surfaces and are implicated in maintaining the mucosal integrity during HIV infections. In addition to the balanced Tregs:Th17 ratio, frequency of Th17 cells in mucosal surfaces prior and also during acute HIV infection contributes to the control of disease progression. Indeed, the degree of Th17 depletion correlates with progression: in HIV controllers, a high number of Th17 cells is associated with slow disease progression (reviewed in [308, 309]).

Finally, another subset of CD4⁺ T cells, the follicular T cells (T_{fh}), was also reported to control HIV replication and to determine disease progression. In humans these cells reside exclusively in germinal centers and are involved in helping B lymphocyte cells to produce high-affinity and broadly neutralizing antibodies against HIV epitopes (Figure 17 A) (reviewed in [310, 311]). Taken together, these cooperative CD4⁺ and CD8⁺ T cell effector functions restrain the viral replication, maintain normal CD4⁺ T cell turnover and mucosal immunity homeostasis, limit chronic immune activation (see below), and select highly-active and broadly neutralizing antibodies, thereby resulting in a slow disease progression and a better immunological control of HIV-1 infection.

In HIV non-controllers, who represent >95% of the HIV-1 infections worldwide, the viral replication is sustained over the long-term, the decline of CD4⁺ T cell counts is irrevocably progressive and the coordinated CD8⁺ T cell responses are ineffective, essentially due to chronic immune activation and exhaustion of CD4⁺ and CD8⁺ T cells, B cell dysfunctions and a higher viral escape capacity without representing a replicative fitness cost for the virus (Figure 17 B). Generalized dysfunction of immune cells (CD4⁺ and CD8⁺ T cells) is observed in most HIV non-controllers. It has been revealed that CD8⁺ T cells are defective in expression of several transcription factors controlling the development and function of these cells, such as T-bet [312, 313], resulting in loss of cytolytic activity (i.e. low expression levels of perforin and granzyme B) [314]. Subsequently, CD8⁺ T cells express the inhibitory protein, PD-1 (programmed cell death protein 1) [315-317]. PD-1 induces an upregulation of BATF (basic leucine transcription factor, ATF-like) expression in these CD8⁺ T cells [281, 318]. This results in chronic antigen stimulation, cellular exhaustion of CD8⁺ T cells. Consequently, CD8⁺ T cells do not proliferate, show a more narrowed CTL activity and fail to efficiently detect and eliminate HIV-infected CD4⁺ T cells (Figure 17 B) (reviewed in [263, 265, 281]).

As indicated above, interdependence of CD4⁺ and CD8⁺ T cell responses is crucial to control HIV replication and disease progression. Like CD8⁺ T cells, HIV-specific CD4⁺ T cells also show high

expression of PD-1 in HIV non-controllers and thus have reduced cytolytic activities. Moreover, these cells no longer maintain virus-specific CD8⁺ T cell functions and responses during the course of HIV-1 infection [297, 319, 320].

Tregs:Th17 ratio was shown to be unbalanced (high frequency of Tregs and low frequency of Th17), which results in a rapid disease progression in HIV-1 non-controllers. Finally, follicular T helper cells (Tfh) were reported to be defective in providing help to B cells to produce highly-active neutralizing antibodies, thereby allowing the viral escape (Figure 17 B) (reviewed in [263]).

Loss of CD4⁺ T cells is a hallmark of HIV-1 infection and a key event in disease progression towards the AIDS stage [253, 264, 270, 321, 322]. Two distinct mechanisms, not necessarily exclusive, have been documented as being the determinants of the progressive decline of CD4⁺ T cell counts generally observed in HIV-1-infected individuals: 1) CD4⁺ T cell destruction by viral infection and replication (“direct virus attack”) that leads to a cytolytic effect, and 2) chronic immune activation that ultimately leads to apoptosis of T cells [323-328]. In the first mechanism, the loss of infected CD4⁺ T cells is initially balanced by production of new CD4⁺ T cells, essentially by the thymus. However, the viruses rapidly spread and infect the memory T cells in the thymus, and these cells undergo a process of elimination, thus contributing to the global loss of CD4⁺ T cell counts during the course of HIV-1 infection [329, 330]. While this direct virus attack/cytolysis mechanism clearly contributes to the loss of infected T cells, the decline of uninfected CD4⁺ and naïve CD8⁺ T cells cannot be explained by this mechanism.

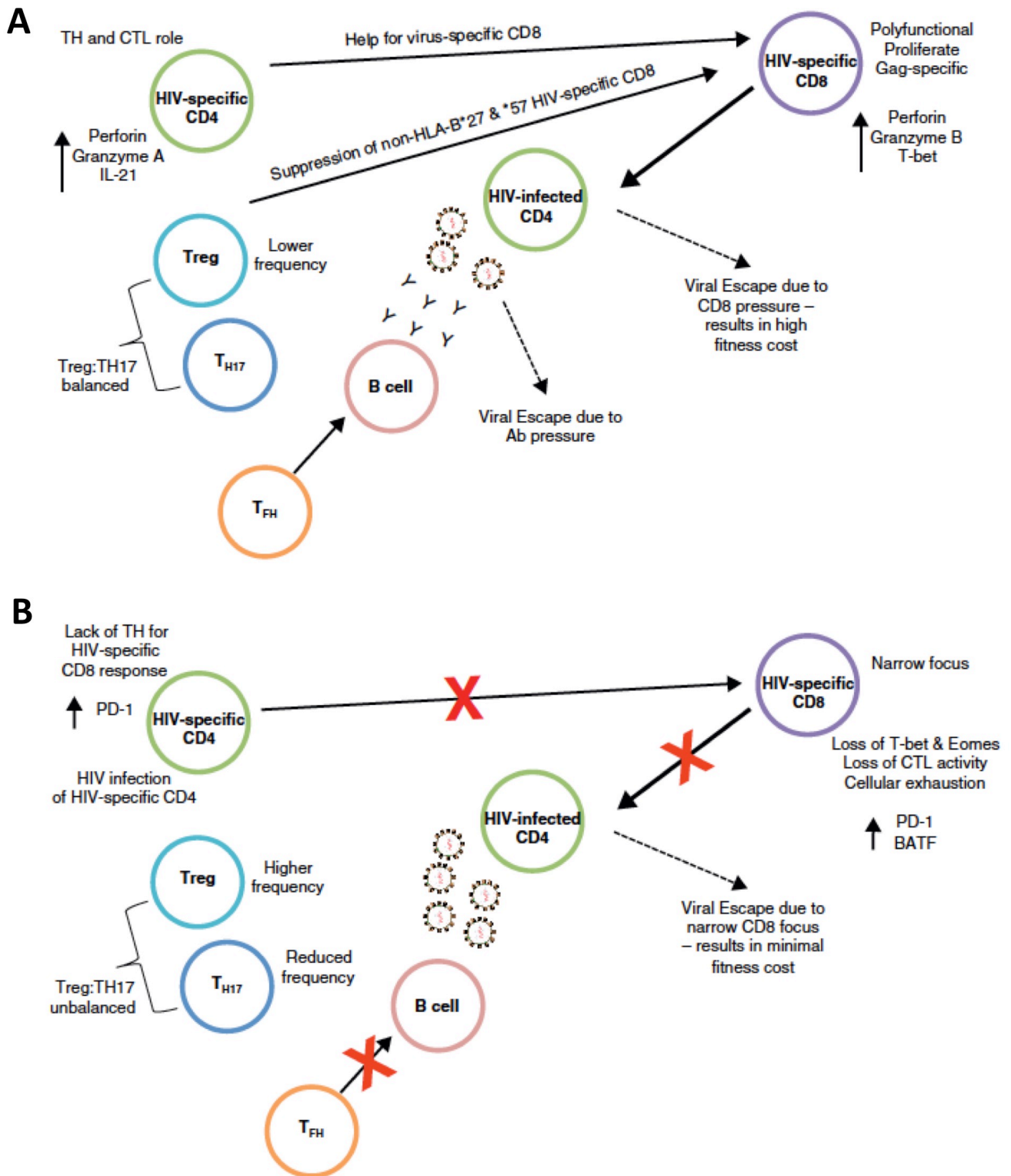


Fig.17: T cell immunity and cellular factors involved in HIV pathogenesis and disease progression. **A.** Overview of an effective T cell immunity to HIV infection, in which HIV-specific CD4⁺ T cells demonstrated high expression of CTL molecules (perforin, granzyme B, as well as IL-21) and provide support to CD8⁺ T cell CTLs. **B.** Overview of an inefficient T cell immunity to HIV Infection. Dysfunctions in CTL responses (e.g. high expression of PD-1) occur because of infection of HIV-specific CD4⁺ and decrease the CD8⁺ T cell responses. Furthermore, there are both unbalanced Treg:Th17 ratio and reduced antibody production, which cause the loss of control of HIV infection (adapted from [263]).

The chronic immune activation (also called the “hyper immune activation”) may also explain the AIDS pathogenesis. In this mechanism, CD4⁺ and CD8⁺ T cells, as well as B cells, demonstrate a high rate of cell division and an upregulation of activation markers (such as CD38, HLA-DR) during the HIV-1 infection. Thus, and paradoxically, the HIV-induced immune activation increases the number of T (i.e. increased turnover) and B cells. In fact, it has been proposed that the activated CD4⁺ T cells have a short lifespan and are likely rapidly lost by cell death or apoptosis, as compared with the non-activated CD4⁺ T cells. This phenomenon is called depletion by activation. The lysis of cells further releases systemic proinflammatory cytokines, and hence contributes to reinforce immune activation and affects HIV pathogenesis [331].

Several other factors strengthen the chronic immune activation, such as the microbial translocation from the gut due to the loss of mucosal integrity during HIV infection [327, 332]. Therefore, the level of activation is a strong indicator of the disease progression during the course of HIV infections [320, 323, 326, 333-336].

SIV infection

Species-specific SIV strains have infected African non-human primates (NHPs) for at least 32,000 years, as revealed by an elegant study conducted by Worobey *et al.* in 2010 [337]. As mentioned above, many NHP species are naturally infected with SIV in the wild, such as sooty mangabeys (SMs) infected with SIV_{smm} and African green monkeys (AGMs) infected with SIV_{agm}, which are referred to as natural SIV hosts. During this long history, SIVs and their respective hosts have evolved to coexistence and appear to maintain an “entente cordiale” since, in marked contrast to HIV-infected humans, these natural hosts generally do not progress to AIDS, typically maintain healthy CD4⁺ T cells, despite high levels of virus replication during both acute and chronic infection. Thus, SIV infections in their natural hosts are considered as nonpathogenic [338-342]. The only exception is the SIV_{cpz} infection which may potentially cause a weak increase in mortality in the chimpanzee host [343].

While SIV_{smm} also gave rise to SIV_{mac} in “non-natural” Asian rhesus macaques (RMs) infected in captivity, free-ranging RMs are not infected with this SIV_{mac} [48-50]. Interestingly, however, SIV_{mac}-infected rhesus macaques experience a pathogenic AIDS-like disease, remarkably similar to the pathogenic HIV-1 infection observed in most humans [344]. Thus, there is an increase in pathogenicity on cross-species transmissions of SIVs from their natural hosts to new hosts, such as humans that took place in the beginning of the 20th century. Understanding and identifying immunological mechanisms by which natural hosts can control the SIV infections, as opposed to HIV-1/SIV_{mac} infections, is a major research topic that could benefit preventive or curative approaches of HIV [1, 345-348].

Although SIV infections in their natural hosts are very different from pathogenic HIV-1/SIV_{mac} diseases, several features are shared by both infections. For instance, SIVs also replicate at high levels in the mucosal gut and these simian viruses are largely distributed in other tissues (e.g. blood, thymus) [338, 349-351]. The SIV infection occurs predominantly in activated CD4⁺ T cells in the mucosal gut and consequently these infected cells demonstrate a short lifespan. Despite the fact that these productively infected CD4⁺ T cells are rapidly eliminated, peripheral CD4⁺ T cells are globally well preserved and present a normal turnover during the chronic phase of SIV infection [339, 349, 352-356]. This is one of the most striking differences between nonpathogenic SIV infections and pathogenic HIV-1/SIV_{mac} infections (Figure 18).

Similar to HIV-1/SIV_{mac} infections, SIV infections also lead to a strong decline of mucosal CD4⁺ T cells in the gut during the acute phase of infection [357, 358]. Interestingly, however, in contrast to HIV-1/SIV_{mac} infections, mucosal CD4⁺ T cell counts are partially (in SMs), even totally (in AGMs) recovered at the end of the acute phase of infection [357, 358] and the intestinal epithelial integrity is conserved over the long-term in nonpathogenic SIV infections [341, 359, 360]. The mucosal barrier is maintained by a preservation of the Th17 cells in the gut, allowing the conservation of a balanced Tregs:Th17 ratio, which is not observed in pathogenic HIV-1/SIV_{mac} infections [359, 361-364]. In infected RMs, the viral persistence, loss of mucosal barrier and immune activation are correlated with the depletion of IL-17-producing cells [365].

The CD8⁺ T cell responses and functions (CTL) have also been reported to be essential in the control of viremia and immune activation in SIV infections, although the exact mechanisms are not fully understood. Chowdhury *et al.* reported that the CD8-depletion in infected RMs strongly increases

viremia in all simian tissues and causes deleterious CD4⁺ T cells activation [366]. Moreover, SIV-specific CD8⁺ T cells were shown to be required to maintain suppression of viral replication in ART-treated RMs [367-369].

Natural SIV hosts such as SMs and AGMs do not undergo microbial translocation and the immune activation remains under control during the chronic phase of infection, thereby preventing progression to (simian) AIDS (Figure 18) [332, 341, 359, 370, 371]. Nonetheless, it is important to note that natural SIV hosts can mount strong and effective innate and adaptive immune responses, through the production of type I IFNs by DCs, up-regulation of interferon-stimulated genes (ISGs) and production of numerous proinflammatory cytokines during the primary infection to such an extent that this may promote immune activation [341, 372-375]. In fact, SIV-infected natural hosts evolved mechanisms and factors to resolve the immune activation after the acute phase of infection, whereas in HIV-1/SIVmac infections, persistence of immune activation leads to AIDS [326, 371, 376-378].

Importantly, the latter observation underscores the crucial influence of both the strong antiviral response in the primary infection and the host's ability to resolve immune activation during the post-acute phase of infection to avoid AIDS [379]. Moreover, the high production of type I IFNs - essentially by HIV/SIV-exposed DCs - in the acute infection induces expression of cellular restriction factors that can specifically inhibit viral replication steps and impede viral spread in natural hosts, as reported by Mir *et al.* [380]. As the restriction factors are the main research topic of this thesis, we dedicate a complete chapter to explain in detail their inhibitory functions in HIV replication. Along the same lines, a recent and elegant study pinpointed the preponderant role of the species-specific host factors (rather than virus-intrinsic virulence) in the lentiviral pathogenicity and disease outcomes [381] (see Chapter II).

The immunological mechanisms by which natural SIV hosts like SMs and AGMs manage the resolution of immune activation in the acute-to-chronic phase of infection have been extensively investigated (reviewed in [370, 371, 382]). Among the proposed mechanisms, the most interesting one – in the context of this thesis research project – is the ability of the SIV Nef proteins to downregulate the CD3-T cell receptor (TCR) complex from infected CD4⁺ T cells. The low levels of CD3-TCR complex at the cell surface decrease the responsiveness of the infected CD4⁺ T cells to further antigenic stimulation and as a consequence, these cells become refractory to subsequent activation

[383, 384]. Indeed, inefficient downregulation of CD3-TCR is associated with loss of CD4⁺ T cells in SIV infection [385].

Interestingly, while SIVs from natural hosts have conserved the Nef-mediated downregulation of CD3-TCR, pathogenic SIVcpz - and therefore HIV-1 - have lost this ability during their evolution following the acquisition of *vpu* gene [103, 386-388]. More interestingly, Nef proteins from HIV-2 have also conserved the ability to downregulate the CD3-TCR receptor in infected cells [6, 383, 389, 390]. As HIV-2 is considered as less pathogenic than HIV-1 (see next section), the CD3-TCR downregulation may represent a key mechanism involved in the disease progression.

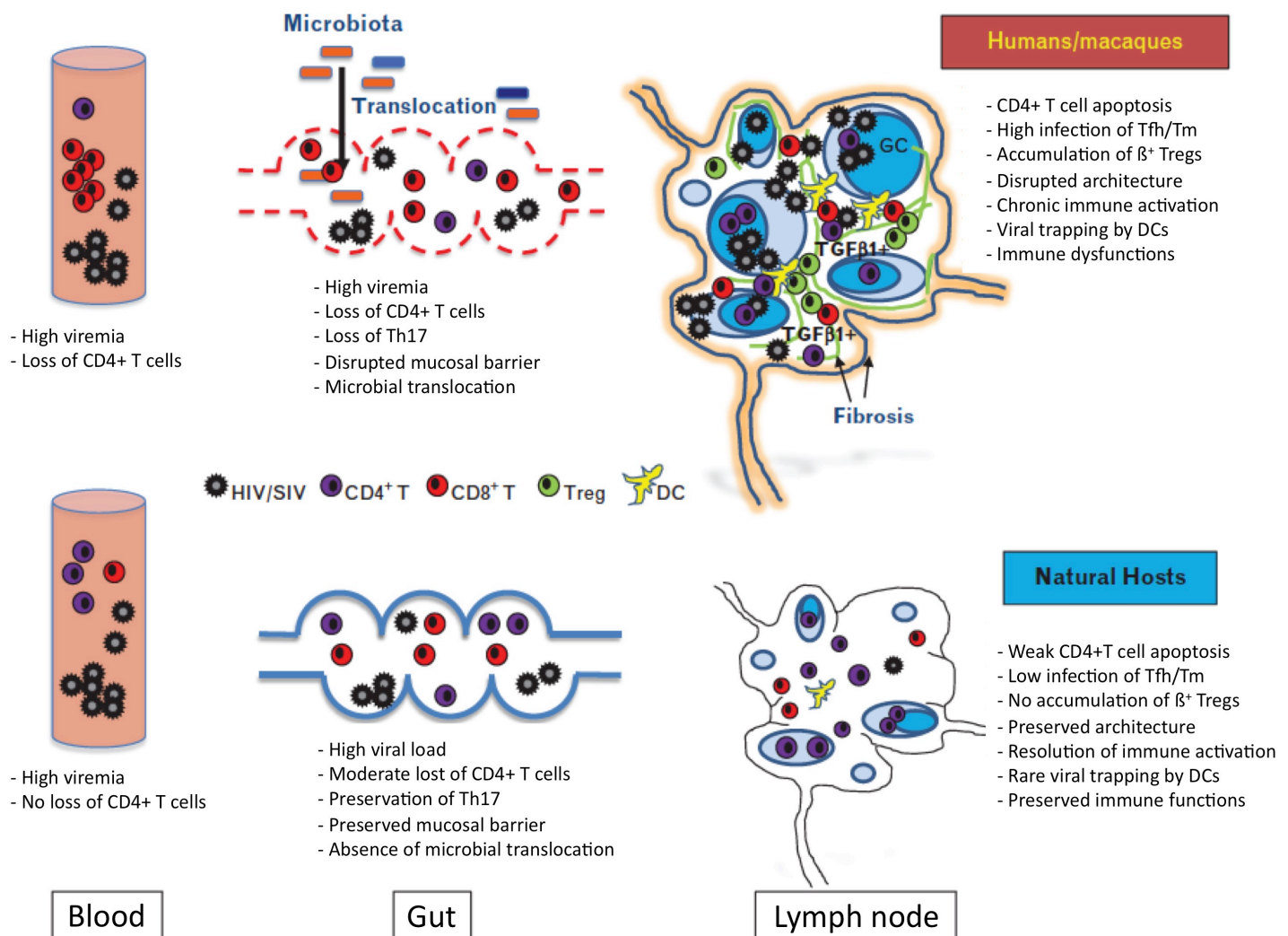


Fig.18: Major differences between pathogenic HIV/SIVmac infections and nonpathogenic SIV infections in their natural hosts. Although the gut mucosa is the site of active replication of retroviruses in all types of infection, natural hosts display a conserved epithelial barrier integrity and a preserved Th17 immunity. In nonpathogenic SIV infections, there is no microbial translocation (i.e. the passage of bacterial products from the

lumen of the gut into the blood circulation) and thus the immune activation is controlled over the long-term, in contrast to pathogenic infections. SIV infection in rhesus macaques is associated with sustained viral replication in the germinal centers (GC) and viral trapping by dendritic cells. This induces inflammation in the lymph nodes and subsequently the disruption of the lymphoid architecture (fibrosis). In striking contrast, nonpathogenic SIV infection in their natural hosts is associated with a rapid control of viral replication lymph nodes and a poor viral replication in germinal centers. Therefore, the lymph nodes present a normal architecture (adapted from [371]).

HIV-2 infection

HIV-2 shares many similarities with HIV-1, including the typical genomic organization of retroviruses (presence of *gag*, *pol* and *env* genes), a tropism for the immune cells, intracellular replication pathways, modes of transmission and may cause AIDS in human host. With disease progression, HIV-2-infected individuals experience similar clinical outcomes and opportunistic infections as those infected with HIV-1 [391-394]. However, in striking contrast to HIV-1, the vast majority of HIV-2-infected individuals does not progress to AIDS. This observation is reminiscent of the small group of LTNPs and ECs representing 1-3% of total HIV-1 cases, but concerning HIV-2 infection, this group of controllers accounts for 70-80% of cases, without resorting to ART [395, 396]. Furthermore, most HIV-2 patients experience a longer asymptomatic phase than those infected with HIV-1 [397]. Therefore, HIV-2 infection is generally considered as an attenuated model of retroviral infection. Nevertheless, it is important to note that this retrovirus cannot be defined as a benign or defective cousin of the highly pathogenic HIV-1 virus, but rather that HIV-2 is better controlled by the immune system of the infected host. Indeed, a recent (and unique) study described, by using a prospective HIV cohort in Guinea Bissau, that the HIV-2 may be more pathogenic than previously reported, and that HIV-2-infected individuals have a high probability of developing AIDS. The major difference would be a delayed onset of acquired immunodeficiency in individuals infected with HIV-2 [398, 399].

HIV-2 infection is characterized by lower viral loads, usually below the detection level (<50 RNA copies/mL) and a slower decline of CD4+ T cell counts than HIV-1 infection [400, 401]. Interestingly, the viral loads at comparable levels of CD4+ T cell counts are significantly lower in HIV-2-infected patients than those infected with HIV-1 despite similar levels of integrated DNA provirus [402-404]. The latter observation argues in favour of a similar capacity of both HIV-1 and HIV-2 to infect the

cells but also suggests that HIV-2 might be associated with lower viral replication at the post-integration steps or that HIV-2 is more susceptible to the immune control [223, 405-407].

The CD4⁺ T cells show a better proliferative capacity in aviremic HIV-2-infected patients and these cells demonstrate normal turnover rates and survival patterns during the course of infection [408]. A recent study demonstrated that low CD4⁺ T cell levels during the post-acute phase of HIV-2 infection were associated with faster disease progression [409]. Several studies have reported that the viral immune control is correlated with maintenance of HIV-2-specific CD4⁺ and CD8⁺ T cell responses and effector functions. Duvall *et al.* found that the HIV-2-specific T cells are more polyfunctional and produce higher levels of IL-2, IFN γ and TNF α , as compared with HIV-1-specific T cells [410, 411]. Moreover, other research groups revealed that HIV-2 infections are associated with efficient CD8⁺ T cell responses specifically directed over HIV-2 Gag epitopes [412-415]. Angin *et al.* described that the CD8⁺ T cells from HIV-2 nonprogressors display a strong capacity to inhibit HIV-2 infection in CD4⁺ T cells *ex vivo*. Moreover, they revealed that HIV-2 nonprogressors also retain robust lymphopoiesis and CD8⁺ T cell production. This may be a key mechanism for the conservation of an efficient CD8⁺ T cell response involved in viral control during the HIV-2 infection [416]. These polyfunctional HIV-2-specific T cell responses are a hallmark of HIV-2 infections and might play a crucial role in both maintaining HIV-2 viral loads and in slowing the disease progression.

The virus-specific CD4⁺ T cell response is also defined by a high proportion of cells producing MIP-1 β chemokine (also known as CCL4), which have a specificity for the CCR5 coreceptor. Thus, besides acting as a chemoattractant for natural killer (NK) cells, MIP-1 β binds the CCR5 coreceptor and impedes its recognition by HIV Env proteins. The high production of MIP-1 β is specific of the HIV-2 infections, since it is not observed in HIV-1 infections [411].

In HIV infections, the immune activation is a key determinant of immunopathology and disease progression [326, 336]. Noticeably, the immune activation levels observed in HIV-2 patients generally lie somewhere between those of seronegative and HIV-1-infected individuals [417, 418]. This is relatively coherent with the levels of immune activation observed in natural hosts infected with SIVs (SIV_{smm} or SIV_{agm}) and also evocative of the “less activation/low disease progression” paradigm. However, such studies enrolled only a few HIV-2 subjects with high viral loads and low CD4⁺ T cell counts. Moreover, these infected patients were in the chronic phase of infection and the levels of immune activation in the acute phase or in the acute-to-chronic phase were not evaluated. Nonetheless

these studies demonstrated that HIV-2 progressors present highly activated immune profiles and soluble markers of immune activation (such as β 2-microglobulin and neopterin), similar to those in HIV-1 infection. High immune activation observed in the subset of HIV-2 displaying high viremia could explain the disease progression to AIDS [417, 419, 420]. A further study conducted by Hegedus *et al.* described that aviremic HIV-2 individuals (with a CD4 count >500 cells/ μ L) present low levels of immune activation and normal rates of T cell turnover, while the viremic HIV-2 subjects (with similar CD4 counts) undergo a much higher immune activation and an accelerated T cell turnover (both CD4⁺ and CD8⁺ T cells), similar or even superior to those infected with HIV-1 [421]. Furthermore, a study reported that the fast disease progression is also correlated with high frequencies of CD4⁺ T cells with an exhausted PD-1 phenotype, as in the HIV-1 non-controllers [422] (see Figure 17).

As aforementioned, microbial translocation from the lumen of the gut to the peripheral blood circulation due to the loss of mucosal integrity during pathogenic HIV-1/SIVmac infections is a determinant of the immune activation. Since HIV-2 nonprogressors present lower levels of immune activation, it is tempting to hypothesize that the mucosal integrity is also conserved in these HIV-2-infected individuals. Recently Fernandes *et al.* found a sustained viral replication in the gut mucosa of HIV-2 nonprogressors, however they also showed that this replication is correlated with high IFN γ -producing CD8⁺ T cell expansion. With an interesting similarity to nonpathogenic SIV infections, the interleukin 17-producing T helper (Th17) cells, as well as Th22 cells, were highly preserved in HIV-2 infections. This research group reported that the epithelium integrity was conserved (essentially the tight junctions). Furthermore, high expression levels of CCL20 and CCL28 were significantly enhanced, suggesting a strong proliferation and recruitment of CD4⁺ T cells. This proliferation may potentially balance the loss of CD4⁺ T cells during the acute and chronic phase of HIV-2 infections [423]. Equally important is the potential ability of Nef proteins from HIV-2, but not HIV-1, to downmodulate the expression of the CD3-TCR complex from the cell surface of virally infected CD4⁺ T cells in order to diminish the responsiveness of these cells to further antigenic stimulation, thereby preventing CD4⁺ T cells hyperactivation and ultimately immune exhaustion. An early study suggested that the HIV-2 Nef-mediated downmodulation of CD3-TCR was not associated with low levels of immune activation or slow progression of the HIV-2 infection [424]. Nef variant sequences from 28 different HIV-2 patients, categorized as progressors or nonprogressors, have been tested for their

ability to downmodulate the CD3-TCR complex. In parallel, expression levels of several immune activation markers (CD38 and HLA-DR) were evaluated in the same individuals. These authors reported a correlation between the efficiency of Nef proteins to downmodulate the CD3-TCR complex and the immune activation in HIV-2 infected patients with a low viral load. However, they also reported that the ability of CD3-TCR downmodulation by Nef proteins was not impaired in HIV-2 progressors. These observations led the authors to conclude that the TCR downregulation does not necessarily avoid the disease progression in HIV-2 infections [424]. A few years later, an elegant study conducted by Khalid *et al.* confirmed that *nef* alleles from both viremic and aviremic HIV-2 patients showed equal efficiency of downregulation of the CD4, CD3-TCR, CD28 and MHC-I (major histocompatibility complex class I) expression. Nonetheless, they also observed that the potency of Nef proteins in downmodulating CD3-TCR was associated with high CD4⁺ T cell counts in viremic HIV-2 individuals, but not in aviremic HIV-2-infected individuals. This observation suggests that the efficient Nef-mediated downmodulation of CD3-TCR may be involved in the conservation (and homeostasis) of CD4⁺ T cell counts by avoiding T cell activation. Finally, these authors reported a role for HIV-2 Nef proteins in inhibiting the expression of PD-1 at the cell surface of CD4⁺ T cells [389].

Another striking feature of the HIV-2 infection is the high production of potent and highly-active neutralizing antibodies (nAbs) that are elicited by HIV-2 patients during the course of infection. Several studies reported that the proportion of HIV-2-infected individuals producing broadly neutralizing antibodies is higher as compared with HIV-1-infected patients (reviewed in [425]). For instance, Kong *et al.* reported the presence of potent neutralizing antibodies against HIV-2 envelopes from 64 HIV-2-infected patient samples [426]. In addition, these authors suggested that the low number of glycosylation sites in the V4 region of HIV-2 Env, in comparison with HIV-1 Env, decreased the shielding of this protein and increased the accessibility of HIV-2 envelope to neutralizing antibodies. It has been also observed that HIV-2-infected patients elicit heterologous and autologous nAb responses and that these antibodies remain active over the course of 15 years of follow-up, which is not observed in HIV-1-infected patients [427], even in elite controllers [428]. Three subsequent studies reported a high frequency of neutralizing antibodies that react potently with epitopes on the V3, V4 and CD4 binding site of the HIV-2 Env protein [429-431]. Therefore, production of antibodies with these specificities largely contributes to the potent neutralization of HIV-2 and thereby viral control observed in HIV-2 infected individuals, in contrast to HIV-1.

HIVs may also infect resting memory CD4⁺ T cells. In these long-lived cells, the integrated provirus is transcriptionally inactive. Therefore, there is no production of new virions. These latently infected cells are called the viral reservoirs and represent one of the major barriers to a functional cure of HIV/AIDS since the infected memory CD4⁺ T cells are not eliminated by the CD8⁺ T cells and the antiretroviral compounds are inefficient in these latently infected cells (reviewed in [432]). It is interesting to note that the size of the viral reservoirs may depend on both the efficacy of the CD8⁺ T cell response and the production of highly active neutralizing antibodies. For example, several studies demonstrated that in HIV-1 controllers, the efficient CD8⁺ T cell response combined with a high production of neutralizing antibodies are correlated to a reduced size of the viral reservoirs [433-435]. As aforementioned, in HIV-2-infected patients, the CD8⁺ T cell response and the production of neutralizing antibodies are increased, but there is no study describing the effect of these responses on the size of the viral reservoirs. Therefore, it would be interesting to assess the correlates of these responses and the size of the viral reservoirs in HIV-2 progressors and non-progressors individuals.

Finally, HIV-2 infection is characterized by a high specificity of the antibody-dependent cellular cytotoxicity (ADCC) response mediated by specific antibodies directed against Env [436], allowing the lysis of infected cells by NK [437]. Interestingly, a recent study observed the presence of cross-reactive ADCC targeting HIV-1 Env in plasma samples from HIV-2-infected individuals [438]. This large magnitude of the ADCC activity - in addition to all the immune mechanisms mentioned above - is certainly related to the protective immunity commonly seen in HIV-2-infected individuals.

Chapter II: The host restriction factors and viral antagonists

2.1 Overview of the innate immune sensing of HIV-1 and HIV-2

Upon virus entry, a variety of cellular proteins may recognize invading pathogens and trigger innate immune responses to inhibit the viral replication and dissemination. These specific proteins, constitutively expressed in almost all cell types, act as pattern recognition receptors (PRRs) and present a high ability to identify several pathogen-associated molecular patterns (PAMPs). PAMPs include viral components, such as viral DNA/RNA molecules or particular motifs within viral proteins [439-442]. The innate immune system represents the first barrier against viral infection. The innate system immediately responds to incoming viruses by sensing PAMPs and triggers potent defense mechanisms that include expression of interferons (IFNs, essentially type I and II), numerous interferon-stimulated genes (ISGs) and proinflammatory cytokines with potent antiviral functions (Figure 19). PRRs can be divided into two groups : Toll-like receptors (TLRs), which are membrane-associated proteins, and cytosolic receptors such as cGAS (cyclic GMP-AMP synthase), IFI16 (IFN γ -inducible protein 16) or RIG-I (retinoic acid-inducible gene I) [443-446].

Toll-like receptors 2, 4, 7, 8 and 9 can sense either the HIV envelope glycoprotein or the viral nucleic acids. TLR2 and TLR4 are expressed at the PM and may sense the HIV envelope glycoproteins, prior to the virus entry. Upon recognition of the Env glycoprotein, these TLRs can directly activate TAK1, which subsequently induces activation of the NF- κ B signaling pathway [447]. In addition to their antigen-presenting function, plasmacytoid DCs are the major producers of type I IFN in response to HIV [448]. In HIV-exposed dendritic cells, TLR7 and TLR8 recognize HIV ssRNA in the endosome and stimulate IFNs expression via activation of interferon regulatory factor 7 (IRF7) and NF- κ B as well [449, 450], while TLR9 recognizes the CpG DNA, a sequence enriched in cytosine-phosphate-guanine dinucleotides (Figure 19) [446, 451]. Upon HIV entry, the reverse transcription occurs and generates some nucleic acid intermediates (RNA, DNA/RNA, DNA molecules) which are sensed by cytosolic sensors in the infected cell. RIG-I activates the NF- κ B signaling cascade upon recognition of viral ssRNA molecules [452]. Three separate research groups reported in 2013 that HIV DNA intermediates are recognized by cGAS, which can produce cyclic GMP-AMP dinucleotides (cGAMP) in the cytosol. These dinucleotides cGAMP activate STING (stimulator of interferon genes) and ultimately induce, via the serine kinase TBK1, the IRF3-dependent expression of IFNs and ISGs [453-455] (Figure 19). Stem-loop containing HIV DNA may also be sensed by another cytosolic sensor,

IFI16, resulting in activation of STING, recruitment of TBK1 and phosphorylation of IRF3. Similar to the cGAS-mediated sensing of DNA intermediates, IRF3 stimulates expression of type I IFNs and numerous interferon-stimulated genes [442, 456].

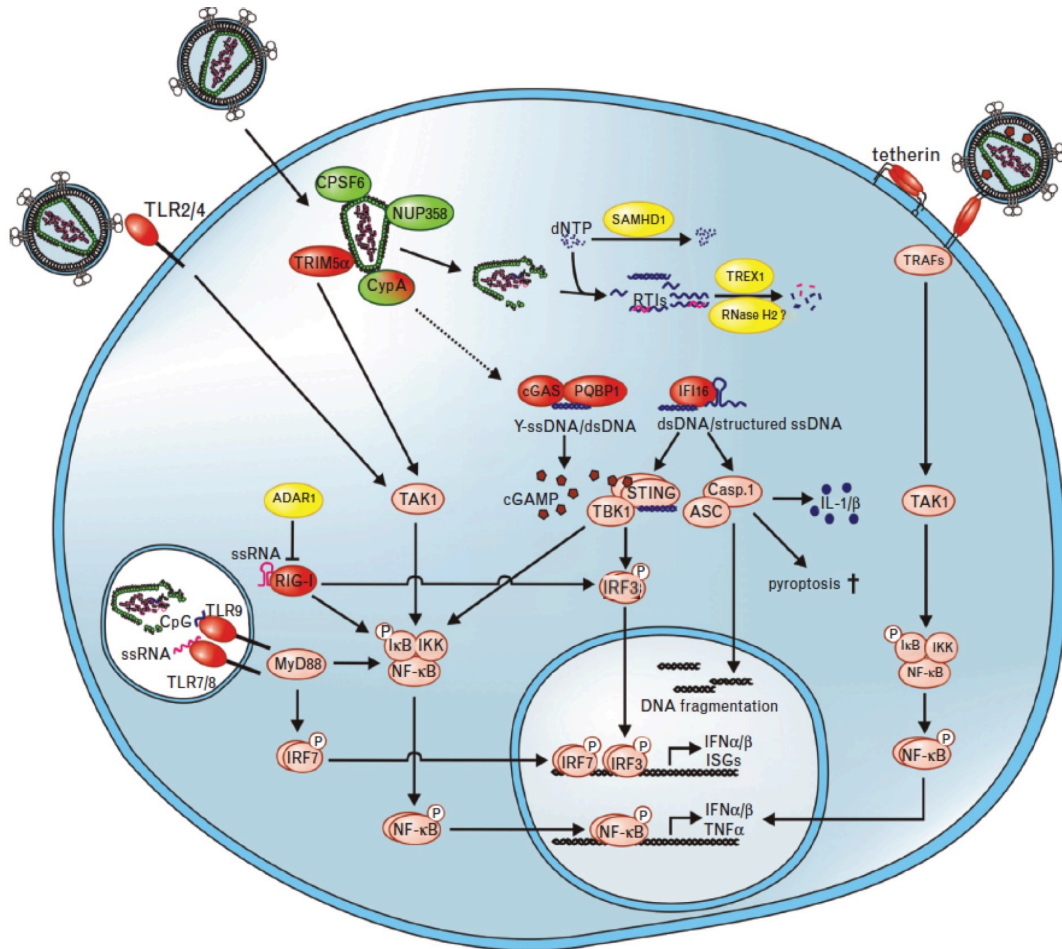


Fig.19: The innate sensing of HIVs. Membrane-bound and cytosolic immune receptors sense viral infections (shown in red). cGAS, IFI16 and RIG-I recognize DNA or RNA intermediates of reverse transcription. Two restriction factors (TRIM5α and BST-2) may sense viral infection upon binding of the incoming viral capsid and the budding of virions, respectively (see next section). The signaling cascades following activation of sensors involve STING, MyD88 or TRAFs and converge in the activation of the IRF3, IRF7 and NF-κB transcription factors. This results in antiviral and proinflammatory gene expression. CypA, CPSF6, and Nup358 (in green) are cellular proteins associated with the viral capsid core. Nucleases (TREX-1, SAMHD1, RNase H2 and ADAR1; in yellow) reduce sensing by depleting nucleic acid intermediates (adapted from [442]).

HIV-1 and HIV-2 are thus recognized by these innate immune sensors, which subsequently prompt the production of type I interferons (IFN-α/β; IFN-I). IFNs are well-known cytokines eliciting extensive antiviral effects by inducing the expression of more than 300 ISGs [457]. Indeed, once the type I IFNs are produced, mainly by plasmacytoid dendritic cells (pDCs), these cytokines bind to their cognate

receptor molecule that is expressed at the surface of a large number of cells. The IFN receptor (known as IFNAR) is composed of two transmembrane proteins, IFNAR1 (IFN- α/β receptor α chain) and IFNAR2 (IFN- α/β receptor β chain). The binding of IFN-I (α or β) to the IFNAR receptor induces the activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway. Phosphorylated STAT1 and STAT2 recruit IRF9 to form ISGF3 complex that can translocate into the nucleus, bind to the ISRE (IFN-stimulated response element) located in the promoter of the ISGs and trigger their expression, resulting in the production of direct antiviral effectors or molecules which build an antiviral state in the cell [458-460]. Therefore, the IFN/ISG responses play a crucial role in the early restriction and confinement of the HIV infections.

The relationship between the IFN/ISG responses and the immunopathogenesis of HIV and SIV infections has been extensively studied during the last three decades. It remains debated until now, as both detrimental and beneficial roles of IFN/ISG responses in HIV and SIV infections have been reported. Indeed, some studies associated IFN/ISG signaling with progression of the HIV-1 disease since a positive correlation between the plasma levels of IFN- α and the plasma viral load has been observed. Furthermore, an inverse correlation between the IFN- α levels and the CD4⁺ T cell counts has also been reported in the chronic phase of HIV-1 infection [461, 462]. Additionally, the production of IFN- α by pDCs may inhibit the proliferation of bystander CD4⁺ T cells and may trigger the apoptosis of uninfected CD4⁺ T cells residing in the lymphoid tissue of HIV-1-infected patients [463]. Several clinical trials based on the administration of exogenous IFN-I to (chronically) HIV-1-infected patients reported a modest and transient decrease of viral replication. The same observations have been made using NHP SIV models, irrespective of the infected hosts (nonpathogenic models like SMs and AGMs, or pathogenic model like RMs) (reviewed in [464-466]). Thus, and despite the antiviral functions of IFN-I, it seems that high IFN/ISG expression during the chronic phase of HIV-1 infection does not inhibit the viral replication, probably due to IFN-I desensitization of the immune cells. Conversely, it may reinforce the CD4⁺ T cells exhaustion and may contribute to the progression towards AIDS [467]. However, all these studies have explored the role of IFN-I in the immunopathogenesis of HIV or SIV during the chronic phase of infection. Thus, it remains uncertain whether the expression of IFN-I/ISG during this phase of the infection is the main cause or rather a consequence of the immune activation and the progression of the disease.

In contrast, accumulating evidence suggest that high IFN-I production during the acute phase of HIV or SIV infections is beneficial to prevent viral replication and dissemination, to maintain healthy CD4+ T cell counts and to slow the progression of the disease (reviewed in [464, 465, 468]). For example, a key study with SIV revealed that the blockade of the IFN-I signaling in RMs prior to infection or during the acute phase of infection has dramatic effects on the disease progression and clinical outcome: reduced antiviral gene expression, higher viral loads and increased SIV reservoirs size. Moreover, an accelerated decline of CD4+ T cells during both acute and chronic infection associated with a more rapid progression to AIDS were observed in these RMs compared to the control RMs [469]. These observations have been recently confirmed by Carnathan *et al.* [470]. In humans, it has been reported that the pDCs from HIV-1 elite controllers have an increased ability to produce IFN-I compared to that of HIV-1 non-controllers [471, 472]. In line with this, it is important to note that the plasma levels of IFN-I in HIV-2-infected individuals - who are considered as nonprogressors - are significantly higher than those of HIV-1, without resulting in higher levels of immune activation [418, 473]. Hence, the timing and the magnitude of the IFN-induced innate responses in the acute phase of SIV and HIV infections may profoundly affect the disease progression and may override the deleterious consequences of increased immune activation. Therefore, we postulate that the efficient and robust IFN/ISG responses during the acute phase of infection in HIV-2-infected individuals may be a major determinant of the lower levels of viral load and immune activation, and also that this strong immune response is involved in the delayed disease progression, as compared with HIV-1 infection. Our second article and our article “in preparation” partly investigate this issue.

Furthermore, a mechanism by which IFN-I may restrain the HIV viral replication during the acute phase of infection is the induction of the expression of an important set of genes belonging to the family of ISGs. These cellular proteins can virtually inhibit each step of the HIV viral replication cycle. These intrinsic factors are called the restriction factors (see next section).

2.2 The host restriction factors and viral antagonists

The restriction factors are cellular proteins and are autonomously acting proteins that exhibit a potent antiviral activity in cells. They are often constitutively expressed in several cell types and can be upregulated by IFN-I. These proteins employ specific mechanisms to inhibit several processes in the viral replication and have unusually diverse amino acid sequences as a consequence of antagonistic co-evolution with viruses. Finally, restriction factors are often, but not always, antagonized by viral proteins [474]. Herein, we provide a brief overview of the major restriction factors that play a role in the inhibition of the HIV replication steps (Figure 20). In the next section, we will focus on a unique restriction factor that we have studied in this research project, called BST-2/Tetherin.

The IFN-inducible transmembrane proteins (IFITMs)

IFITMs reside either at the PM or in the intracellular endosomal compartments and inhibit the viral entry of several enveloped viruses. IFITMs are essentially expressed in CD4⁺ T cells [475]. IFITMs reduce the fluidity of the cell membrane and impair the fusion between the cell membrane and the viral membrane, although the exact mechanism is currently unknown. Actually, IFITMs are incorporated in the nascent virions, thereby resulting in the production of viral progeny with a decreased viral infectivity [476]. It has been proposed that IFITMs may interact with the Env incorporation into the new virions, but this remains to be determined (review in [477, 478]) (Figure 20).

SERINC proteins

It has long been known that the accessory protein Nef of HIVs enhances the viral infectivity, but again the mechanism underpinning this phenotype was undetermined [479, 480]. However, in 2015, two separate groups described that this improvement of infectivity conferred by Nef relies on its ability to counteract a host restriction factor: the serine incorporator family proteins (essentially SERINC3 and 5). These proteins are highly expressed in CD4⁺ T cells, macrophages and dendritic cells, as well as several endothelial cell types [481, 482]. The Nef proteins exclude the SERINC3 and SERINC5 proteins from the membrane upon the assembly and budding of the virions from the infected cells [483].

The molecular mechanism of this countermeasure mediated by Nef is currently not fully

understood, while several studies suggested, again, that the HIV envelope glycoproteins may be the target of the human SERINC3 (reviewed in [478]).

TRIM5 α

Upon virus entry, the viral capsid proteins are recognized by the TRIM5 α proteins (Tripartite motif protein 5 α) in the cytosol. TRIM5 α is mainly expressed in CD4⁺ T cells. This potent restriction factor promotes the premature disassembly of the capsid core during the reverse transcription process [484, 485]. The C-terminal domain of TRIM5 α contains a SPRYPRY motif that can bind to the proline-rich domain of the retroviral CA. HIVs may circumvent the restriction of TRIM5 α by selecting mutations of these proline residues in the CA. In contrast, HIV-2 viruses harboring a proline at the position 119 within the CA are more sensitive to TRIM5 α [486].

APOBEC proteins

The reverse transcription process is targeted by the apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC)-3 family proteins. APOBEC3G and APOBEC3F are cytidine deaminase enzymes incorporated in the nascent virions and are highly expressed in all immune cells (CD4⁺ T cells, monocytes, macrophages and dendritic cells). In the newly infected cells, APOBECs catalyze cytosine-to-uracil deamination during the viral DNA synthesis in the reverse transcription complex [487]. This deamination leads to a high frequency of G-to-A hypermutations in the proviral DNA, thereby inducing lethal amino acid substitutions and/or premature STOP codons within the viral proteins (reviewed in [488, 489]). The accessory protein Vif is the viral antagonist of APOBEC proteins [490]. In the producer cells, Vif binds to APOBECs and promotes the recruitment of the Elongin B/C-Cullin-5 E3 ubiquitin ligase complex, leading to the ubiquitination and proteasomal degradation of APOBECs. This results in a lower rate of APOBEC incorporation within the newly produced virions (reviewed in [491]).

SAMHD1

Another restriction factor is involved in the impairment of the reverse transcription process during the HIV replication cycle. SAMHD1 (Sterile alpha motif and histidine aspartate domain containing

protein 1) exerts its antiviral function by depleting the pool of intracellular dNTPs (deoxynucleotide triphosphate), leading to very low levels of the dNTPs available for the viral reverse transcription [492-494]. SAMHD1 is only active in non-dividing cells, such as macrophages and dendritic cells (myeloid cells). Interestingly, only HIV-2 is able to counteract SAMHD1 by using its accessory protein Vpx. HIV-1, that lacks Vpx, is sensitive to this restriction factor [495-497]. This striking difference of antagonism between HIV-1 and HIV-2 will be discussed in the discussion section (chapter V).

Mx protein

The IFN-inducible Mx2 protein (Myxovirus resistance protein 2) is a member of the family of dynamin-like guanosine triphosphatases (GTPases) and is highly expressed in CD4+ T cells. Similar to TRIM5 α , Mx2 is a post-entry restriction factor that can bind the retroviral CA directly or via interaction with human cyclophilin A (CypA) [498-500]. Mx2 interferes with both the uncoating process and the nuclear import of the RTC/PIC (Figure 20) (reviewed in [501, 502]). To date, there is no evidence of a virus-encoded antagonist able to counteract Mx2.

Schlafen 11

SLFN11 (Schlafen protein 11) is expressed in all human cell types and prevents the modification in the composition of tRNA pool required for efficient translation of HIVs [503]. In physiological conditions, retroviral protein translation is hampered by the codon bias of lentiviral genomes, which have a high frequency of A/T nucleotides. To overcome this limitation to replication, HIV-1 induces a change in the composition of the tRNA pool, increasing the frequency of tRNAs decoding A-ending codons [504]. Thus, by maintaining the physiological composition of tRNA pool, SLFN11 exploits viral codon preferences to specifically attenuate viral protein synthesis [503].

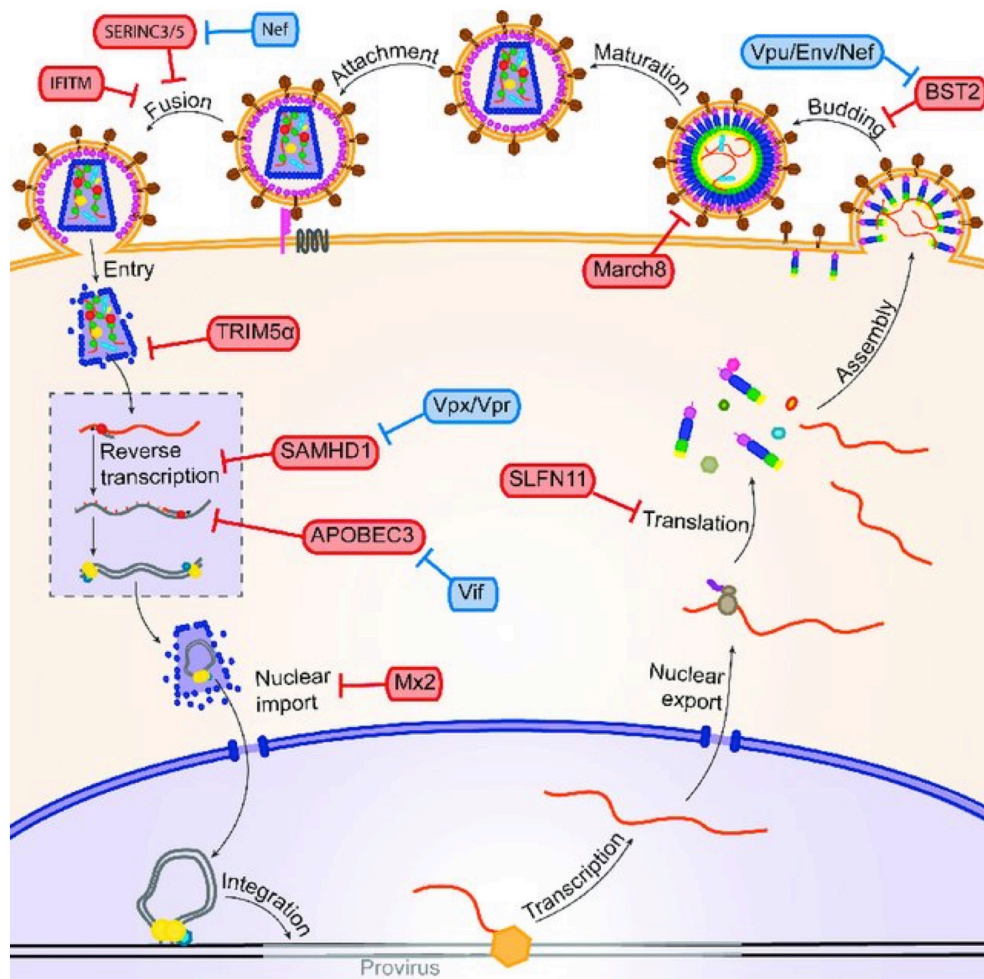


Fig.20: Schematic representation of the major restriction factors. All steps of the replication cycle are indicated and the restriction factors impairing or blocking these steps are represented in red. The virus-encoded proteins acting as viral antagonists of the restriction factors are highlighted in blue (adapted from [505]).

MARCH8

Recently, Tada *et al.* reported that the membrane-associated RING-CH8 (MARCH8) E3 ubiquitin ligase, a protein that is highly expressed in differentiated myeloid cells, possesses a potent anti-HIV-1 activity *in vitro* [506]. MARCH8 blocks the incorporation of the HIV-1 envelope glycoprotein into virus particles, resulting in a substantial reduction in the efficiency of virus entry, thus inhibiting its infectivity. Intriguingly, viruses are normally released, but are rendered non-infectious in the presence of MARCH8. Neither HIV-1 Vpr, Vpu, nor Nef has detectable anti-MARCH8 activity, suggesting that HIV-1 lacks a counter mechanism that dampens the effects of MARCH8. The same inhibitory effect was observed in virions containing envelope proteins from HIV-2 and SIVs [506].

Further studies are needed to unveil the precise mechanism by which MARCH8, as well as IFITMs and SERINC5 exert their antiviral function and whether the Env glycoproteins from HIV-1 and HIV-2 are involved in this restriction.

Finally, at the very end of the replication cycle, BST-2/Tetherin impairs the release of the neoformed virions by maintaining them at the cell surface, and subsequently promotes endocytosis and degradation of the viral particles. This potent restriction factor is antagonized by at least three different viral antagonists (see next section).

2.3 BST-2/Tetherin

Structure and functions of BST-2/Tetherin

Bone marrow stromal antigen 2 (BST-2, also known as tetherin or CD317) is an IFN-inducible transmembrane protein with an unusual topology. BST-2 is composed of 160 aa and displays a N-terminal domain that resides in the cell's cytoplasm, an alpha-helical transmembrane domain, an extracellular coiled-coil domain, and a glycosyl-phosphatidylinositol (GPI) anchor at the C-terminal side (Figure 21). Hence, BST-2 is a single-pass type II transmembrane glycoprotein and possesses two structural domains associated with the plasma membrane [507, 508]. Mature BST-2 forms homodimers through three disulfide bounds mediated by three highly conserved cysteine residues in the N-terminal part of the extracellular domain [509]. The N-terminal cytoplasmic tail of BST-2 includes an evolutionarily conserved dityrosine motif (YxY) which can recruit AP-2 to mediate clathrin-dependent internalization of BST-2 from the PM to the TGN (Figure 21) [510].

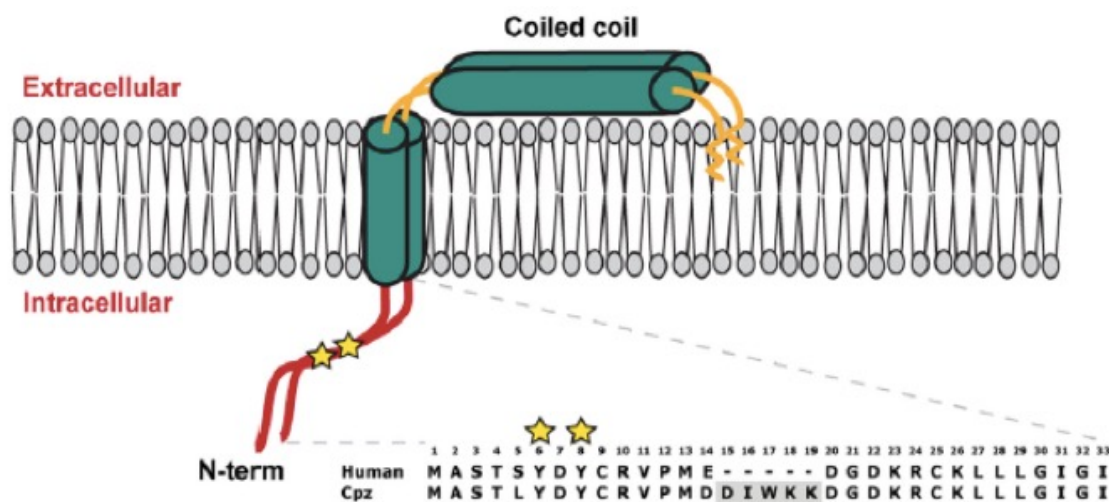


Fig.21: Structure and topology of BST-2/Tetherin in the PM. The cytoplasmic N-terminal amino acid sequences of the human and chimpanzee BST-2 are depicted. The dityrosine motifs are represented by the yellow stars (adapted from [478]).

Interestingly, the methionine residue at the position 13 of the BST-2 may serve as an alternative start codon, and thus BST-2 co-exists in two isoforms: the short and the long isoform. The former lacks the

first 12 aa and the endocytic di-tyrosine motif. Therefore, this short isoform preferentially localizes at the PM [511]. The extracellular domain also encompasses two asparagine residues which are glycosylated in the ER and the Golgi apparatus in order to address BST-2 at the PM (reviewed in [512]). Prior to the characterization of its antiviral functions, BST-2 had been identified in the late 1990's as a cell surface marker of bone marrow stromal and mature B cells, and had also been reported as a tumor antigen highly expressed on several types of myeloma cells [513]. Although a precise role was not attributed to BST-2, further studies revealed that this protein was constitutively expressed and also IFN-inducible in a large number of human cells, such as CD4⁺ T cells, macrophages and DCs, monocytes, as well as hepatocytes, gastrointestinal, renal and lung cells, suggesting a role in the innate and/or adaptive immune response [514, 515].

The BST-2 antiviral function was discovered as the target of the HIV-1 accessory protein Vpu, which is long known to facilitate the release of virions from the infected cells. In 2003, Bour *et al.* observed that *vpu*-defective mutants of HIV-1 are unable to be released from CD4⁺ T cells or macrophages, and remain attached at the PM or in endosomes [516]. Moreover, it has been reported that Vpu was also capable of increasing the release of other retroviruses or even filoviruses, suggesting an active restriction of different enveloped viruses in these cells [517-519]. In 2008, two separate groups concomitantly reported that BST-2/Tetherin is the host restriction factor counteracted by Vpu [520, 521]. The mechanism by which BST-2 tethers the budding virions lies on its topology. Indeed, BST-2 does not target any virus-encoded component, but rather the plasma membrane of the infected cells during the viral release. The GPI anchor of BST-2 is linked into the cholesterol-rich microdomains of the PM [522] and interestingly, HIVs predominantly assemble and bud at these sites of the PM [523] (Figure 22 A). Therefore, BST-2 is a potent antiviral factor that resides “at the right place” to retain the budding virions by trapping them at the PM and to perform subsequent AP-mediated endocytosis and degradation of the viral particle into endo-lysosome compartments (reviewed in [491, 524-526]) (Figure 22 B and C).

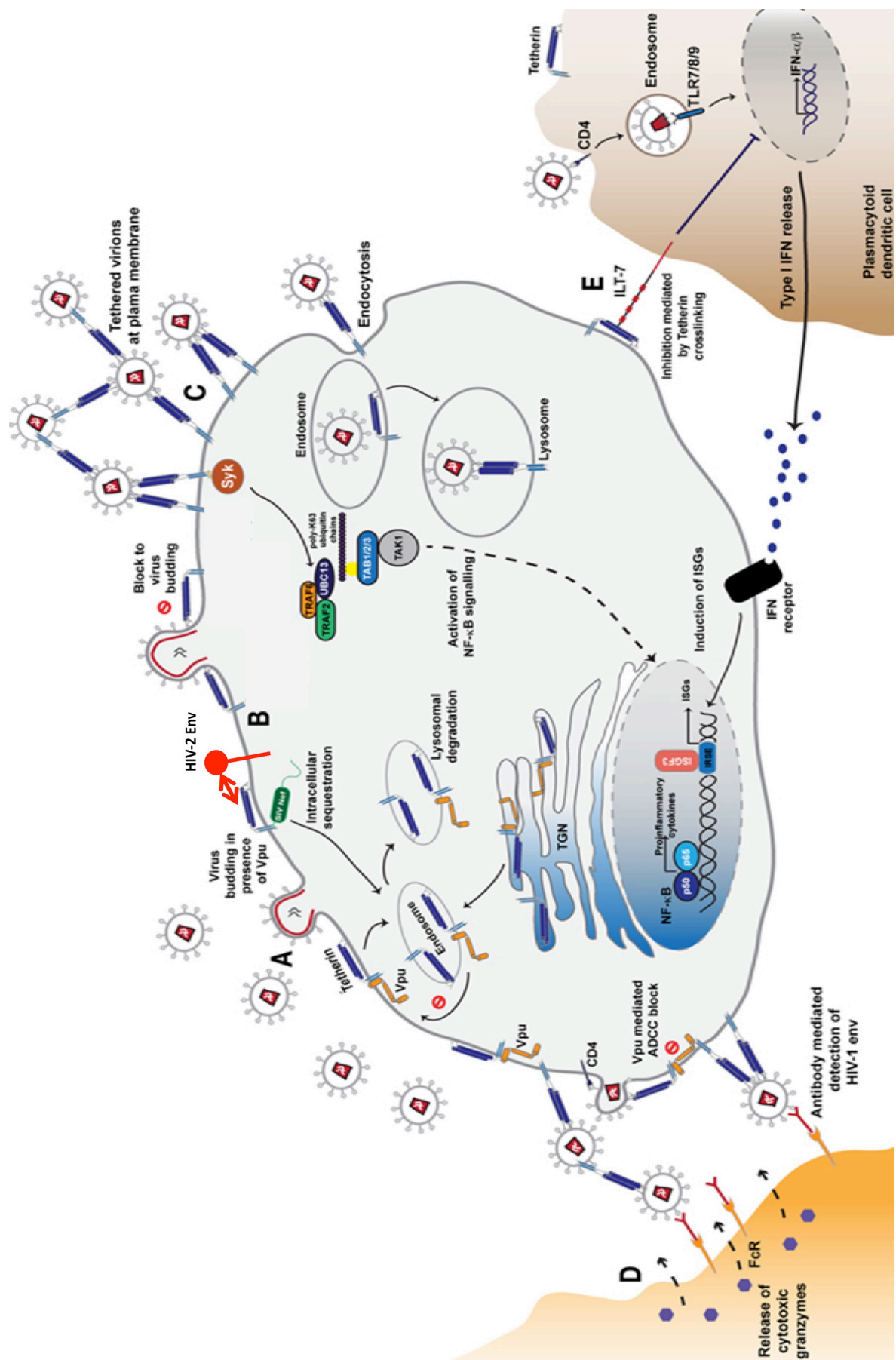


Fig.22: Topology and cellular functions of human BST-2. **A.** In presence of HIV-1 Vpu, HIV-2 Env or SIV Nef, BST-2 is removed from the cell surface and is subsequently sequestered in the TGN and/or degraded in lysosomes. **B and C.** In absence of viral antagonists, the viral particles are tethered at the cell surface by the human BST-2. The physical retention of retroviruses also leads to the phosphorylation of the diytrosine motif in the cytoplasmic domain of BST-2 and the recruitment of a Syk kinase. This Syk kinase ultimately activates TAK1 through TRAF6. TAK1 can activate the NF- κ B transcription factor in the cytosol. NF- κ B is translocated to the cell nucleus and binds the κ B binding sites localized in the promoter of numerous antiviral genes (see next section). The viruses retained at the cell surface undergo a BST-2-mediated endocytosis and subsequent degradation. **D.** By blocking the viral particles at the cell surface, BST-2 increases the antibody-mediated recognition of HIV or SIV Env glycoproteins. By this way, BST-2 promotes the recognition of infected cells by NK or FcR-expressing immune cells, thereby increasing the release of cytotoxic granzymes and lysis of the infected cells. **E.** BST-2 is able to bind the ILT-7 receptor at the cell surface of dendritic cells (DCs). DCs are the major producer of IFN-I. In order to avoid aberrant or uncontrolled production of IFN-I by DCs, BST-2 regulates it through this interaction with the ILT-7 receptor (adapted from [474]).

Interestingly, it has been reported that the viral particles retained at the PM were more exposed to the anti-Env antibodies, thereby sensitizing the cells to the anti-HIV ADCC responses mediated by the Fc-receptor found in myeloid and NK cells [527-529] (Figure 22 D). Another function of BST-2 concerns the ability of modulating the expression of IFNs by engaging the ILT-7 receptor at the PM of DCs. The BST-2/ILT-7 interaction results in a decrease of IFN-I expression in the DCs in order to avoid aberrant IFN-I production (Figure 22 E) [530].

Viral antagonists of BST-2/Tetherin

Given its broad antiviral activities, BST-2 has been selectively targeted by different viral antagonists to overcome the BST-2-mediated viral restriction, and these antagonisms occur in a species-specific manner in humans or monkeys. Although the virally-encoded antagonists and mechanism of action may differ, the anti-tetherin countermeasure is a conserved property among the primate lentiviruses (SIVs and HIVs). The fact that HIVs and SIVs have evolved three different proteins able to antagonize human or simian BST-2 highlights the importance of BST-2 as both antiviral protein and potent barrier of cross-species transmissions [512, 531].

Almost all SIVs use their Nef proteins to overcome tetherin and remove it from the sites of virus release. SIV Nef targets five amino acids (G/DIWKK) in the cytoplasmic domain of simian tetherin, and subsequently sequesters tetherin in a perinuclear compartment [531-534] (Figure 23 B). Exception to this are the Vpu-expressing SIVs from greater spot-nosed, mustached and mona monkeys (SIVgsn/mus/mon) that are able to use Vpu proteins to degrade tetherin of their hosts. In contrast, despite the presence of a *vpu* gene, SIVcpz and SIVgor also rely on Nef proteins to inhibit tetherin restriction [531]. Notably, the five amino acids (G/DIWKK) have been lost by the human tetherin during evolution (see Figure 21 for sequence comparison), thereby conferring resistance of the human BST-2 to the SIV Nef antagonism, representing an obstacle for successful cross-species transmissions of SIV to humans [535-537]. In humans, pandemic HIV-1 group M strains evolve Vpu to handle this species barrier and inhibit human BST-2 by targeting several conserved residues in the transmembrane domain of this restriction factor [538-540]. Subsequently, BST-2/Vpu complexes are directed to late endosomes for degradation. Vpu can recruit clathrin adaptor proteins and concomitantly with this process, the conserved DSGxxS motif in the cytoplasmic domain of Vpu interacts with a SCF (Skp1,

Cullin, F-box complex) E3 ubiquitin ligase through the adaptor proteins β -TrCP, leading to the ubiquitination of the cytoplasmic domain of Vpu and the targeting of the Vpu/BST-2 complex for degradation [541-543] (Figure 23 A).

HIV-1 group N strains (~ 20 cases of infections documented to date) also use their Vpu proteins to counteract the restriction of BST-2 but, in contrast to HIV-1 group M, Vpu proteins show only a weak ability to interact with and remove BST-2 from the infected cells [531]. In line with this, Vpus from the endemic HIV-1 Group O (~ 100,000 cases) are poor antagonists of BST-2 [544], although a single example of an efficient inhibition of BST-2 restriction by HIV-1 group O Vpu exists [545]. Instead, HIV-1 group O strains may use Nef proteins as antagonists of BST-2, mainly because these Nef proteins target several residues in the cytoplasmic tail of BST-2 that are adjacent to those typically used by SIV Nefs (the G/DIWKK motif) [546]. Nevertheless, this Nef-mediated antagonism of BST-2 is not as effective as that mediated by the Vpu proteins from HIV-1 group M [544].

HIV-1 group P viruses (two cases of infections) do not appear to use Vpu, Nef or Env proteins as a countermeasure of the BST-2 restriction [547]. Therefore, it is interesting to note that only the HIV-1 group M viruses, which encode a potent antagonist of BST-2, have efficiently spread into the human population [6, 512].

Concerning HIV-2 strains, which all lack Vpu, the viral antagonist of BST-2 is the envelope glycoprotein, as revealed by an elegant study [242]. Prior to the discovery of BST-2, it has been observed that the HIV-2 Env proteins can enhance the viral release from infected cells [548]. Le Tortorec *et al.* reported that the Env-mediated countermeasure of BST-2 relies primarily on the interaction with BST-2 through their respective extracellular domain (Figure 23 C). Nevertheless, these authors did not provide more precision on which motifs are involved in this interaction [242]. In 2016, we sought to determine this (or these) motif(s) involved in the Env-mediated countermeasure and we reported an important role for an asparagine residue in position 659 within the extracellular domain of HIV-2 Env in the antagonism of human BST-2 (see chapter III and our first article) [549]. We also revealed that the cytoplasmic tail (CT) of the HIV-2 Env protein cooperates in this ability to overcome BST-2.

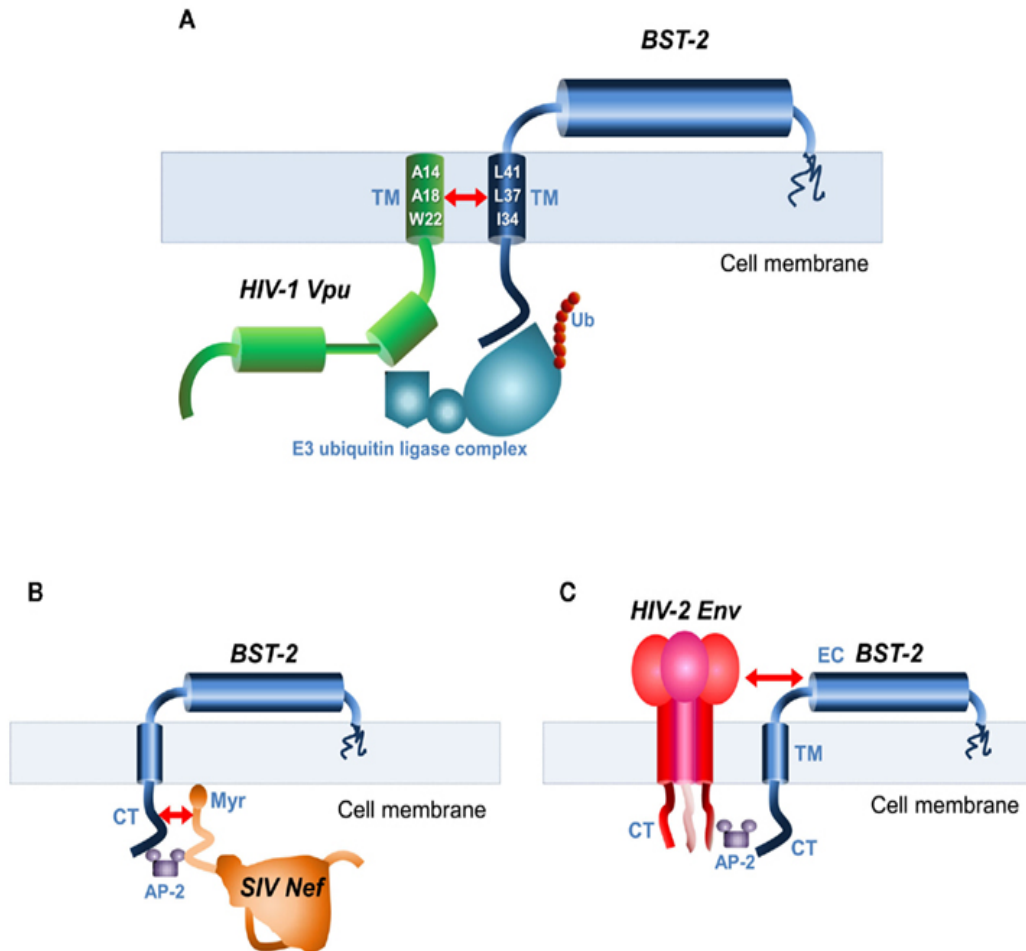


Fig.23: Overview of the viral antagonists used by HIVs and SIVs to overcome BST-2. HIV-1 uses Vpu (A), SIVs rely on Nef (B), while HIV-2 uses Env proteins (C) to antagonize BST-2/Tetherin. CT: cytoplasmic tail; TM: transmembrane domain; EC: ectodomain; Ub, ubiquitination chain; Myr: myristoylation (adapted from [550]).

Interestingly, Serra-Moreno *et al.* have described a similar role for the cytoplasmic tail of Env in the antagonism BST-2, by using a Nef-deleted SIV strain. This lentivirus is thus unable to antagonize tetherin. After several passages in tetherin-expressing cells, the virus has selected several compensatory changes in the Env CT to rescue the antagonism of BST-2 [551].

Endocytosis of BST-2 by the HIV-2 Env also employs the clathrin-dependent endocytosis process and requires the endocytic motif in the Env CT, GYXXΦ, that is highly conserved in HIV and SIV envelopes. Importantly, there is no BST-2 degradation upon endocytosis of BST-2/Env complexes, suggesting that the antagonism of BST-2 by the Env protein is less effective than Vpu from HIV-1 [242, 552].

In this thesis research project, we also looked at another function of BST-2 which consists of activating the NF-κB signaling pathway during HIV infections. This crucial function underscores that

on the one hand, BST-2 is a potent restriction factor belonging to the intrinsic immunity, but on the other hand, BST-2 can also modulate the adaptive immune response upon HIV infections by activating NF- κ B (see next section). In the second part of this research project, we investigated how the antagonism of BST-2 by the HIV-2 envelope glycoprotein affects the modulation of this signaling pathway (see chapter III, our second article as well as our “article in preparation”).

2.4 BST-2 and the NF- κ B signaling pathway in HIV-1 and HIV-2 infections

Upon retroviral retention at the cell surface, BST-2 promotes IFN-I and proinflammatory gene expression via the activation of the NF- κ B signaling pathway in the infected cells [249, 250]. In addition to its direct antiviral role mentioned above, BST-2 may also act as an immune sensor of viral infections and is involved in the adaptive immune response toward HIVs [524, 526]. The physical retention of viral particles induces the phosphorylation of the conserved tyrosine residues in the cytoplasmic domain of BST-2, which allows subsequently the recruitment of spleen tyrosine kinase Syk [553]. Syk promotes the activation of TAK1 through TRAF6 and/or TRAF2. Consequently, TAK1 phosphorylates the IKK complex, which in turn induces the phosphorylation of the inhibitors of κ B (I κ Bs). I κ Bs are also ubiquitinated by the SCF/ β -TrCP ubiquitin ligase complex and are then degraded [249-251]. This degradation of I κ Bs allows the translocation of the NF- κ B transcription factors into the cell nucleus. NF- κ B binds to its specific binding sequences localized in the promoter of a large number of genes involved in cell survival, expression of cytokines and chemokines including IFNs, as well as some ISGs (including restriction factors), thereby building a robust antiviral state in the infected cell and the surrounding cells. NF- κ B may also be activated by various stimuli or by the PRRs upon HIV entry into the cell (see also Figure 19). An overview of these diverse stimuli that can activate NF- κ B is outlined in the Figure 24. The roles and functions of NF- κ B in the innate and adaptive immune responses are extensively reviewed in several papers [244-247].

Interestingly, HIVs and SIVs contain a few NF- κ B binding sites in the enhancer region of their LTRs : just a single for the HIV-2 isolates, and two or three for the HIV-1 isolates [554]. Therefore, activation of NF- κ B may result in the improvement of LTR-driven proviral gene transcription and may be beneficial to viral replication. However, the activation of NF- κ B prompts the transcription of numerous antiviral genes, so that the balance between benefit and cost may not favour the retroviruses [555]. In accordance with this, it has been proposed that HIV-1 may regulate the NF- κ B pathway during the replication cycle, but the results of the studies are often contradictory (reviewed in [248]).

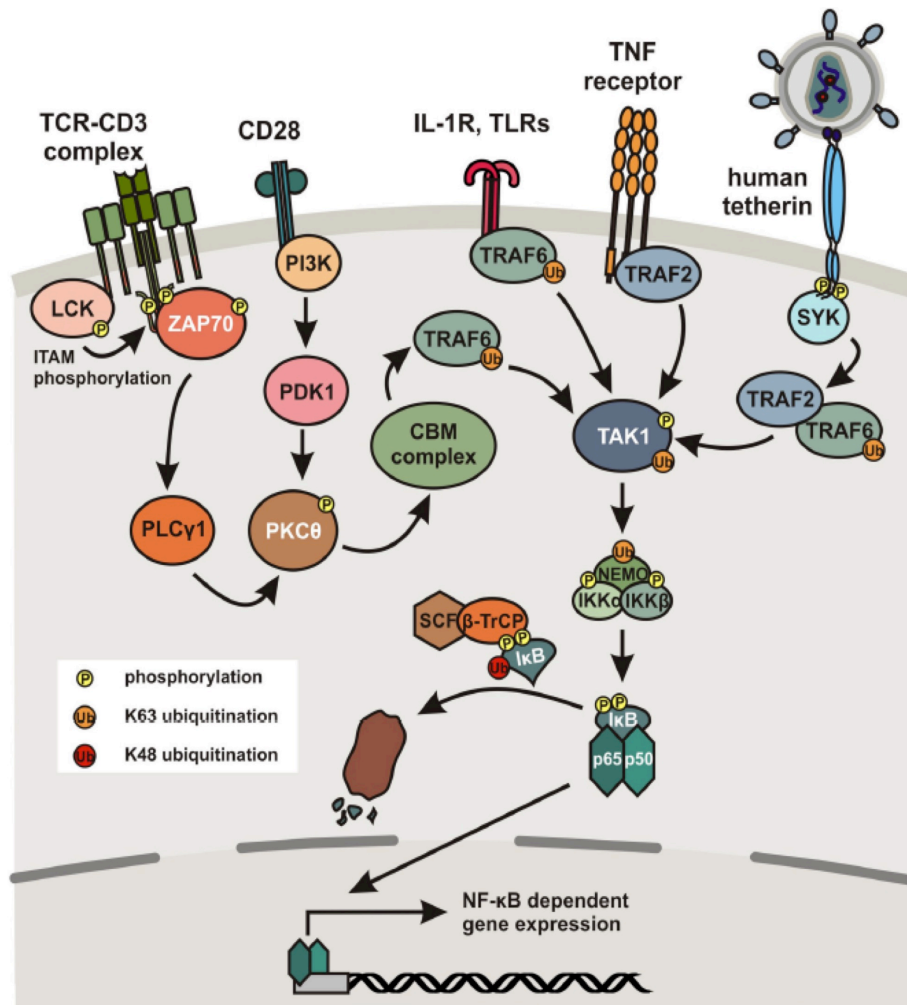


Fig.24: Schematic presentation of the NF- κ B activation by different cellular receptors. The binding of the TCR-CD3 complex by MHC molecules triggers LCK-dependent phosphorylation of ITAMs in the cytoplasmic domain of CD3. ITAMs recruit ZAP70, which induces activation of PLC γ 1 and PKC θ . PKC θ can also be activated through a signal cascade including PI3K after binding of the costimulatory factor CD28. PKC θ subsequently activates the CARD11, BCL10 and MALT1 complex (CBM), which activates TAK1 in a TRAF6-dependent manner. Ligand binding to TLRs or TNF receptors also induces TAK1 activation in a TRAF6- or TRAF2-dependent manner, respectively. In humans, the cellular restriction factor BST-2/Tetherin can also activate NF- κ B (p50/p65 heterodimers) which translocates to the nucleus where it binds to specific κ B binding sites and initiate NF- κ B-dependent gene expression (adapted from [248]).

HIV-1 Nef, Tat and Vpr proteins may promote the early activation of NF- κ B [556-560], while the late Vpu proteins potently inhibit the NF- κ B signaling pathway by both antagonizing BST-2 and stabilizing the inhibitors of κ B (I κ Bs) [249-251, 561]. This combined effect of Vpu results in a strong inhibition of the NF- κ B signaling pathway during the late stages of the replication cycle and allows HIV-1 to avoid the NF- κ B-mediated antiviral responses [251, 562]. Importantly, Postler and

Desrosiers described an important role of the HIV-1 (and SIVmac) Env CT in the activation of NF- κ B signaling pathway [243]. In this study, these authors revealed that the motif Y₇₆₆HRL in the cytoplasmic tail of HIV-1 Env (GenBank accession no. AF324493.2) is crucial for the activation of NF- κ B, and that this activation is TAK1-dependent. Further studies revealed that the inhibitory effect of Vpu on the NF- κ B is dominant over all other potential effects from other stimuli [251].

Therefore, since the ability of regulating NF- κ B activity appears to be crucial for the HIV-1 to dampen the innate and immune antiviral responses, it was tempting to study whether and how the HIV-2 Env protein was also able to activate the NF- κ B pathway and to analyze the potential effect of the anti-BST-2 function of HIV-2 Env in this activation, in comparison with HIV-1 Vpu. This is detailed in our second article as well as our “article in preparation”.

Chapter III: Objectives of the research project

3.1 Determination of the motifs in HIV-2 envelope glycoprotein involved in BST-2 antagonism

The first objective of this thesis is to identify the motifs in the extracellular domain of the HIV-2 Env protein involved in the antagonism of the host restriction factor BST-2. In HIV-1, it is well known that Vpu can bind BST-2, remove it from the virus budding sites and promote its degradation [520, 521, 542, 563]. Vpu binds human BST-2 mainly through interaction of their transmembrane domain upon recognition of several highly conserved residues in this domain of BST-2. Several alanine residues in the transmembrane domain of Vpu are involved in this interaction with BST-2 [564-566]. This potent countermeasure allows an increase of the release of HIV-1 viruses and also a downregulation of the BST-2-mediated innate and immune responses against the retroviruses. Indeed, the ability of Vpu to antagonize BST-2 in the initial phase of infection governs the viral replication and the successful dissemination, as well as the viral evasion of the immune system. Furthermore, Vpu-mediated antagonism of BST-2 is thought to be a crucial determinant of the IFN-I resistance of HIV-1, allowing the spread in human population [567, 568]. Since HIV-2 is better controlled by the host immune system compared to HIV-1, we aim to study the contribution of the HIV-2 Env-mediated antagonism of BST-2 in the modulation of the immune responses. To this end, we first seek to determine the residues within the extracellular domain of HIV-2 Env glycoprotein that are mandatory to antagonize BST-2. When starting this research project (2014), little was known about this (or these) motif(s) involved in this anti-tetherin function. An early study suggested that the interaction may be mediated by amino acid residues within the ectodomain of Env gp36 [242]. A subsequent study also revealed that the HIV-2 Env can induce the endocytosis and sequestration of BST-2, but notably and in contrast to HIV-1 Vpu, it does not result in the degradation of BST-2 [552].

3.2 Analysis of the regulation of the NF- κ B signaling pathway during the HIV-2 replication cycle

Besides its role of restriction of the viral release, BST-2 can act as an immune sensor of the viral assembly and budding. Upon sensing of these retroviral infection steps, the dityrosine motif in the cytoplasmic domain of BST-2 may recruit a Syk kinase that ultimately promotes NF- κ B activation via TAK1 [249, 250, 553]. NF- κ B subsequently translocates into the cell nucleus and triggers the expression of numerous antiviral genes, including some restriction factors and IFN-I (essentially IFN- β) (reviewed in [244-247]). Therefore, retroviruses have evolved means to inhibit this strong NF- κ B-mediated immune response. HIV-1 Vpu inhibits NF- κ B activation in the late phase of the replication cycle by two means: degradation of BST-2 and stabilization of the inhibitor of NF- κ B (I κ Bs) (reviewed in [248]). Interestingly, it has been reported that the HIV-1 Env protein can activate the NF- κ B signaling pathway [243], but the inhibitory effect of Vpu is dominant in order to dampen the NF- κ B-mediated antiviral response [251, 562].

Since the viral antagonist of BST-2 in HIV-2 is the Env protein, we aim to assess whether this protein could also activate the NF- κ B signaling pathway, and whether and how the anti-tetherin function of HIV-2 Env could modulate or influence this signaling pathway. As HIV-2 is better controlled by the host immune system than HIV-1, we hypothesize that several differences may exist in the modulation of the NF- κ B-mediated antiviral response during the HIV-1 and HIV-2 replication cycle.

3.3 Roles of the HIV-2 envelope glycoproteins in the viral replication

As mentioned above, the cytoplasmic tail of the envelope glycoprotein of retroviruses have several cellular and viral partners to ensure the viral replication and the production of fully infectious virions (see chapter I, section 1.4). During this research project, we have observed several roles of Env in the replication cycle of HIV-2. First, we have demonstrated in our first article that the cytoplasmic tail of HIV-2 Env protein cooperates in the antagonism of BST-2. Indeed, a complete cytoplasmic tail is necessary for the anti-tetherin function. Second, we revealed that the HIV-2 Env is involved in both the NF- κ B activation and in the modulation of this signaling pathway through the BST-2 antagonism during the replication cycle.

A third role has been observed by investigating the impact of insertions within the cytoplasmic tail of HIV-2 Env. Before the beginning of this thesis, our laboratory conducted a study in which we have obtained the sequences of several *env* genes from HIV-2-infected patients [569]. We have observed that the patients who presented the highest viral loads were infected with viruses harboring insertions of 7 or 3 aa in the cytoplasmic tail of Env proteins. Therefore, we aim to assess the impact of these insertions in the replication capacity and infectivity of these HIV-2 variants.

Chapter IV: Results

4.1 First article: “Single amino acid substitution N659D in HIV-2 envelope glycoprotein (Env) impairs viral release and hampers BST-2 antagonism”

The first article is related to the first objective of this research project and concerns the determination of the motifs within the gp36 ectodomain of the HIV-2 Env involved in the anti-tetherin function (see chapter III, section 3.1). This article has been published in *Viruses* in October 2016 (doi: <https://doi.org/10.3390/v8100285>). In this article, we demonstrate that the asparagine residue in position 659 of the Env glycoprotein is essential in the anti-tetherin function and is involved in the interaction between HIV-2 Env and BST-2. Moreover, we reveal that the cytoplasmic tail of HIV-2 Env cooperates in this anti-tetherin function [549].

Note that the references cited in this paper are shown at the end of the article and are not linked to the numbers of references cited in the text of the present thesis.

Article

Single Amino Acid Substitution N659D in HIV-2 Envelope Glycoprotein (Env) Impairs Viral Release and Hampers BST-2 Antagonism

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Abstract: BST-2 or tetherin is a host cell restriction factor that prevents the budding of enveloped viruses at the cell surface, thus impairing the viral spread. Several countermeasures to evade this antiviral factor have been positively selected in retroviruses: the human immunodeficiency virus type 2 (HIV-2) relies on the envelope glycoprotein (Env) to overcome BST-2 restriction. The Env gp36 ectodomain seems involved in this anti-tetherin activity, however residues and regions interacting with BST-2 are not clearly defined. Among 32 HIV-2 ROD Env mutants tested, we demonstrated that the asparagine residue at position 659 located in the gp36 ectodomain is mandatory to exert the anti-tetherin function. Viral release assays in cell lines expressing BST-2 showed a loss of viral release ability for the HIV-2 N659D mutant virus compared to the HIV-2 wild type virus. In *bst-2* inactivated H9 cells, those differences were lost. Subtilisin treatment of infected cells demonstrated that the N659D mutant was more tethered at the cell surface. Förster resonance energy transfer (FRET) experiments confirmed a direct molecular link between Env and BST-2 and highlighted an inability of the mutant to bind BST-2. We also tested a virus presenting a truncation of 109 amino acids at the C-terminal part of Env, a cytoplasmic tail partial deletion that is spontaneously selected *in vitro*. Interestingly, viral release assays and FRET experiments indicated that a full Env cytoplasmic tail was essential in BST-2 antagonism. In HIV-2 infected cells, an efficient Env-mediated antagonism of BST-2 is operated through an intermolecular link involving the asparagine 659 residue as well as the C-terminal part of the cytoplasmic tail.

Keywords: HIV-2; BST-2; tetherin; gp36; envelope glycoprotein; Env; restriction factors; viral antagonist; FRET; CRISPR/Cas9 knockout

1. Introduction

Human immunodeficiency virus type 2 (HIV-2) belongs to the *Retroviridae* family and can cause acquired immune deficiency syndrome (AIDS) as HIV-1 does. Whereas the latter virus is ubiquitous, HIV-2 is mainly localized in West Africa and was isolated for the first time in 1986 [1–3]. The highest prevalence of HIV-2 occurs primarily in Guinea-Bissau and Senegal [4]. Both types of HIV arose from different interspecies transmissions of simian immunodeficiency viruses (SIVs) naturally infecting African primates [2,5]. In untreated individuals, HIV-2 plasma viral load is generally lower compared to HIV-1, resulting in lower transmission rates [6]. Importantly, in most of the HIV-2-positive individuals

the disease does not progress to AIDS, although those who reach this disease state present clinical symptoms identical to HIV-1 infected patients [7–10]. In the HIV-2 infected group, many individuals appear to manage or control the infection and are therefore called long-term non-progressors [2,8,9,11]. A recent study explained, at least partly, the disparity of this evolution by showing the importance of host genetic factors in disease progression: two different individuals infected with a closely related strain of HIV-2 could be either viraemic or long-term non-progressors [12].

Some human protein families act as host cell restriction factors or cellular antiviral factors. Among those, APOBEC3G (apolipoprotein B mRNA-editing enzyme), a cytidine deaminase, introduces several lethal G to A substitutions during viral RNA retrotranscription in the cell cytoplasm [13]. TRIM5 α (tripartite motif 5- α) disrupts the viral uncoating [14]. BST-2 (also called tetherin or CD317), which restricts the viral budding of enveloped viruses at the cell surface [15–18], is an interferon-inducible factor specifically expressed in plasmacytoid dendritic cells, plasma cells, mature B and CD4⁺ T cells [15,19–22]. Consequently, BST-2 promotes endocytosis of the attached virions by recruiting the clathrin adaptor protein AP-2. Viral particles are then susceptible to internalization into endosomal compartments and undergo subsequent degradation [17,18,23–25]. Structurally BST-2 is a type II integral membrane protein, with the N-terminus inside the cell cytoplasm, a single membrane spanning domain, and a C-terminus modified by the addition of an unusual GPI (glycosylphosphatidylinositol) anchor [26–30].

Genetic analyses have demonstrated that BST-2 was under high positive selective pressure during mammalian evolution [31–34]. As BST-2 is a potent antiviral factor, mechanisms against tetherin activity were selected in the course of evolution in viruses. In HIV-1, the viral antagonist is the accessory protein Vpu (viral protein unique) [15,16,24,35] which interacts physically with BST-2 through its transmembrane domain and internalizes BST-2 via an ubiquitination mediated by β -transducin repeat-containing protein 2 (β -TrCP) [36–42]. Subsequently, BST-2 is sequestered in the intracellular compartments leading to a downregulation at the cell surface facilitating viral release [21,24,43,44]. Although most of SIVs use Nef (negative regulatory factor) as tetherin antagonist [35,43,45–47], HIV-2 relies on its envelope glycoprotein (Env) [48]. The HIV-2 *env* gene encodes two envelope glycoproteins from a proteinic precursor, cleaved into an external protein (HIV-2 gp105) and a transmembrane protein (HIV-2 gp36, or gpTM) [48,49]. The interaction between the viral protein and BST-2 at the cell surface leads to the endocytosis of the complex. However, Env does not promote degradation of BST-2 through the proteasome pathway as Vpu does [36,44]. Experiments with chimeric viruses showed that the Env regions required to antagonize BST-2 lie mostly in the HIV-2 gp36 ectodomain. However, amino acid residues involved in this antagonistic role are not clearly defined, except for the endocytosis motif (GYRPV) in the Env cytoplasmic tail (CT) that allows the internalization of the Env-BST-2 protein complexes [24,27,43,48]. A recent study [50] conducted with the HIV-2 ROD14 strain demonstrated that the residues K422 and A598 in the ectodomain of the gp are mandatory for BST-2 antagonism. Nevertheless this strain is poorly released from some cell types as compared to the other HIV-2 ROD strains [51] and ROD14 Env differs from the ROD reference strain by several mutations including seven amino acid substitutions and a large deletion in the CT [52].

In the present study, we mapped the residues involved in the binding ability and the antagonism of BST-2 by site-directed mutagenesis in the gp36 ectodomain coding region of a functional HIV-2 ROD molecular clone. This clone encodes an Env protein with a full cytoplasmic tail (158 amino acids [53]) and does not harbor the I312T or V536L substitutions observed in the ROD10 molecular clone [1,52,54]. In the present study we demonstrated the importance of the asparagine residue at position 659 in the HIV-2 gp36 ectodomain for the anti-tetherin function.

The cytoplasmic tail of the envelope protein could be required for effective BST-2 antagonism, because some regions interact with clathrin adaptors and are involved in the protein localization in the cell. Furthermore, the hypothesis of a full cytoplasmic tail required for anti-tetherin function is supported by a recent study reporting that a Nef-deleted SIVmac virus could reacquire functional BST-2 antagonism by introducing some compensatory changes in the Env cytoplasmic tail [27,55–58].

Therefore, we also tested here a virus presenting a C-terminal 109 amino acid truncation in the CT that is spontaneously selected in vitro when HIV-2 reference strains are cultured [49].

2. Materials and Methods

2.1. Cell Lines

HEK293T and H9 cells were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program (NIH ARRRP). HEK293T cells were maintained in Dulbecco's modified Eagles's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, HyClone™ FetalClone™ from GE Healthcare Life Sciences, Fairfield, CT, USA) and 1% gentamicin (Thermo Fisher Scientific). H9 cells were maintained in RPMI-1640 (Thermo Fisher Scientific) supplemented with 10% FBS and 1% gentamicin. Jurkat cells (clone E6-1) were obtained through the ATCC organization (Manassas, VA, USA) and maintained in RPMI-1640 GlutaMAX™ media (Thermo Fisher Scientific). All cells lines were tested for mycoplasma contamination before use.

2.2. Plasmids, Cloning and Site-Directed Mutagenesis

The pKP59 HIV-2 ROD proviral expression plasmid was obtained from Dr. Isabel Barahona (Instituto Superior de Ciências da Saúde-Sul, Portugal) [53]. This infectious clone encodes the whole genome of HIV-2 ROD reference strain (GenBank: M15390.1) and an envelope glycoprotein displaying a full cytoplasmic tail (158 amino acids). To generate all HIV-2 Env mutants tested in this study, site-directed mutagenesis was performed in order to introduce substitutions at the sites of interest in the pKP59-ROD infectious clone using the QuickChange II kit (Agilent Technologies, Santa Clara, CA, USA) and sequences were verified by nucleotide sequencing. HIV-2 envelope glycoprotein and *nef* sequences were amplified from the pKP59-ROD plasmid (primers *env*: 5'-ATGATGAATCAGCTGCTTATTGCC-3'; 5'-TCACAGGAGGGCGATTCTGCTCC-3' and primers *nef*: 5'-ATGGGTGCGAGTGGATCCAAG-3'; 5'-TTAACTAAATGGTATTCCTCTTGC-3'). Human *bst-2* sequence was amplified using cDNA obtained by reverse transcription of H9 cells mRNAs using the Transcriptor First Strand cDNA Synthesis Kit from Roche (Mannheim, Germany) (5'-ATGGCATCTACTTCGTATGAC-3'; 5'-TTACTGCAGCAGAGCGCTGAG-3'. NCBI Reference Sequence: NM_004335.3).

To customize and process the flow cytometry-based Förster resonance energy transfer (FRET) assay, pcDNA3-Clover (donor fluorochrome) and pcDNA3-mRuby2 (acceptor fluorochrome) expression plasmids were used. Both these FRET-adapted plasmids were a gift from Michael Lin (Departments of Pediatrics and Bioengineering, Stanford University, USA; Addgene plasmid # 40259, # 40260, respectively; [59]). The pcDNA3.1-Clover-mRuby2 was donated by Kurt Beam (Department of Physiology & Biophysics, University of Colorado, USA; Addgene plasmid # 49089) and served as a positive control. In order to fuse the Clover fluorochrome at the N-terminal part of protein, the tetherin sequence was amplified with primers including the restriction sites *Bsr*GI and *Apa*I (5'-TGACAAGATGGCATCTACTTCGTATGAC-3'; 5'-GGCCCTTACTGCAGCAGAGCGCTGAG-3'). *Bsr*GI digestion of pcDNA-Clover removed the initial stop codon of the fluorochrome coding sequence, thereby enabling the correct expression of the Clover-BST-2 fused protein. Since the membrane localization signal of BST-2 is determined by both the anchor signal located in the transmembrane domain and the GPI-anchor, addressing of the BST-2 fusion protein is not disrupted in those conditions [26,28].

HIV-2 Env was fused with the mRuby2 fluorochrome on its C-terminal part. Env sequences from HIV-2 wild type (WT), Env 1-749 and Env N659D were amplified with primers including the restriction sites *Hind*III and *Nhe*I (for env WT and env N659D: 5'-AAGCTTATGATGAATCAGCTGCTTATTGCC-3'; 5'-GCTAGCCAGGAGGGCGATTCTG-3', and for env 1-749: 5'-GCTAGCGGGCCAGTATCTGTCTCCAC-3' reverse primer) by taking care to remove the stop codon in the *env* sequence in order to enable the correct expression of the fused protein.

For cloning HIV-2 *nef* sequence in pcDNA3-mRuby2, an analogous protocol was used, including *HindIII-NheI* sites. HEK293T cells (5×10^5) were co-transfected with the following combination of constructed expression plasmids: 500 ng of plasmid expressing BST-2-Clover fusion protein and 1 μ g of plasmid expressing Env-mRuby2 (or Env 1-749-mRuby2 or Env N659D-mRuby2) fusion proteins. A configured BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) was used to assess the binding capacity of HIV-2 Env WT and Env mutants proteins to BST-2 based on the FRET principle (a complete analysis of the cytometry platform configuration is detailed in the results section).

To perform *bst-2* knockout, a pL-CRISPR.EFS.GFP plasmid encoding *Streptococcus pyogenes* CRISPR associated protein 9 (SpCas9) and green fluorescent protein (GFP), a gift from Benjamin Ebert (Harvard Medical School, Boston, USA; Addgene plasmid # 57818, [60]), was used to transduce single guide RNA (sgRNA) targeting *bst-2* gene. Sequence fidelity for all constructed plasmids was verified by sequencing.

2.3. Cell Infections and Viral Release Quantification

The HIV-2 Env mutants selected using the comparison of the HIV-1 (HXB2, MN, BRU/LAI and BH10 strains) and HIV-2 (ROD, BEN, EHO and D205 strains) gp41/gp36 amino acid sequences were generated using site-directed mutagenesis from the pKP59-ROD infectious clone. HEK293T cells were transfected with these clones harboring mutations in Env. Briefly, 3×10^5 cells were harvested and seeded in a 24-well plate 24 h before transfection. One microgram of pKP59-ROD was used to transfect cells with TurboFect Transfection Reagents according to the manufacturer's instructions (Thermo Fisher Scientific). Three days post-transfection, supernatant was collected and centrifuged 10 min at $800 \times g$. Then, 5×10^5 H9 cells were infected with 250 μ L of infectious supernatant. Infected cells were washed three times with phosphate-buffered saline (PBS) (Thermo Fisher Scientific) after four hours of infection and then seeded in fresh RPMI-1640 media supplemented with 10% FBS and 1% gentamicin. Three days post-infection, the cell-free supernatant was filtered (0.45 μ m) in order to quantify the viral particles in the cell culture medium and to establish virus stocks. For virus quantification, viral RNA was extracted with the MagNA Pure Compact Nucleic Acid Isolation Kit (Roche) and eluted in 50 μ L. Three microliters of RNA served as a template for the reverse transcription (RT) reaction (Transcriptor First Strand cDNA Synthesis Kit from Roche). Three microliters of cDNA were added to a FastStart Essential DNA Green Master (Roche, Mannheim, Germany) containing primers specific to HIV-2 long terminal repeat (LTR; primers JR72 and JR73 [61]). Quantitative RT-PCR (RT-qPCR) was performed on a LightCycler® 96 platform from Roche and absolute quantification (based on in vitro transcribed RNA calibrators [62]) was performed with the LightCycler® 96 software.

For the viral release assays, a multiplicity of infection (MOI) of 2 was chosen to infect H9 and Jurkat cells (3×10^5 cells infected with 6×10^5 viral particles). Viral release was quantified by RT-qPCR at two, three and six days post-infection for the HIV-2 Env mutants tested in comparison to the HIV-2 WT virus. A negative control (supernatant from uninfected H9 or Jurkat cells) was tested in every viral quantification experiment.

2.4. Subtilisin Treatment

Three days post-infection, the infected H9 cells were centrifuged ($300 \times g$ for 5 min). While the viral RNA present in the supernatant was stored for quantification, cell pellets were gently washed once with PBS, resuspended in 30 μ L of subtilisin buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 5 mM CaCl₂ in PBS 1X; concentration: 1 mg of subtilisin in 1 mL of buffer) and placed at 37 °C for 15 min. After incubation, 170 μ L of phenylmethylsulfonyl fluoride (PMSF) (stock solution: 200 mM in 50 mL of culture medium) were added to the reaction to stop proteolysis, preserve cellular integrity and inactivate subtilisin protease (purchased from Sigma-Aldrich, St. Louis, MO, USA). Cells were centrifuged again and supernatant was collected to quantify the number of viral particles tethered at the cell surface. Two negative controls were tested for viral quantification: a medium from uninfected H9 cells treated with subtilisin, and a medium from infected H9 cells not treated with subtilisin.

2.5. CRISPR/Cas9 Knockout

The pL-CRISPR.EFS.GFP plasmid produced *bst-2* gene expression knockout in H9 cells. *BsmBI* (*Esp3I* isoschizomer, Thermo Fisher Scientific) restriction sites were used to clone the sgRNA sequence (5'-CAGCGAGGCCTGCCGGGACGGCCTTCG-3') downstream of a hU6 promoter in the pL-CRISPR.EFS.GFP plasmid. Its transcription products target the first exon of *bst-2* and allow Cas9 to produce a double-strand break in *bst-2*. Pseudotyped viruses were created by transfecting lentivectors in HEK293T cells: 750 ng of pL-CRISPR.EFS.GFP (encoding sgRNA of interest), 500 ng of psPAX2 (psPAX2 was a gift from Didier Trono (EPFL, Lausanne, Switzerland; Addgene plasmid # 12260) and 300 ng of pMD2.G encoding vesicular stomatitis virus envelope protein (VSV-G; Addgene plasmid # 12259). At 24 h after transfection, supernatant was collected, centrifuged to discard cells (or debris) and stored at 4 °C. Fresh cell culture medium was added to the virus-producing HEK293T cells for an additional 24 h. The pseudovirus-containing supernatants were pooled and used to infect H9 cells. After several passages, infected cells were sorted using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) according to GFP emission since Cas9 protein was fused to GFP. This transduction remained stable for approximately one month. Furthermore, Western blot was performed to confirm *bst-2* knockout. The viral release assay performed with these *bst-2*^{-/-} H9 cells was the same as previously described. As a negative control, pL-CRISPR.EFS.GFP encoding the initial sequence between *BsmBI* restriction sites, which recognized the human cyclic adenosine monophosphate (cAMP) and cAMP-inhibited cyclic guanosine monophosphate (cGMP) 3',5'-cyclic phosphodiesterase 10A intron sequence, was also used in this experiment.

2.6. Statistics and Software

Graphs and statistical analyses (Kruskal–Wallis one-way analysis of variance followed by Dunn's multiple comparisons test) were performed using the GraphPad™ Prism 7.0 (GraphPad Software, La Jolla, CA, USA). A *p*-value ≤ 0.05 was considered to be statistically significant.

UniProt™ alignment tool allowed the comparison of the HIV-1 and HIV-2 gp41/36 sequences.

To analyze the fluorescence-activated cell sorting (FACS)-based FRET assay, FlowJo™ Data Analysis Software (version 10.1, Ashland, OR, USA) was used to generate multiple graphs.

3. Results

3.1. Residue N659 in the HIV-2 ROD Env Protein Is Involved in the Efficiency of Viral Release

Since HIV-1 Env does not antagonize BST-2 [27], we selected 47 potential amino acids in HIV-2 Env by a comparison of the HIV-1 and HIV-2 gp41/36 amino acid sequences. Each conserved amino acid in HIV-2 reference strains but differing from HIV-1 reference strains HXB2, BRU/LAI, MN and BH10 was substituted with the corresponding HIV-1 residue. We used site-directed mutagenesis to introduce mutations at the sites of interest in a pKP59 (HIV-2 ROD) infectious clone and generated 32 different HIV-2 Env mutants (Figure 1).

The first objective was to find a mutant virus unable to antagonize BST-2, presenting thus a significant lack of release from infected cells since BST-2 inhibits the viral budding step in the viral cycle. HEK293T cells, which do not express BST-2, were transfected with the clones produced by mutagenesis and the released viral particles were collected in order to infect H9 cells, a cell line expressing BST-2 (MOI = 2). A RT-qPCR was performed to quantify viral particle release in the H9 cell culture medium. Since the HIV-2 Env cytoplasmic tail could be important in the Env-mediated anti-tetherin function as previously suggested [55,57,63], in this experiment we also tested the HIV-2 Env 1–749 mutant (mutant 32, Figures 1 and 2) virus that expressed a truncated cytoplasmic tail (having a 49 amino acid CT length), in order to determine the impact of this size variability on the viral release ability and on the anti-tetherin activity.

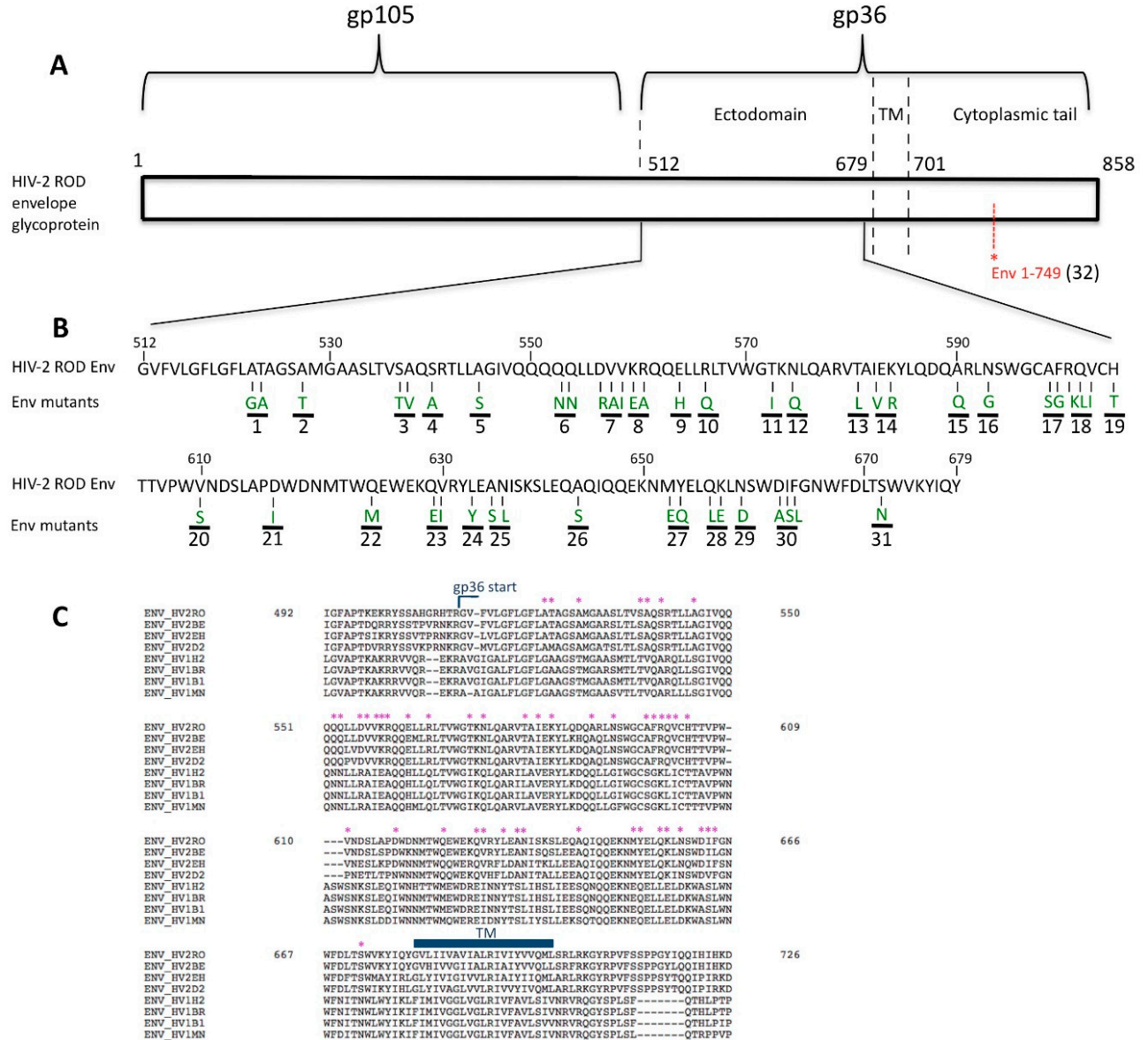


Figure 1. Schematic representation of human immunodeficiency virus type 2 (HIV-2) ROD envelope glycoprotein (Env). (A) The functional HIV-2 ROD Env protein is composed of an external domain (gp105) and a transmembrane domain (gp36). Three regions comprise gp36: the ectodomain, the membrane spanning domain and the cytoplasmic tail (158 amino acids). To generate a mutant virus with a truncated cytoplasmic tail (Env 1–749), the tryptophan residue (TGG) at the *env* position 749 was replaced with a stop codon (TAA) using site-directed mutagenesis. This position is indicated in red in this figure (TM: transmembrane domain); (B) Amino acids 512 to 679 composing the HIV-2 ROD gp36 ectodomain are shown in this panel. Based on a comparison of the HIV-1 and HIV-2 gp41/36 amino acid sequences, 47 positions were selected (Env mutants). All the selected amino acids mutations are highlighted in green, each corresponding to the related HIV-1 Env residues used to generate substitutions by site-directed mutagenesis of the pKP59 HIV-2 ROD plasmid. The 32 HIV-2 Env mutants generated in this study are numbered and highlighted with the black lines in this figure; (C) Alignment and comparison of the HIV-1 and HIV-2 gp41/36 amino acid sequences. Selected amino acids are pointed with the symbol * (Env_HV2RO: HIV-2 ROD strain; Env_HV2BE: HIV-2 BEN strain; Env_HV2EH: HIV-2 EHO strain; Env_HV2D2: HIV-2 D205 strain; Env_HV1H2: HIV-1 HXB2 strain; Env_HV1BR: HIV-1 BRU/LAI strain; Env_HV1B1: HIV-1 BH10 strain and Env_HV1MN: HIV-1 MN strain).

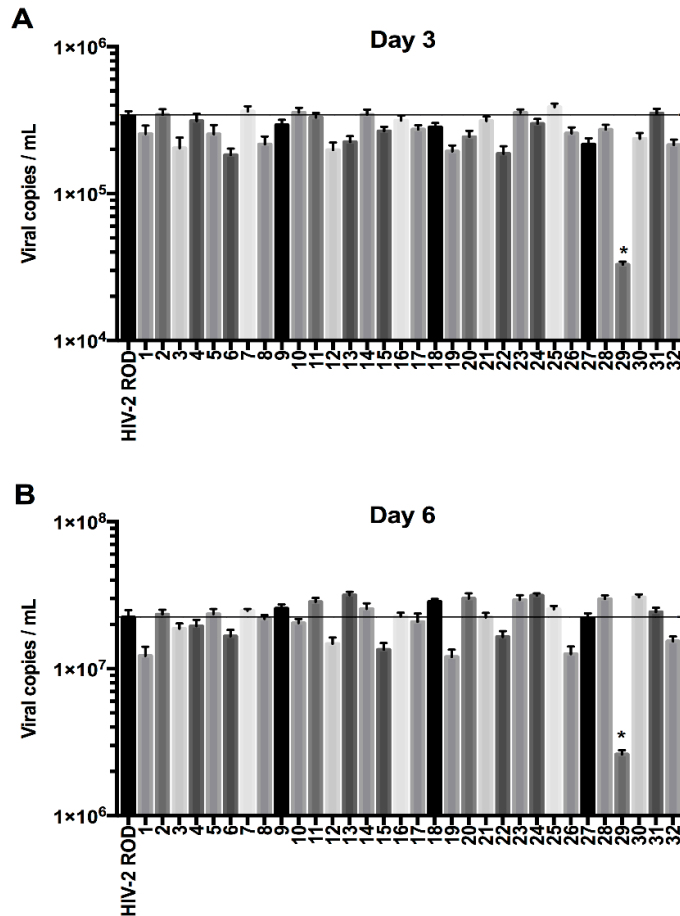


Figure 2. Absolute quantification of viral release ability among thirty-two HIV-2 Env mutants. The different clones were generated by site-directed mutagenesis and transfected in HEK293T cells. The viral particles were then used to infect H9 cells, a BST-2 producing cell line. Quantification was performed at three (**A**) and six (**B**) days post-infection ($n = 3$ independent experiments). The mutant numbers 1 to 32 are the same as highlighted substitutions in Figure 1B. The HIV-2 Env N659D mutant (mutant 29) showed a lower viral release compared to the HIV-2 Env WT. Error bars indicate mean $\pm$ standard deviation (SD) and statistical tests give statistical significance with a $p < 0.05$ (*).

In addition, we also tested a mutant carrying a similar mutation to HIV-2 Env ROD14 (substitution of the Env A598 residue) that was crucial for BST-2 antagonism in a recent study [50]. Intriguingly, in our hands this point mutation (mutant 17, Figures 1 and 2) did apparently not cause a lack of viral release from H9 cells compared to HIV-2 Env WT in the context of the present study.

Among the 32 HIV-2 Env mutants tested, the one harboring the N659D substitution showed a lower viral release from infected H9 and Jurkat cells compared to HIV-2 wild type virus at two, three and six days post-infection (Figure 3).

In order to link the poorer release observed for the HIV-2 Env N659D virus to a viral inability to bud from the cell surface, we performed a subtilisin treatment of the infected H9 cells three days post-infection. As BST-2 inhibits the viral budding and retains the virions at the cell surface by embedding its GPI anchors into the budding virus membrane [21,29], subtilisin protease cleaves BST-2 peptides and allows the release of tethered virions. We performed RT-qPCR to quantify the number of tethered virions for H9 cells infected with the HIV-2 Env WT, HIV-2 Env 1-749 or HIV-2 Env N659D viruses. This experiment suggested a deficit of BST-2 antagonism for the N659D mutant compared to the Env WT and Env 1-749 viruses. Indeed, the HIV-2 N659D virus was eleven-fold more tethered at the cell surface (Figure 4).

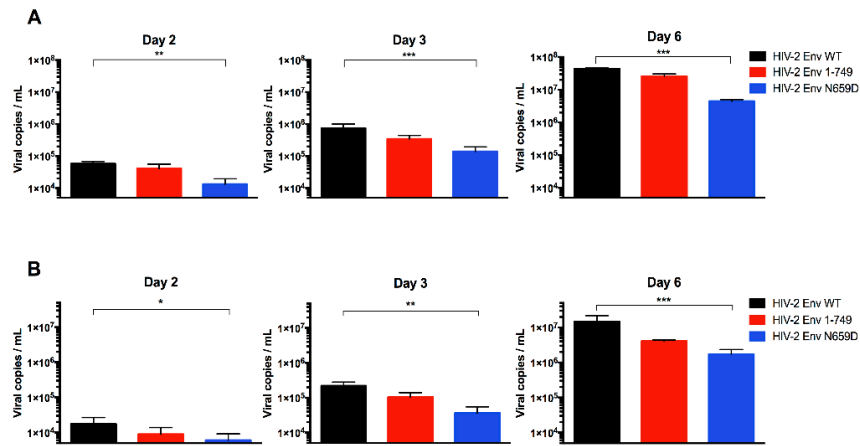


Figure 3. Absolute quantification of the HIV-2 Env wild-type (WT), Env 1-749 and Env N659D viral release capacity. (A) Quantification of the number of viral particles released from infected H9 cells in the supernatant at two, three and six days post-infection; (B) Quantification of the number of viral particles released from infected Jurkat cells in the supernatant at two, three and six days post-infection. Graphs present the number of viral copies in a logarithmic scale. Error bars indicate mean $\pm$ SD for $n = 6$ independent experiments. Statistical tests give statistical significance for HIV-2 Env WT and HIV-2 Env N659D with a $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

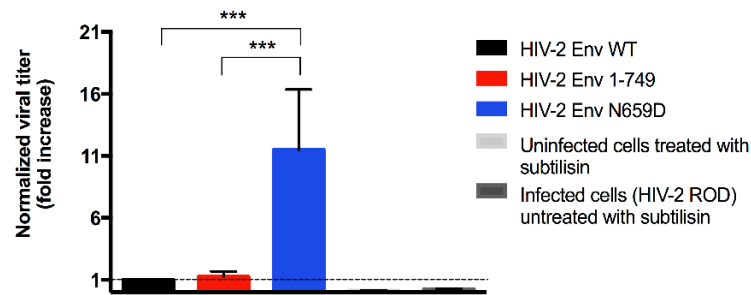


Figure 4. At three days post-infection, infected H9 cells were conserved and then treated with the bacterial protease subtilisin that released the tethered virions. Graph shows relative quantification of viral particles tethered at the cell surface. Data for the HIV-2 Env WT has been normalized to 1 (dashed line in the graph). Data for the others viruses has been compared to this normalized value and has been expressed as folds increase. Error bars indicate mean $\pm$ SD for $n = 4$ independent experiments. Statistical tests (one-way ANOVA followed by Tukey's multiple comparisons test) give statistical significance with a $p < 0.001$ (***) both for Env WT and Env N659D viruses, and for Env 1-749 and Env N659D viruses. There is no statistical significance in titer between the Env WT and Env 1-749 viruses. Two negative controls were also tested: a medium from uninfected H9 cells treated with subtilisin, and a medium from infected H9 cells untreated with subtilisin.

3.2. Viral Release of the Env N659D Mutant Is Restored in BST-2-Depleted H9 Cells

In order to prove that the lack of viral release of the Env N659D mutant is linked to a deficit of BST-2 antagonism, we generated *bst-2* knockout in H9 cells using the CRISPR/Cas9 system. Pseudotyped viruses were used to transduce a sgRNA and the Cas9 endonuclease gene from *Streptococcus pyogenes* into H9 cells that naturally express BST-2. The single guide RNA is a synthetic RNA composed of a scaffold sequence mandatory for Cas9-binding and a 25 nucleotide sequence which targets the first exon of *bst-2* gene. Cas9 protein induced a double-strand break in the gene sequence and compelled the cells to introduce insertions or deletions (InDels). These InDels in the *bst-2* gene caused frameshift mutations within the open reading frame and ensured complete BST-2 knockout.

As expected, we found that particle release at two, three and six days post-infection of the Env N659D virus came back to a level comparable to the wild type (Figure 5A). These results confirmed

that the N659 residue is involved in the Env-mediated anti-tetherin function in the HIV-2 ROD virus. Since BST-2 was not expressed in those H9 clones, the viral release efficiency was increased regardless of the three viruses tested when compared to the viral release observed in H9 cells that express BST-2 (Figure 3A). It is also interesting to note that the Env 1–749 virus, with a truncated cytoplasmic tail spontaneously selected after several passages during replication in vitro in some cell types [49], appeared to have a significant replicative advantage when produced in BST-2-depleted H9 cells.

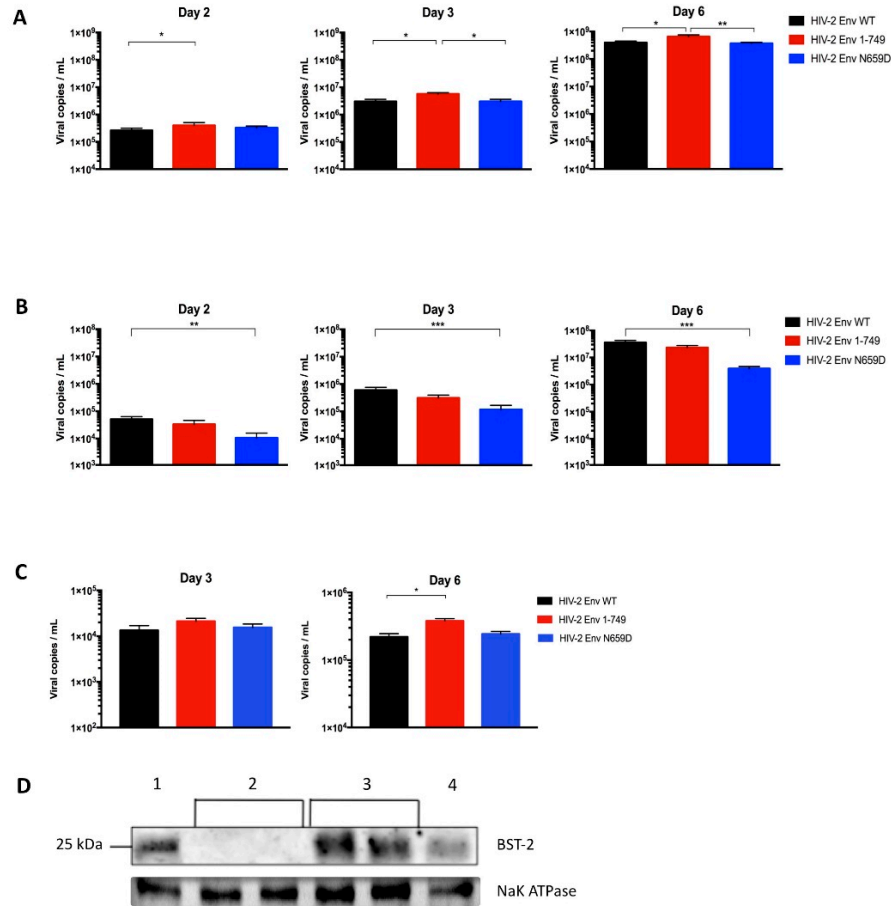


Figure 5. (A) Absolute quantification of the HIV-2 Env WT, Env 1–749 and Env N659D viral release capacity from BST-2-depleted H9 cells at two, three and six days post-infection; (B) Absolute quantification of viral particles released from H9 cells transduced with pseudotyped viruses that encoded Cas9 protein and a single guide RNA (sgRNA) sequence recognizing an intron sequence. The replicative capacity of the three viruses remained equivalent to the viral release assay using H9 cells that expressed BST-2 (Figure 3A); (C) Absolute quantification of viral release capacity from transfected HEK293T cells, which do not express BST-2, at three and six days post-transfection; (D) BST-2 expression in those cells was verified by immunoblotting proteins from H9 cells lysates (1), two different populations of BST-2-depleted H9 cells (2), two different populations of H9 cells transduced with pseudotyped viruses that encoded a sgRNA sequence recognizing an intron sequence (3) and Jurkat cells (4). Graphs present the number of viral copies in a logarithmic scale. Error bars indicate mean $\pm$ SD for $n = 4$ independent experiments (except for the panel C, $n = 3$). Statistical tests give statistical significance with a $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

As a control, we also tested our viruses in H9 cells transduced with pseudotyped viruses that encoded a sgRNA sequence recognizing the human cAMP and cAMP-inhibited cGMP 3',5'-cyclic phosphodiesterase 10A intron sequence. BST-2 remained constitutively expressed in those cells and the replicative capacity was comparable to the viral release assay using untransduced H9 cells (Figure 5B).

This assay represents a convincing control for ensuring that results obtained using BST-2-depleted cells were not dependent on or specific to the CRISPR/Cas9 knockout system. BST-2 expression in those cells was verified by immunoblotting proteins from H9 cells, from the different populations of BST-2-depleted H9 cells and from Jurkat cells lysates. A loading control was performed to conclude that BST-2 knockout was successful. Na⁺/K⁺-ATPase is also a membrane protein constitutively expressed in human cells (Figure 5D).

3.3. HIV-2 Env N659D Mutant Is Unable to Bind BST-2

We determined the binding capacity of this recombinant protein with BST-2 using a flow cytometry-based FRET assay. FRET is a robust and non-invasive approach to analyze protein interactions in living cells and is based on energy transfer between two compatibles fluorochromes. This transfer is efficient only if both fluorochromes are at a distance of less than 10 nm and the fluorescence emission intensity of the acceptor fluorochrome is enhanced [64].

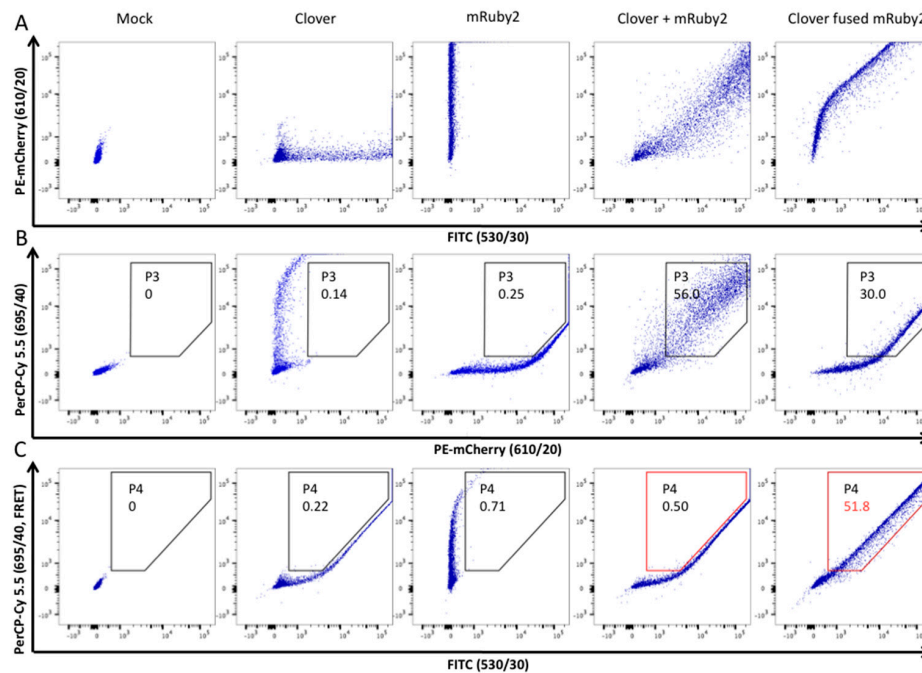


Figure 6. BD FACSaria™ III cell sorter configuration and experimental setup of Förster resonance energy transfer (FRET)-measurements. Firstly, gate P1 selecting living cells (according to forward and sideward scatter FSC-A/SSC-A) and gate P2 selecting cells in P1 (according to forward and sideward scatter FSC-W/SSC-W in order to exclude joined or grouped cells), have been applied (not shown in this figure). Secondly, HEK293T cells expressing Clover or mRuby2 individually, in combination or as a fusion protein, were analyzed with three different filters in order to construct analysis gates and to define the FRET-positive signal. Clover (green fluorescent protein variant) was excited with the laser 488 nm and the FITC filter was used to examine fluorescence emission. mRuby2 (red fluorescent protein variant) was excited with the laser 561 nm and the PE-mCherry filter was used (panel A). Importantly, mRuby2 and Clover showed some emission in the FRET-channel (PerCP-Cy 5.5 filter). Thus, a gate (P3) was constructed to exclude cells that emitted a false-positive signal in the FRET-channel (panel B). As described in this image, cells co-transfected with Clover and mRuby2 exerted an aleatory FRET signal that should also be excluded. Therefore, an analysis gate (P4, in red) was applied to determine the FRET-positive cells when the FRET-adapted filters were selected (PerCP-Cy 5.5 and FITC filters, panel C). This P4 gate excluded cells that were co-transfected with Clover and mRuby2 and thus are FRET-negative (0.5% of P2), while including cells that showed a FRET-positive signal (Clover fused mRuby2; 51.8% of P2). This gating strategy allowed for assessment the enhanced emission of the acceptor fluorochrome and therefore demonstrated the energy transfer between the two fluorochromes; namely the protein interactions.

Initially, FACS was not set up to assess FRET signal. To establish this experiment, we configured a BD FACS Aria™ III cell sorter (BD Biosciences) as shown previously [65]. Two plasmids encoding each a FRET-adapted fluorochrome were used: pcDNA3-Clover and pcDNA3-mRuby2 encoding respectively Clover (a GFP variant) and mRuby2 (a red fluorescent protein (RFP) variant) [59]. As a positive control, we used a plasmid that expressed Clover fused to mRuby2 and an unmodified pcDNA3.1(+) as a negative control. Those four previous plasmids were transfected individually or in combination into HEK293T cells in order to select the cell sorter filters, exclude double false-positive and aleatory FRET signals, and finally to construct analysis gates to assess the specific FRET-measurements indicating relevant protein interactions (Figure 6).

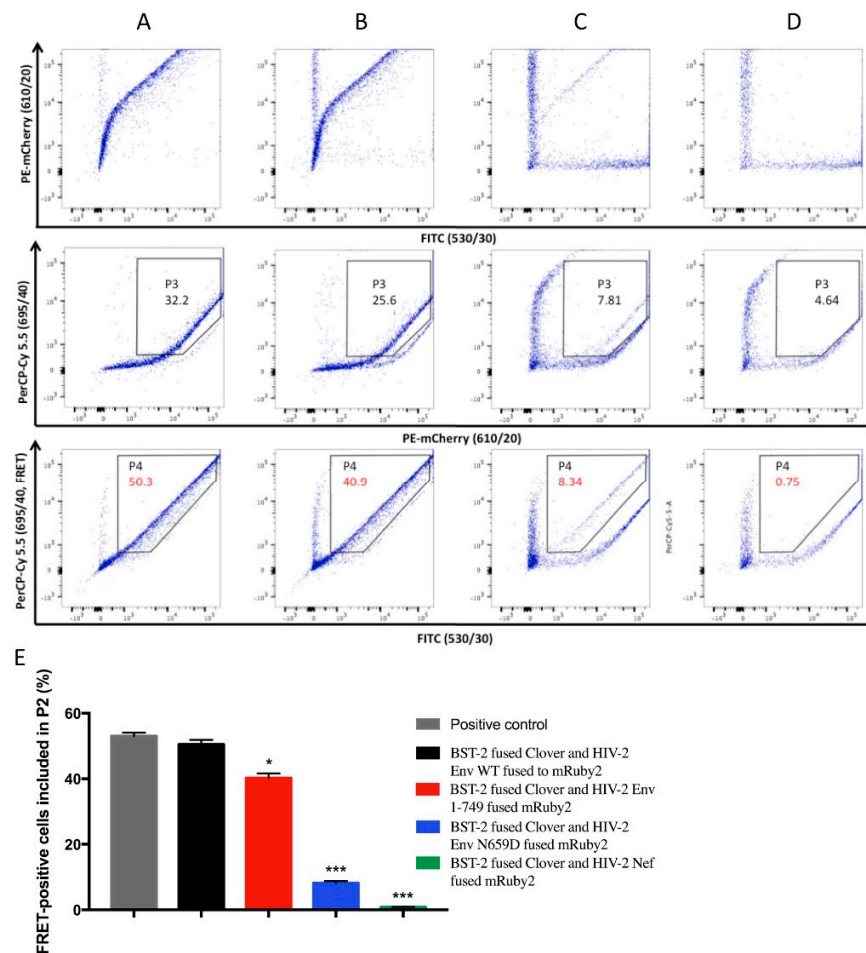


Figure 7. HEK293T cells co-expressing the fusion proteins in the following combinations were analyzed with the configured BD FACS Aria™ III cell sorter. (A) BST-2 fused Clover and HIV-2 Env WT fused to mRuby2; (B) BST-2 fused Clover and HIV-2 Env 1-749 fused mRuby2; (C) BST-2 fused Clover and HIV-2 Env N659D fused mRuby2 and (D) BST-2 fused Clover and HIV-2 Nef fused mRuby2. Numbers indicated in gate P4 give the percentage of FRET-positive cells included in P2 according to the previous fluorescence-activated cell sorting (FACS) configuration for the different co-expression in living cells (Figure 6); (E) Bar diagram summarizing the FRET-positive signals from four independent experiments ($n = 4$). Statistical tests give a significance with a $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

Once the cytometer was configured, we exploited this methodology in order to evaluate the capacity of the HIV-2 Env WT, Env 1-749 and Env N659D proteins to link with BST-2. We therefore constructed expression plasmids encoding the following fusion proteins: BST-2 fused to Clover on its N-terminal part and the three studied Env proteins each fused to mRuby2 on their C-terminal part. In this way, both FRET-adapted fluorochromes were localized in the cytoplasmic compartment of the

living cells and thus allowed potential energy transfer between the fluorochromes. In addition, we constructed an expression plasmid that encoded HIV-2 Nef protein, since this myristoylated HIV-2 protein does not antagonize or interact with BST-2 [45–47,55]. These plasmids were co-transfected in combinations into HEK293T cells and the FRET-measurements were performed after thirty-six hours (Figure 7).

Firstly, this assay confirmed an effective intermolecular link between HIV-2 Env and human BST-2 proteins. Compared to the FRET signal exhibited by the positive control used to configure the FACS (Clover fused mRuby2, Figure 6C, plot 5), the FRET signal provided by cells co-expressing BST-2 and HIV-2 Env fusion proteins was comparable (50.3% of cells included in P2, Figure 7A); Secondly, a full cytoplasmic tail was required for the Env-mediated anti-tetherin function, as the FRET signal emanating from cells co-expressing BST-2 and HIV-2 Env 1–749 revealed a loss of binding capacity compared to HIV-2 Env WT (40.9% of cells in P2, Figure 7B). Conversely, the FRET signal was considerably diminished from cells co-expressing BST-2 and HIV-2 Env N659D, therefore illustrating a consistent lack of binding capacity (8.34%, Figure 7C). Thirdly, the absence of FRET signal (0.75%, Figure 7D) when BST-2 was co-expressed with HIV-2 Nef protein confirmed that these two proteins did not interact, as previously described in several studies [45–47,55].

This co-transfection was a robust negative control to evaluate our flow cytometry-based FRET assay and therefore allowed us to precisely determine protein interactions in living cells.

4. Discussion

BST-2 is part of the interferon (IFN)-dependent antiviral response pathway and acts as a potent host restriction factor [19–21,25]. Embedded into the cell membrane, mainly in cholesterol-rich domains where virions preferentially bud, this small protein blocks the viral release of enveloped viruses and therefore impairs viral spread [19,29,43]. Retroviruses evolved in turn several counteractions to evade this antiviral factor. Whereas HIV-1 relies on Vpu, HIV-2 uses its envelope glycoproteins to overcome BST-2 restriction and promote a downmodulation of the number of BST-2 molecules at the cell surface, facilitating the later viral budding from infected cells [44,48]. Env regions and residues involved in anti-tetherin activity are still less known and, to date, studies that aimed to define these regions have used particular Env proteins from the HIV-2 ROD10 or ROD14 molecular clones. These Env proteins displayed several specific mutations and both harbored a truncated cytoplasmic tail, which might alter structural conformation and functionalities of the envelope glycoprotein [50–52,54,66]. In contrast, replicating HIV-2 viruses from infected individuals encode a full cytoplasmic tail [49]. In this study, we pointed out a residue in the gp36 ectodomain essential for the antagonism of human BST-2.

We selected potential amino acids mutations by comparing the HIV-1 and HIV-2 gp41/36 sequences, since HIV-1 Env does not antagonize BST-2. Substitutions of HIV-2 Env residues with the corresponding HIV-1 amino acids allowed for preservation of functionalities and the tertiary structure of the envelope glycoprotein. We generated thirty-two Env mutant viruses and measured the impact of each mutation on their replicative capacity and ability to bud, allowing the identification of residues involved in the antagonism of BST-2. Our protocol was based on the quantification of HIV-2 viral particles released from human cell lines, which differs from a previous publication observing the stimulation of HIV-1 Gag-Pol virus-like particles (VLPs) release by HIV-2 Env variants [50]. Interestingly, we demonstrated that only a single substitution of the asparagine in position 659 (Env N659D) markedly impaired the viral release ability compared to the HIV-2 ROD wild type virus in two different T lymphocytes cell lines.

Subtilisin treatment revealed that the HIV-2 Env N659D virus was eleven-fold more tethered at the cell surface than HIV-2 Env WT or Env 1–749 viruses and thus presented an inability to be released from H9 cells. This experiment proved that the decrease of viral release was not due either to a lack of viral production or to a disruption of the Env addressing, as virions were actually localized at the cell surface. HIV-2 Env N659D virus is therefore unable to antagonize BST-2 and does not promote the

endocytosis and sequestration of BST-2. Consequently, BST-2 remains localized at the cell surface and retains a large amount of budding viruses.

As a deficiency of viral release is not necessarily assigned to a lack of BST-2 antagonism, and as entry defaults could potentially influence viral titers in multiple rounds of infections, we performed a *bst-2* knockout in H9 cells using the CRISPR/Cas9 system. This key experiment indicated that the viral replication and release of the Env N659D virus were restored to a comparable level as with the wild type virus. These results also proved that the infectivity of the mutant virus was maintained, excluding an influence of the Env mutation on entry steps. Therefore, that asparagine residue in the gp36 ectodomain of the HIV-2 ROD virus is implicated in the Env-mediated anti-tetherin activity.

The N659 residue in Env might lie in the putative membrane-proximal external region (MPER). In HIV-1, this 22 amino acid peptide is involved in the incorporation of Env proteins into the membrane, allows membrane fusion, promotes viral infectivity and finally the MPER region is also an immunogenic peptide [67–70]. The N659D substitution could potentially have an impact on the HIV-2 predicted MPER functions. But when the asparagine residue was replaced with the equivalent HIV-1 residue, the viral assay using BST-2-depleted cells indicated that this mutant virus conserves its viral infectivity since viral release ability remained the same as with the HIV-2 Env WT virus. Moreover, it has been reported that only tryptophan residues in the MPER region are essential for membrane fusion and thus for viral infectivity [71].

Even though the HIV-2 Env region interacting with human BST-2 lies in the ectodomain, the cytoplasmic tail is also required for a correct antagonism: in this study we demonstrated that HIV-2 Env 1–749 presenting a truncated cytoplasmic tail partially lost its ability to bind BST-2. Interestingly, we previously showed that this mutant virus was spontaneously selected after several passages when cultured in some cell types [49], despite the fact that such a modified CT was not observed in clinical samples, suggesting that this shorter form is more vulnerable in vivo. This mutant with a truncated CT showed here a non-significant decline of its ability to be released from infected cells (H9 and Jurkat) compared to HIV-2 Env WT virus, while it was not more tethered at the cell surface as revealed by the subtilisin treatment. Conversely, that mutant had a significant replicative advantage when the replication occurred in BST-2-depleted H9 cells, suggesting that the spontaneous selection of this large C-terminal deletion occurs in cell lines that do not express large amounts of BST-2, such as MT-2 or MT-4 cells. The cytoplasmic tail is probably necessary in cells that constitutively express large amounts of BST-2. The argument that a full cytoplasmic tail is required for an adequate anti-tetherin function is supported by a recent study reporting that a Nef-deleted SIVmac virus could revert to a functional BST-2 antagonist by introducing some compensatory changes in its Env cytoplasmic tail [55]. In accordance with these insights, we demonstrated with a FRET assay the influence of the CT in the binding ability to BST-2, as cells co-expressing Env 1–749 and BST-2 showed a partial loss of protein interaction compared to cells co-expressing BST-2 and WT proteins. Moreover, our sensitive FRET experiments also confirmed the absence of interaction between HIV-2 Nef and BST-2 proteins. Although the experiments presented here all implied membrane localization of the Env variant proteins, further investigations would be needed to clarify the Env trafficking and addressing inside the cell, especially for the Env 1–749 proteins.

In summary, an efficient Env-mediated antagonism of BST-2 is operated through a direct intermolecular link, and an asparagine residue at the position 659 together with a normal full cytoplasmic tail are mandatory.

5. Conclusions

Among a series of 32 Env mutants generated by site-directed mutagenesis, we demonstrated the importance of the asparagine residue at position 659 in the HIV-2 gp36 ectodomain for the anti-tetherin function. Viral release ability in cell lines expressing BST-2 in comparison to *bst-2* knockout clones, subtilisin treatment of infected cells as well as FRET assays all point out the importance of this residue for antagonizing BST-2.

We also demonstrated the involvement of the Env cytoplasmic tail in this antagonistic role as viruses harboring a truncated cytoplasmic tail lost partially their function. Further investigations would be helpful to assess the envelope glycoprotein topology and structural conformation of those mutants, as well as the relevance of the various BST-2 antagonism abilities of HIV-2 strains in disease progression and non-progressing phenotypes.

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Author Contributions: F.E.D., P.G., and J.R. conceived and designed the study. F.E.D. performed the experiments. F.D. analyzed the data and wrote the manuscript with the help of P.G. and J.R. C.L. contributed to design the flow cytometry-based FRET assay. All the authors read and approved the final manuscript.

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4.2 Second article: “Modulation of the NF- κ B signaling pathway by the HIV-2 envelope glycoprotein and its incomplete BST-2 antagonism”

The second article is related to the second objective of the present thesis (see chapter III, section 3.2). This study has been published in *Virology* in October 2017 (doi: <https://doi.org/10.1016/j.virol.2017.09.024>). In this article, we reveal that the HIV-2 Env is able to activate NF- κ B in HEK293T cells and that the antagonism of BST-2 by this viral protein does not result in a complete inhibition of the NF- κ B signaling pathway, in contrast to HIV-1 [570].

Note that the references cited in this paper are shown at the end of the article and are not linked to the numbers of references cited in the text of the present thesis.

Modulation of the NF- κ B signaling pathway by the HIV-2 envelope glycoprotein and its incomplete BST-2 antagonism

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Abstract

The HIVs have evolved by selecting means to hijack numerous host cellular factors. HIVs exploit the transcription factor NF- κ B to ensure efficient LTR-driven gene transcription. However, NF- κ B is primarily known to act as a key regulator of the proinflammatory and antiviral responses. Interestingly, retroviruses activate NF- κ B during early stages of infection to initiate proviral genome expression while suppressing it at later stages to restrain expression of antiviral genes. During HIV-1 infection, diverse viral proteins such as Env, Nef and Vpr have been proposed to activate NF- κ B activity, whereas Vpu has been shown to inhibit NF- κ B activation. It is still unclear how HIV-2 regulates NF- κ B signaling pathway during its replication cycle. Here we confirm that human BST-2 and HIV-1 Env proteins can trigger potent activation of NF- κ B. Importantly, we demonstrate for the first time that the HIV-2 Env induces NF- κ B activation in HEK293T cells. Furthermore, the anti-BST-2 activity of the HIV-2 Env is not sufficient to completely inhibit NF- κ B activity.

Abbreviations: HIV, human immunodeficiency virus; Env, envelope glycoprotein; BST-2, Bone Marrow Stromal Cell Antigen 2; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; I κ Bs, inhibitors of κ B proteins; IKKs, I κ B kinases.

Keywords: HIV, HIV-2, Env, envelope glycoprotein, NF- κ B, BST-2, Tetherin, Vpu, antagonism, restriction factor.

1. Introduction

The transcription factor NF- κ B plays pivotal roles in the innate immune and antiviral responses by inducing expression of proinflammatory cytokines, type I interferons (IFN- α and IFN- β), interferon-stimulated genes (ISGs) as well as expression of genes involved in cell growth and survival (Chan and Greene, 2012; Gosh and Hayden, 2012; Sen and Baltimore, 1986). In absence of stimuli, NF- κ B is maintained in an inactive form in the cell cytoplasm by the inhibitors of κ B (I κ Bs). A variety of stimuli promote degradation of I κ B proteins : viral antigens, cytokines and specific cell surface receptors such as interleukin 1 receptor, IFN receptors, T cell TCR-CD3 complex, Toll-like receptors (TLRs) or tumor necrosis factor (TNF) receptors (Chan and Greene, 2012; Pfeffer, 2011; Vallabhapurapu and Karin, 2009). Furthermore, human BST-2/Tetherin acts as an innate sensor of viral assembly and budding. A dityrosine motif (Y₆X Y₈) in the BST-2 cytoplasmic tail recruits Syk kinase to prime the NF- κ B signaling pathway (Arias and Evans, 2014; Cocka and Bates, 2012; Galao et al., 2012; Galao et al., 2014; Tokarev et al., 2013). The induction of the NF- κ B signaling pathway requires activation of the IKK complex (IKK α , IKK β and NEMO subunits). This complex phosphorylates I κ B regulatory domains, thus allowing its ubiquitination and degradation.

Consequently, NF- κ B is translocated into the cell nucleus and binds to specific DNA sequences located in the promoters of NF- κ B-dependent target genes (Chan and Greene, 2012; Heusinger and Kirchhoff, 2017; Kanarek and Ben-Neriah, 2012; Pfeffer, 2011; Vallabhapurapu and Karin, 2009). Interestingly, most of the HIV long terminal repeats (LTRs) include κ B binding sequences, typically one in HIV-2 and two or three in HIV-1 (Bachu et al., 2012; Chen-Park et al., 2002; Hiebenthal-Millow et al., 2004; Jeeninga et al., 2000; Stroud et al., 2009). Although NF- κ B is a key regulator of the host immune and antiviral responses, it is manipulated by retroviruses for promoting effective transcription of viral genes (Chan and Greene, 2012; Hiscott et al., 2001). However, even though NF- κ B activation is beneficial for initial viral replication, this leads to rapid antiviral immune responses against the retroviruses. HIVs overcome this challenge by activating NF- κ B at early stages of the viral cycle while inhibiting this transcription factor at later stages, restraining therefore expression of various antiviral genes (Heusinger and Kirchhoff, 2017; Hiscott et al., 2001).

While many studies attempted to identify the proteins used by HIV to fine-tune NF- κ B activity throughout the replication cycle, it is still unclear and controversial if the HIV-1 envelope glycoprotein (Postler and Desrosiers, 2012), the HIV-1 and HIV-2 Nef proteins (Mangino et al., 2011; Sauter et al., 2015) or the Vpr proteins (Liang et al., 2015; Liu et al., 2013; Roux et al., 2000) activate the NF- κ B signaling pathway. At later stages of infection, it is well defined that the HIV-1 Vpu protein - which is not encoded by HIV-2 - inhibits the NF- κ B activity by three means : internalization and degradation of BST-2/Tetherin (Galao et al., 2012; Tokarev et al., 2013), impeding the I κ B degradation through sequestration of β -TrCP proteins (Akari et al., 2001; Bour et al., 2001) and blocking the NF- κ B nuclear translocation (Sauter et al., 2015).

To date, no study investigated neither the effects of the HIV-2 Env protein in the NF- κ B activation, nor the impacts of the Env-mediated anti-BST-2 antagonism in the modulation of the NF- κ B signaling pathway. In the present study, we tested the potential impacts of HIV-2 Env in the NF- κ B activity.

2. Results

2.1. The HIV-2 envelope glycoprotein induces NF- κ B activation

Since it has been described that the cytoplasmic domain of the HIV-1 glycoprotein gp41 enhances NF- κ B activation (Postler and Desrosiers, 2012), we first sought to determine whether the HIV-2 envelope glycoprotein was capable of inducing the NF- κ B pathway. To test this hypothesis, we generated a derivative of HEK293T cells stably expressing firefly luciferase gene under the control of an NF- κ B-dependent promoter (HEK293T-NF- κ B cells). Transfections of the transduced cells with plasmids encoding viral Env or Nef proteins of either HIV-1 or HIV-2, followed by a luciferase reporter assay allowed to assess the effect of these proteins on NF- κ B activity. We observed that HIV-1 Env activated NF- κ B ~12- to 20-fold compared to the negative control (Fig. 1A). These results confirmed that the HIV-1 Env protein stimulates NF- κ B activity in HEK293T cells, in agreement with published data (Postler and Desrosiers, 2012). Interestingly, expression of HIV-2 Env protein potently enhanced NF- κ B activation with a ~17- to 28-fold increase compared to the negative control (Fig. 1A). Therefore, we showed for the first time that the HIV-2 envelope glycoprotein may activate the NF- κ B signaling pathway in HEK293T cells.

A recent study conducted by Sauter et al. assigned the NF- κ B activation ability to the viral accessory Nef protein in both HIV-1 and HIV-2 (Sauter et al., 2015). We partly reproduced these experiments

and we tested the potential activation of NF- κ B pathway by the Nef proteins. Transfections with plasmids encoding HIV-1 or HIV-2 Nef both showed a weak and non-significant induction of NF- κ B activity, as previously reported (Fig. 1A) (Heusinger and Kirchhoff, 2017; Sauter et al., 2015).

As previously mentioned, human BST-2 can activate the NF- κ B pathway, triggering thereby proinflammatory responses. This restriction factor acts as an innate immune sensor of the assembly and budding of mammalian enveloped virions (Cocka and Bates, 2012; Galao et al., 2012; Galao et al., 2014; Tokarev et al., 2009; Tokarev et al., 2013). In our experiments, overexpression of BST-2 indicated, as expected, that this protein induced the NF- κ B signaling pathway (Fig. 1A).

To validate the specificity of the NF- κ B pathway activation by BST-2 and HIV-2 Env proteins, we used a dominant negative form of IKK β . Dominant negative IKK β (DN-IKK β) is unable to phosphorylate the I κ B protein, therefore preventing its proteasomal degradation and nuclear translocation of NF- κ B subunits. Thus, NF- κ B is consistently maintained in its inactive form in the cytoplasmic compartment (Cocka and Bates, 2012). Firstly, co-transfection of expression vectors encoding BST-2 and DN-IKK β revealed an extensive and significant inhibition of NF- κ B activity initially induced by BST-2. Secondly, co-transfection of plasmids encoding HIV-2 Env and DN-IKK β showed a similar inhibition (Fig. 1B). These results demonstrated that the HIV-2 Env-mediated NF- κ B activation observed was specific of the NF- κ B pathway and ruled out the potential involvement of another transcription factor.

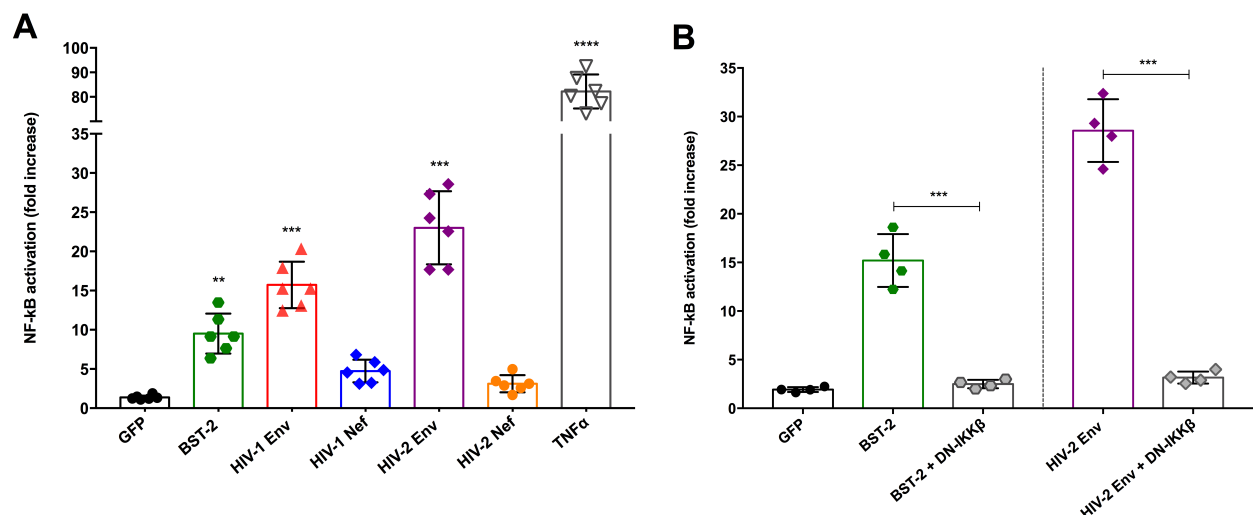


Fig. 1. Stimulation of NF- κ B activity by human BST-2 and lentiviral proteins. (A) Transduced HEK293T cells were transfected with expression vectors encoding GFP (as a negative control), human BST-2 or viral proteins of HIV-1 and HIV-2 (Env or Nef proteins). HEK293T cells were lysed and luciferase activities were determined 24 hours post transfection. As a positive control, TNF α was added into the cell culture medium (25 ng/ml) to induce a potent activation of NF- κ B pathway (Chan and Greene, 2012). (B) Specificity of the NF- κ B pathway activation by BST-2 and HIV-2 Env proteins. Transduced HEK293T cells were co-transfected with BST-2 or HIV-2 Env plasmids alongside with a dominant negative form of IKK β (DN-IKK β). NF- κ B activity is represented as fold increase, being the ratio between the luciferase activity (RLU) of the corresponding measure and that of transduced but non-transfected HEK293T cells. These results were derivated from six independent experiments (n = 6) in (A) and four independent experiments (n = 4) in (B). Data were analyzed by one-way ANOVA statistical test followed by a multiple comparison test which compares each mean with that of the negative control (GFP) in (A) or compares each mean with all other means in (B) (** p -value < 0.01 , *** < 0.001 and **** < 0.0001). Error bars indicate mean $\pm$ standard deviation (SD).

2.2. Differential modulation of the NF- κ B activation by the anti-BST-2 HIV-1 Vpu and HIV-2 Env

HIV-1 utilizes the accessory Vpu protein to overcome BST-2 host restriction factor by removing BST-2 from the cell surface and facilitates therefore the budding of nascent virions from infected cells

(Dube et al., 2010; Goffinet et al., 2009; Neil et al., 2008; Van Damme et al., 2008). Vpu binds BST-2 through its membrane and cytoplasmic domain, causing subsequent proteasomal degradation of this restriction factor (Douglas et al., 2009; Goffinet et al., 2009; Kueck and Neil, 2012; Vigan and Neil, 2010). As previously reported (Bour et al., 2001; Galao et al., 2012; Sauter et al., 2015; Tokarev et al., 2013), co-expression of HIV-1 Vpu and human BST-2 nearly abolished the NF- κ B activity (Fig. 2A and 2B).

However, the HIV-2 genome does not encode *vpu* gene and relies on the envelope glycoprotein to antagonize BST-2 (Hauser et al., 2010; Le Tortorec and Neil, 2009; Le Tortorec et al., 2011). As shown in the previous experiment, HIV-2 Env and BST-2 both activated the NF- κ B signaling pathway when expressed separately (Fig. 1A). Thus, we sought to determine whether co-expression of both proteins might modulate the NF- κ B signaling. Interestingly, co-expression of HIV-2 Env and BST-2, at 24 and 48 hours post transfection, resulted in an incomplete inhibition of the NF- κ B activation compared to the potent Vpu-mediated inhibition (Fig. 2A and 2B).

Furthermore, we showed in a previous study that N659D substitution in the extracellular domain of the HIV-2 Env protein, as well as a truncated cytoplasmic tail, impaired the anti-BST-2 ability (Dufrasne et al., 2016). Here we observed that the HIV-2 Env N659D activated NF- κ B in a similar way to HIV-2 Env wild-type when expressed alone. Nevertheless, in the presence of BST-2, the resulting NF- κ B activation was not reduced significantly (Fig. 2A and 2B).

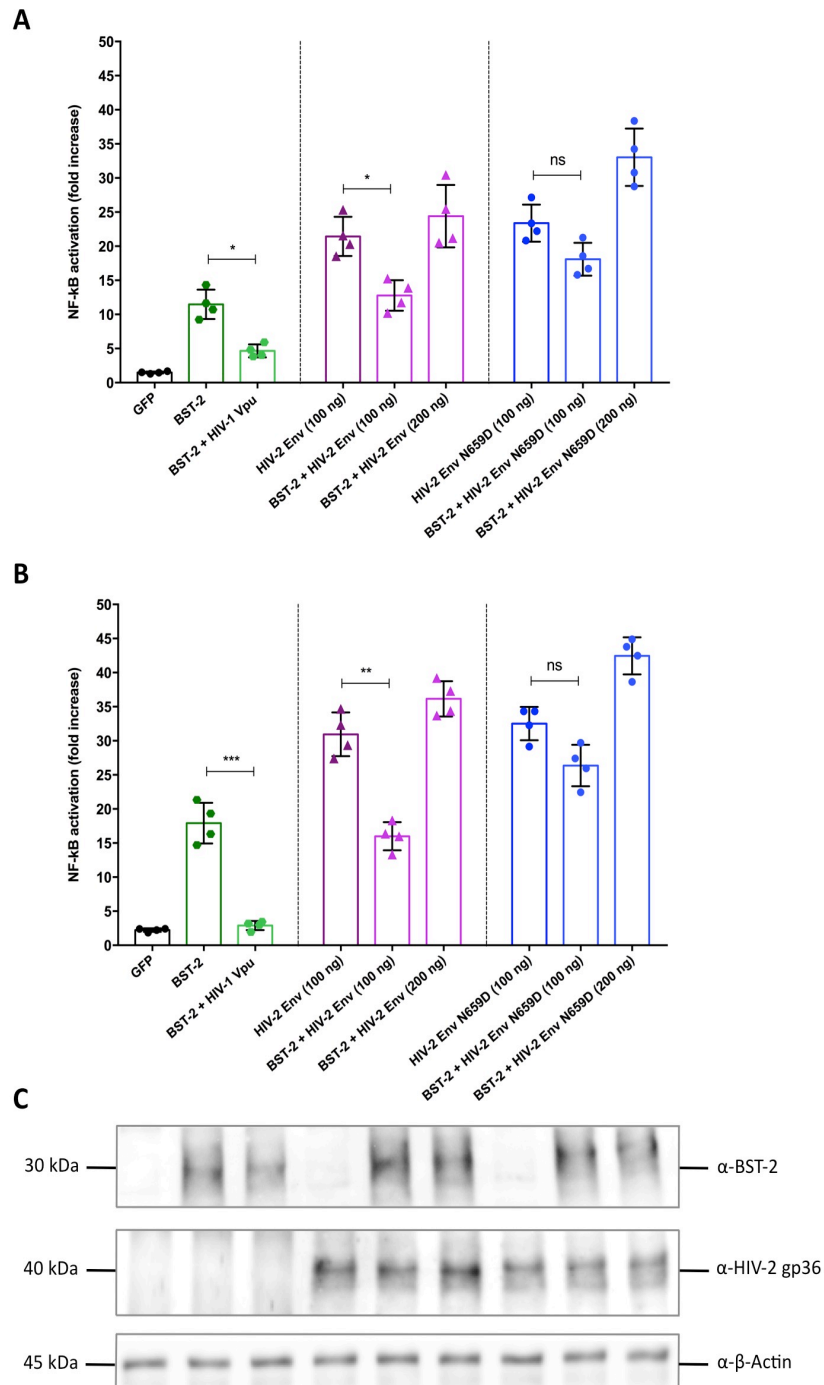


Fig. 2. Modulation of the NF- κ B activity by the anti-BST-2 HIV-1 Vpu and HIV-2 Env.

Transduced HEK293T cells were transfected or co-transfected with expression vectors encoding GFP, BST-2, viral proteins of HIV-1 (Vpu) or HIV-2 (Env or Env N659D) as indicated. Co-transfections were carried out by varying Env and Env N659D plasmid amounts (100 ng or 200 ng) while maintaining constant the BST-2 plasmid amounts (70 ng). Transfected HEK293T cells were lysed and luciferase activities were determined at

(A) 24h and (B) 48h post transfection. Results were derived from four independent experiments ($n = 4$) and were analyzed by one-way ANOVA followed by a multiple comparison test (* p -value < 0.05 , ** < 0.01 , *** < 0.001 , ns = non-significant). Error bars indicate mean $\pm$ SD. (C) Expression levels of BST-2 and HIV-2 Env proteins were evaluated by Western blots. The order of the samples is the same as that of the co-transfections in (B). Expression levels of HIV-2 Env and Env N659D proteins were equivalent and HIV-2 Env proteins did not degrade BST-2, in contrast to HIV-1 Vpu proteins.

2.3. Activation of the NF- κ B signaling pathway by BST-2 upon HIV-2 assembly

Human BST-2 can act as a viral sensor of HIV-1 assembly and release by inducing a potent activation of NF- κ B (Galao et al., 2012; Tokarev et al., 2013). Therefore, we next sought to determine whether assembly of HIV-2 virions stimulates BST-2 to activate NF- κ B, and whether the HIV-2 Env-mediated anti-BST-2 antagonism modulates this additional activation of NF- κ B. We generated a HEK293T cell population that encoded the NF- κ B binding sequence while constitutively expressing BST-2 (called HEK293T-NF- κ B-BST-2 cells) to prevent the NF- κ B activation due to transient expression of BST-2 (Galao et al., 2012; Galao et al., 2014). We used these cells to perform transfections of HIV-2 wild type, HIV-2 Env N659D or HIV-2 Δenv proviral clones and we compared levels of NF- κ B activation obtained from these cells with those from the HEK293T-NF- κ B cells that did not express BST-2. First, the NF- κ B activation induced by the expression of HIV-2 ΔEnv virions was significantly reduced compared to expression of HIV-2 wild type or HIV-2 Env N659D in the HEK293T-NF- κ B cells (Fig. 3A). To evaluate the activation of NF- κ B resulting only from the viral sensing by BST-2, we also transfected HEK293T-NF- κ B-BST-2 cells with the HIV-2 Δenv proviral clone. As shown in the figure 3A, levels of NF- κ B activation by BST-2 were slightly but significantly increased upon sensing of the

assembly of HIV-2 Δ Env virions. Secondly, the transfection of HEK293T-NF- κ B-BST-2 cells with the HIV-2 Env N659D proviral clone, unable to antagonize BST-2, induced important NF- κ B activity, probably due to BST-2 viral sensing and additional NF- κ B stimulation by HIV-2 Env. In addition, transfections of these cells with the HIV-2 wild type proviral clone demonstrated a significant but incomplete inhibition of the NF- κ B activity when compared to the results of HIV-2 Env N659D transfections.

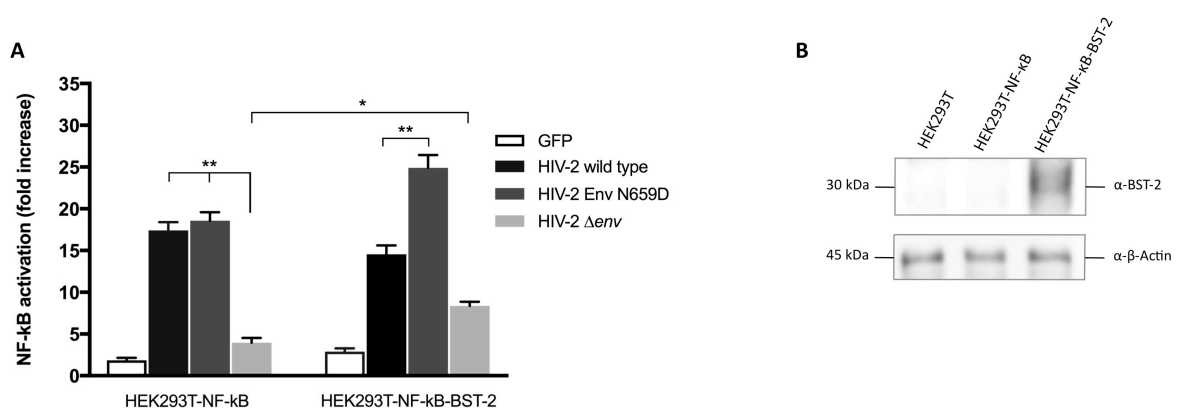


Fig.3. NF- κ B activation by BST-2 upon HIV-2 assembly. (A) HEK293T-NF- κ B-BST-2 cells were generated by stable transfection and used to perform transfections of HIV-2 wild type, HIV-2 Env N659D or HIV-2 Δ env proviral clones (300 ng). Thirty hours post transfection, luciferase assays were performed as previously described. Results were derived from three independent experiments ($n = 3$) and were analyzed by one-way ANOVA followed by a multiple comparison test (* p -value < 0.05 , ** < 0.01). Error bars indicate mean $\pm$ SD. (B) Expression of BST-2 was verified in the HEK293T-NF- κ B-BST-2 cells by Western blots.

3. Discussion

HIV-2-infected individuals develop immunodeficiency with a substantial delay and a majority do not progress to the AIDS disease state compared to HIV-1-infected persons. These are called long-term

non progressors (de Silva et al., 2008; Rowland-Jones and Whittle, 2007; Thiébaud et al., 2011). HIV-2-infected patients present a lower viral load as well as a slower decline in CD4⁺ T cell counts observed during asymptomatic infection. The contributing factors of these differences are attributed to the immune responsiveness of HIV-1- and HIV-2-infected persons, which can explain the differences in pathogenesis between the two types of infection. It has been proposed that better immune control and lower replication capacity of HIV-2 could account for these discrepancies (Damond et al., 2002; de Silva et al., 2013a; de Silva et al., 2013b; Leligidowicz et al., 2010; Leligidowicz et al., 2007; Makvandi-Nejad and Rowland-Jones, 2015; Michel et al., 2000; Nyamweya et al., 2013; Ozkaya Sahin et al., 2013; Rowland-Jones and Whittle, 2007).

The host cellular factor NF- κ B is a key factor in the immune antiviral responses but can also be manipulated by primate lentiviruses to promote proviral genome transcription. In this context, study of the interplay between HIV-2 and NF- κ B could provide new insights to better define, at least partly, molecular mechanisms that underpin the particular HIV-2 pathogenesis. In this study, we demonstrated that the HIV-2 Env was capable of inducing NF- κ B activation, similarly to the HIV-1 Env protein. Secondly, we confirmed that human BST-2 also primed stimulation of NF- κ B and that co-expression with HIV-1 Vpu nearly abolished this activation, essentially through the potent Vpu-mediated anti-BST-2 activity (Cocka and Bates, 2012; Galao et al., 2012; Tokarev et al., 2013) and stabilization of I κ B proteins (Akari et al., 2001; Bour et al., 2001; Sauter et al., 2015). Importantly, HIV-2 does not encode the *vpu* gene and relies on its envelope glycoprotein to overcome the BST-2 host restriction factor but contrary to HIV-1 Vpu protein, this does not lead to BST-2 degradation (Hauser et al., 2010; Le Tortorec and Neil, 2009; Le Tortorec et al., 2011; Neil, 2013). Thus, we tested the effectiveness of HIV-2 Env anti-BST-2 activity to inhibit the activation of NF- κ B. In presence of BST-2, HIV-2 Env

protein failed to thoroughly inhibit NF- κ B activation, contrary to the multifunctional HIV-1 Vpu protein.

In this study, we only used HEK293T cells. Reproduction of our experiments using CD4⁺ T cells single-round infections could strengthen these conclusions.

Overall, our results suggest that the HIV-2 Env-mediated BST-2 antagonism was not sufficient to completely inhibit NF- κ B activity, in contrast to HIV-1 Vpu antagonism. In our experimental design, although the cells triggered a strong NF- κ B activation in response to the HIV-2 Env, this late protein failed to completely restrict antiviral gene expression, as revealed by the experiments of NF- κ B activation upon HIV-2 assembly. Furthermore, our results would also suggest that HIV-2 does not express a potent inhibitor of the NF- κ B activity during its replication cycle, in contrast to HIV-1. Moreover, it has not yet been demonstrated whether the HIV-2 Env protein was capable of sequestering β -TrCP proteins, or whether this protein prevented the nuclear translocation of NF- κ B. This study highlights the importance of an efficient anti-BST-2 activity in the regulation of NF- κ B throughout the viral cycle and could provide new insights in the understanding of the enhanced immune antiviral responses in HIV-2 disease as compared to HIV-1.

4. Materials and Methods

4.1. Cell lines and viral transduction

HEK293T cells were obtained from the ATCC and maintained in Dulbecco's modified Eagles's medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA). This medium was supplemented

with 10% fetal bovine serum (FBS, HyClone™ FetalClone™ from GE Healthcare Life Sciences, Fairfield, CT, USA) and 1 % gentamicin (Thermo Fisher Scientific).

To establish HEK293T reporter cells stably expressing luciferase gene under the control of NF- κ B binding sequence (HEK293T-NF- κ B), we generated VSV-pseudotyped viruses upon transfection of three lentivectors into 3.5×10^5 HEK293T cells : the pHAGE NF κ B-TA-LUC-UBC-GFP-W plasmid (750 ng) (Wilson et al., 2013), the packaging psPAX2 plasmid (400 ng) and the pMD2.G plasmid encoding vesicular stomatitis virus envelope protein (200 ng) (all obtained from Addgene ; #49343, #12260 and #12259 respectively). Forty-eight hours post transfection, supernatant was collected and precleared by centrifugation 10 min at 800 x g. Cell-free supernatant containing viral particles was centrifuged at 32000 x g at 4°C for 2h. Pseudotyped viral particles were then used to infect and transduce HEK293T cells. The pHAGE NF κ B-TA-LUC-UBC-GFP-W plasmid encodes four copies of the NF- κ B consensus sequence. NF- κ B activation and binding trigger promoter activity and drive expression of the firefly luciferase reporter gene. The green fluorescent protein (GFP) is constitutively expressed by the ubiquitin C (UBC) promoter, enabling sorting of transduced cells by flow using a BD FACSAria™ III Cell Sorter (BD Biosciences, San Jose, CA, USA) (Wilson et al., 2013).

To obtain a cell line encoding NF- κ B binding sequence while constitutively expressing BST-2 (HEK293T-NF- κ B-BST-2), HEK293T-NF- κ B cells were stably transfected with pcDNA-BST-2 and pSF-MinCMV-Puro (OG579) Minimal CMV Puromycin vector (Oxford Genetics, Oxford, UK), using a ratio 10:1. Forty-eight hours post transfection, cells were incubated in culture medium supplemented with puromycin (1 μ g/ml) to select, after several passages, resistant cells that stably expressed human BST-2 protein.

All cell lines were tested for mycoplasma contamination using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

4.2. Plasmids and transfections

HIV-1 *nef* and *env* coding sequences were obtained by subcloning PCR technique from the HIV-1 NL4-3 molecular clone (pNL4-3 plasmid obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH) (Adachi et al., 1986). Amplified fragments were purified and inserted into the mammalian expression vector pcDNA3.1(+) (Invitrogen™ ; Thermo Fisher Scientific) with the *HindIII*-*ApaI* restriction sites for *nef* and with the *EcoRI*-*ApaI* for *env* sequence. The codon-optimized *vpu* sequence cloned into pcDNA3.1(-) expression plasmid was also obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (pcDNA-Vphu) (Nguyen et al., 2004).

HIV-2 *nef* and *env* sequences were amplified using the proviral expression clone pKP59 HIV-2 ROD (molecular clone obtained from Dr. Isabel Barahona, Instituto Superior de Ciências da Saúde-Sul, Portugal) (Ribeiro et al., 2005). This infectious clone encodes the whole genome of HIV-2 ROD reference strain (GenBank: M15390.1) and an Env protein harboring a complete cytoplasmic tail (158 amino acids) (Dufrasne et al., 2016). DNA fragments were inserted into the pcDNA3.1(+) expression vector.

The HIV-2 Δenv proviral clone was generated using a site-directed mutagenesis reaction from the pKP59 HIV-2 ROD clone (QuickChange II Site-Directed Mutagenesis kit from Agilent, Santa Clara, CA, USA).

The human *bst-2* sequence was amplified from cDNA obtained by reverse transcription of H9 cell mRNAs using the Transcriptor First Strand cDNA Synthesis Kit from Roche (Mannheim, Germany). Amplicons were also inserted in the pcDNA3.1(+) plasmid.

All plasmid constructs were verified by nucleotide sequencing using an ABI 3500 genetic analyser (Applied Biosystems™, Thermo Fisher Scientific).

All HEK293T cell lines were transfected using TurboFect Transfection Reagents (Thermo Fisher Scientific). For luciferase reporter gene assays, 2×10^5 cells were harvested and seeded in a 24-well plate twenty-four hours before transfection and transfected with 100 ng pcDNA-Env, 150 ng pcDNA-Nef, 150 ng pcDNA-Vphu (HIV-1), or 100 ng pcDNA-Env, 150 ng pcDNA-Nef (HIV-2), or 70 ng pcDNA-BST-2. Luciferase activities were measured 24h after transfections (and 48h for co-transfections), as indicated in figure legend.

The dominant negative IKK β (K44M) plasmid was obtained from Addgene (plasmid #11104). This plasmid expresses an inactive kinase of I κ B (Mercurio et al., 1997).

4.3. Reporter gene assay

Transduced HEK293T cells were used to assess the NF- κ B activation by expression of different HIV-1, HIV-2 or human proteins. Twenty-four hours (or 48h) post transfection, cells were washed once with DPBS (Gibco™; Thermo Fisher Scientific) and lysed with 1x passive lysis buffer (Promega, Madison, WI, USA) for 15 min. Cell lysates were centrifuged 5 min at 15000 x g and 20 μ l of cell-free lysates were added to 100 μ l of Luciferase Assay Reagent II (LAR II) containing luciferin. Bioluminescent signals were measured with a GloMax™ 20/20 luminometer (Promega).

4.4. Western blots and antibodies

Expression levels of HIV-2 Env and BST-2 proteins were determined from cell lysates used to perform the luciferase assays. Samples were added to 2x Laemmli sample buffer, boiled for 5 minutes, separated by electrophoresis on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 for 1 hour, and probed overnight at 4°C with a rabbit monoclonal anti-BST-2 antibody (Abcam, cat #ab134061, dilution 1:5000) or a mouse monoclonal anti-HIV-2 gp36 antibody (OriGene Europe-Acris antibodies, cat #BM3335, dilution 1:1000). Endogenous β -Actin was detected with the rabbit monoclonal anti- β -Actin antibody (Cell Signaling, cat #4970, dilution 1:1000). Membranes were rinsed three times for 5 min in TBS 0.1% Tween-20 and the blots were probed with either HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Cell Signaling, cat #7074 and #7076, respectively) at a dilution of 1:2000 for 1 hour at room temperature. Membranes were then rinsed three times and treated with the Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences), and blots were analysed with the ChemiDoc™ XRS+ System (Bio-Rad).

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4.3 Article in preparation: “HIV-1 and HIV-2 differentially regulate NF- κ B activation and inhibition through the BST-2 antagonism”

This article in preparation provides the last results obtained during the past months. This article brings new data concerning the NF- κ B activation by HIV-2 in CD4+ T cells and is the continuation of the second article. The present article has not been submitted and could be still modified.

Note that the references cited in this paper are shown at the end of the article and are not linked to the numbers of references cited in the text of the present thesis.

HIV-1 and HIV-2 differentially regulate NF- κ B activation and inhibition through the BST-2 antagonism

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Keywords: HIV-1, HIV-2, NF- κ B, BST-2, Tetherin, restriction factor, immune response, disease progression

Abstract

HIV-2 is the second etiological agent of AIDS and is considered as a naturally attenuated form of human retroviral infection. HIV-2-infected individuals display a slow-progressing disease, lower viral loads and a stronger immunological control by the host as compared with HIV-1. The main hypothesis for the difference in disease phenotype between HIV-1 and HIV-2 implies a more efficient T cell-mediated immunity in the immune control of HIV-2 infection. Here, we investigate the effects of the HIV-2 envelope glycoprotein (Env) in the modulation of the NF- κ B signaling pathway in single-round infection of CD4⁺ T cells. We first report an essential role of the Env cytoplasmic tail (CT) in this signaling pathway. Interestingly, HIV-2 Env CT activates NF- κ B in a TRAF6-dependent but TAK1-independent manner. We reveal that HIV-2 Env strongly activates NF- κ B and that HIV-2 is unable to inhibit it in the late stages of infection, in striking contrast to

HIV-1. Indeed, HIV-1 Vpu acts as a potent inhibitor of NF- κ B by mainly antagonizing the restriction factor BST-2. In contrast, HIV-2 appears to be devoid of inhibitor of the NF- κ B activity, although the Env's anti-tetherin function can slightly restrict NF- κ B activation. We observe that this inefficiency of HIV-2 in inhibiting NF- κ B promotes the expression of numerous host genes implicated in the antiviral immune response, as *il-6*, *il-21*, *ifn- β* and *bcl-2* genes. Therefore, HIV-1 and HIV-2 differentially modulate the NF- κ B-mediated innate immune responses. These new insights highlight the mechanisms determining, at least partly, the distinct immune control and disease outcome of HIV-1 and -2 infections.

Introduction

Tetherin (BST-2 or CD317) is an interferon (IFN)-induced restriction factor that inhibits the release of diverse mammalian enveloped viruses by mediating their physical retention at the surface of infected cells [1-3]. Tetherin poses a potent barrier to successful cross-species transmission of primate lentiviruses, such as simian immunodeficiency viruses (SIVs), to humans [4]. It has been reported that an efficient viral countermeasure to BST-2 restriction was a prerequisite for the emergence of the human immunodeficiency virus type 1 (HIV-1) group M, which is responsible for the global HIV/AIDS pandemic [5]. Although SIV from chimpanzee (SIVcpz), which is the immediate precursor of HIV-1, and almost all SIVs use Nef to target tetherin of their natural hosts [6-8], HIV-1 employs its accessory protein Vpu to antagonize human BST-2 [2, 4, 9, 10]. This shift of Nef to Vpu occurred mainly because the human BST-2 presents a deletion of five amino acids (G/DIWKK) in the cytoplasmic domain that disrupts the susceptibility of BST-2 to Nef [4, 11-13].

Vpu promotes surface removal, sequestration and subsequent lysosomal degradation of BST-2 via a β -TrCP-dependent mechanism, thereby reducing cell surface and total steady-state BST-2 levels and improving the budding of the virions at the site of viral release [14-18]. In contrast to HIV-1, HIV-2 derived from the sooty mangabey SIV (SIVsmm) that does not encode a *vpu* gene. Interestingly, HIV-2 relies on the envelope (Env) glycoproteins to antagonize BST-2, similarly to several other viruses such as Ebola virus (EBOV), Herpes simplex viruses 1 and 2 (HSV-1/2) or Japanese encephalitis virus (JEV) [12, 19-21]. BST-2 and HIV-2 Env interact through their ectodomain and the sequestration of the complex requires an endocytosis motif (GYxx Φ) in the cytoplasmic domain of HIV-2 Env [19]. In addition to the aforementioned motif, several

amino acids in the ectodomain of HIV-2 Env, such as T568, A598 and N659 residues, are involved in the ability of HIV-2 Env to counteract human BST-2 [22, 23]. Interestingly, a recent study reported that these residues were highly conserved in both HIV-2 and SIVsmm strains, and that the Env proteins of some SIVsmm strains were capable of antagonizing human BST-2 [24]. These results explain how the SIVsmm has been transmitted to humans on at least eight independent cross-species transmissions and reveal a preadaptation of the SIVsmm for the Env-mediated antagonism of human BST-2 by HIV-2 [24].

Remarkably, the HIV-2 Env-mediated antagonism removes BST-2 from the cell surface but, contrary to HIV-1 Vpu, does not promote BST-2 degradation [15, 19]. Thus, HIV-2 Env may be considered as a poor BST-2 antagonist, likewise the Vpu proteins of HIV-1 group O or P that demonstrate a weak activity against human BST-2 [25-27].

Besides the virus retention activity of BST-2, this host restriction factor can promote innate immune response by triggering the NF- κ B signaling pathway upon sensing of the virus assembly and budding at the cell surface [28, 29]. A dityrosine motif (Y_xY_s) in the cytoplasmic domain of BST-2 is involved in this NF- κ B activation mostly via the recruitment of a Syk kinase and activation of TRAF6 [30]. NF- κ B is a key regulator of the innate and adaptive immune response by inducing the expression of proinflammatory cytokines, type I IFNs, interferon-stimulated genes (ISGs), anti-apoptotic proteins, several potent restriction factors (including BST-2) and thus induces an antiviral state in infected and surrounding cells, which impedes viral replication and spread [31-35]. Intriguingly, HIVs have selected NF- κ B binding sequences in their long terminal repeat (LTR) region and thus can use this transcription factor to promote the LTR-driven viral gene transcription [35-38]. Nevertheless, the NF- κ B activation primes the expression of several hundred genes implicated in the antiviral response, which are harmful for the viral replication and spread [39, 40]. HIV-1 copes with this challenge by fine-tuning the NF- κ B activation during the viral cycle [28, 41]. Previous studies reported that HIV-1 Nef [40, 42], Vpr [43-45] and Env [46] may activate NF- κ B in early stages of infection, but the results remain often speculative or contradictory (reviewed in [40]). In contrast, it is well-established that HIV-1 Vpu strongly inhibits NF- κ B activity in the late stages of infection by antagonizing BST-2, by stabilizing the I κ Bs and preventing the translocation of NF- κ B in the cell nucleus [41, 47]. This inhibition of NF- κ B activity mediated by HIV-1 Vpu is dominant and allows immune evasion of the retrovirus and thereby promotes the viral replication [48]. Recently, we reported that HIV-2 Env can also activate NF- κ B in HEK293T cells on the

one hand, and we also revealed that, on the other hand, the HIV-2 Env anti-tetherin activity was not sufficient to inhibit NF- κ B during the HIV-2 viral cycle [49].

To better describe the molecular mechanisms of NF- κ B modulation by HIV-2, we first investigate in this study the potential involvement of another accessory protein in the regulation of NF- κ B activity by co-expressing HIV-2 Env with Vif, Vpr, Vpx or Nef. We observe that none of the accessory protein takes part in the NF- κ B inhibition, excepted HIV-2 Vpr expression leading to a slight decrease of NF- κ B activation when co-expressed with HIV-2 Env. We also reveal, using siRNA-mediated downregulation experiments, that the HIV-2 Env activates NF- κ B in a TRAF6-dependent but TAK1-independent manner. Furthermore, we describe the region in the envelope cytoplasmic tail involved in this activation. To further define the regulation of NF- κ B by HIV-2 during its viral cycle and compare this regulatory ability to that of HIV-1, we monitor the NF- κ B activity in virally infected CD4⁺ T cells with HIV-1 or HIV-2 during the first 48 hours of infection. Using wild-type HIV-1 or *vpu*-deleted HIV-1 virus, we confirm that HIV-1 Vpu is a potent inhibitor of the NF- κ B signaling pathway in the late stages of infection. Vpu exerts this role partly through its anti-tetherin activity. In contrast, HIV-2 Env is a potent activator of NF- κ B in infected CD4⁺ T cells and, at the late stages, HIV-2 fails to completely downregulate the NF- κ B activity through the BST-2 antagonism or by any other mechanism in our experimental design. As a consequence, the NF- κ B activity remains important at the end of the HIV-2 viral cycle and primes the expression of several NF- κ B targeted genes involved in the antiviral response or cell survival, as we reveal by measuring *il-6*, *il-21*, *ifn- β* and *bcl-2* mRNA expression levels. This study highlights the innate immune role of NF- κ B in the viral replication and the differential immune evasion capacity of the HIV-1 and HIV-2 viruses and may partly explain the contrasting pathogenesis and host immune control of these two retroviruses.

Results

In a recently published paper, we have showed that HIV-2 envelope glycoprotein activates the NF- κ B pathway in HEK293T cells stably expressing firefly luciferase under the control of an NF- κ B-dependent promoter. Here, we reproduced similar experiments but this time using HEK293T stably expressing a short-lived version of the firefly luciferase gene (*Luc2P*) under the control of an NF- κ B promoter. The

corresponding luciferase protein encodes a PEST sequence that decreases its stability. This short-lived luciferase protein is therefore a suitable model to accurately monitor the activation and inhibition of NF- κ B in the cells. Transduced HEK293T cells were transfected with plasmids encoding viral Env or Nef proteins of either HIV-1 or HIV-2. Luciferase activity was used to assess the effect of these proteins on NF- κ B activity. Results show that HIV-1 Env activates NF- κ B ~ 15- to 19-fold compared to the negative control (Figure 1A). In agreement with published data, we found that the HIV-1 Env protein stimulates NF- κ B activity in HEK293T cells [46]. As shown in our previous paper [49], expression of HIV-2 Env protein potently enhances NF- κ B activation with a ~ 20- to 25-fold increase compared to the negative control (Figure 1A). These findings confirm that HIV-2 Env is a potent activator of the NF- κ B pathway in HEK293T cells.

We also wanted to reassess the ability of viral accessory HIV-1 and -2 Nef protein to activate the NF- κ B pathway and found that NF- κ B activity is not significantly induced by HIV-1 or -2 Nef (Figure 1A). Human BST-2 is a restriction factor that can activate the NF- κ B pathway, triggering thereby proinflammatory responses [28]. Here we confirm that overexpression of BST-2 induces the NF- κ B signaling pathway (Figure 1A and B).

Several studies described that HIV-1 Vpu is able to hamper BST-2 restriction by removing it from the cell surface and by enabling budding of nascent virions from infected cells. Vpu binds BST-2 through its membrane and cytoplasmic domain, causing subsequent proteasomal degradation of this restriction factor [14, 50, 51]. In correlation to these findings, we observed that NF- κ B activity was markedly abolished when co-expressing HIV-1 Vpu and BST-2 (Figure 1B). According to our previous data, we could confirm that co-expression of HIV-2 Env and BST-2 resulted in an incomplete inhibition of the NF- κ B activation compared to the potent Vpu-mediated inhibition (Figure 1B).

Moreover, we showed in a previous study that the N659D substitution in the extracellular domain of the HIV-2 Env protein, as well as a truncated cytoplasmic tail, impaired the anti-BST-2 ability [22]. Here we observed that the HIV-2 Env N659D activates NF- κ B in a similar way to HIV-2 Env wild-type when expressed alone. Nevertheless, in the presence of BST-2, the resulting NF- κ B activation was not reduced significantly (Figure 1B). Given that the induction of the NF- κ B pathway by other HIV-2 proteins (Vpx, Nef, Vif and Vpr) has still not been fully elucidated, we wanted to clarify if these viral proteins have the ability to induce NF- κ B signaling. Intriguingly, we observed that none of these HIV-2 accessory proteins could significantly modulate

the NF- κ B pathway (Figure 1C). Nevertheless, results show a modest but not significant NF- κ B activation by HIV-2 Vpr. Then, we wanted to investigate if these HIV-2 proteins could modify the induction of NF- κ B by HIV-2 Env. Our data shows that the potent activation of NF- κ B by HIV-2 Env is not significantly up- or downregulated when HIV-2 Vpx, Nef, Vif or Vpr are co-expressed with HIV-2 Env (Figure 1C). Nevertheless, our results indicate a weak but not significant reduction of NF- κ B activity when HIV-2 Env is co-expressed with HIV-2 Vpr.

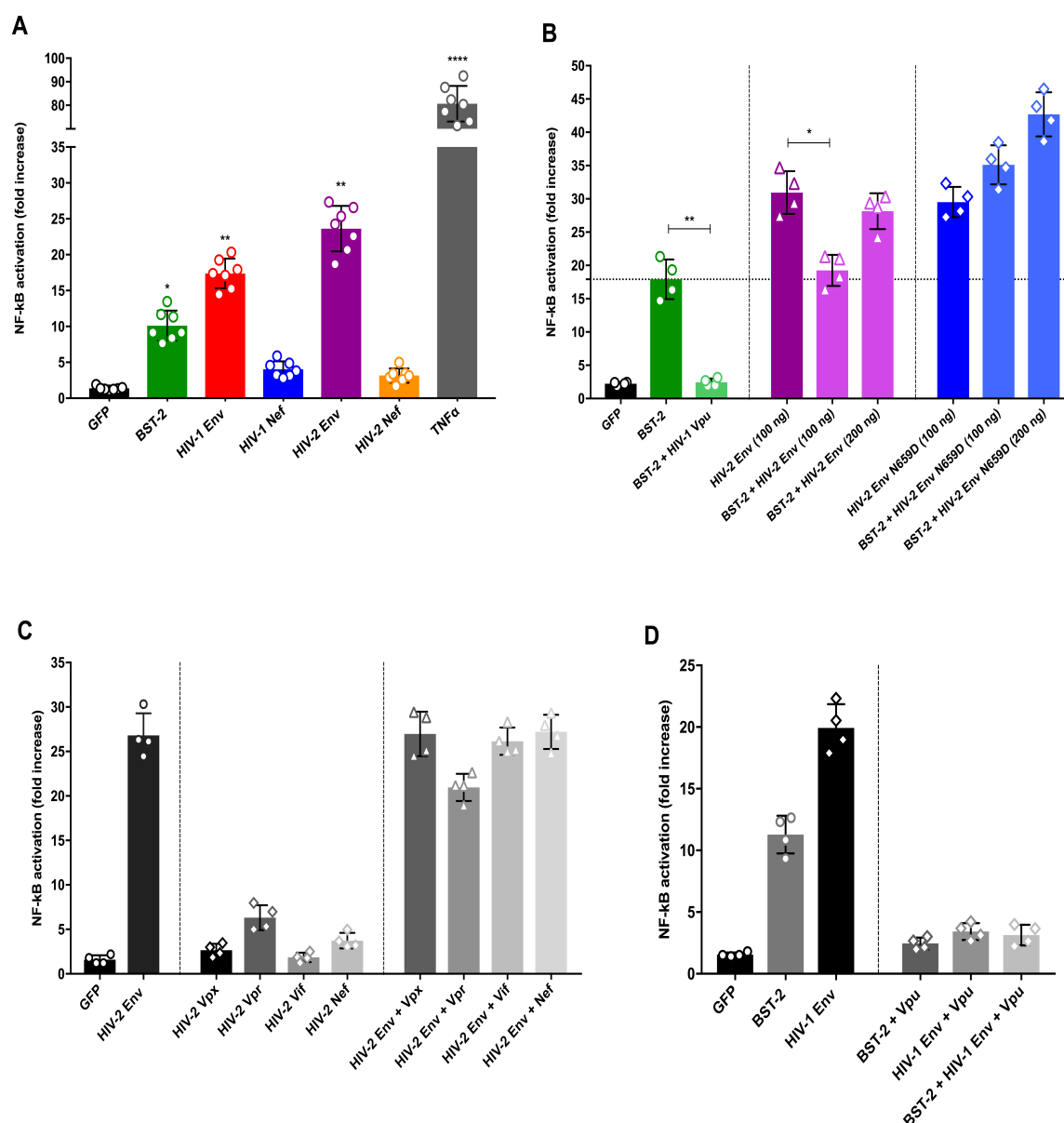


Figure 1: Modulation of the NF- κ B activity by BST-2, HIV-1 or HIV-2 viral proteins. (A) HEK293T stably expressing a short-lived version of the firefly luciferase under the control of an NF- κ B promoter were transfected with expression vectors encoding GFP (as a negative control), human BST-2 or viral proteins of HIV-1 and HIV-2 (Env or Nef proteins).

HEK293T cells were lysed and luciferase activities were determined 24 hours post-transfection. As a positive control, TNF α was added into the cell culture medium (20 ng/ml) to induce a potent activation of NF- κ B pathway. (B) Transduced HEK293T cells were co-transfected with expression vectors encoding GFP, BST-2, viral proteins of HIV-1 (Vpu) or HIV-2 (Env or Env N659D). Co-transfections were carried out by varying Env and Env N659D plasmid amounts (100 ng or 200 ng) while maintaining constant BST-2 plasmid amounts (70 ng). HEK293T cells were lysed and luciferase activities were determined 36 hours post-transfection. (C) Transduced HEK293T cells were either transfected with expression vectors encoding GFP, HIV-2 viral proteins (Vpx, Vpr, Vif or Nef) or co-transfected with HIV-2 Env expression vector and an HIV-2 viral protein expression vector as indicated. (D) Transduced HEK293T cells were transfected with expression vectors encoding GFP, BST-2 or HIV-1 viral proteins (Env or Vpu). All panels show NF- κ B activity represented as fold increase, being the ratio between the luciferase activity (RLU) of the corresponding measure and that of transduced but non-transfected HEK293T cells. These results derive from seven independent experiments (n = 7) in (A) and four independent experiments (n = 4) in (B), (C) and (D). Data were analyzed by one-way ANOVA statistical test followed by a multiple comparison test that compares each mean. (* p-value < 0.05, ** p-value < 0.01 and **** < 0.0001). Error bars indicate mean $\pm$ standard deviation (SD).

Sauter *et al.* reported that Vpu suppresses NF- κ B activation in HIV-1 infected cells in a dominant manner [41]. As expected, our results show that NF- κ B is not induced when HIV-1 Env and Vpu are co-expressed (Figure 1D). Interestingly, we could not observe a NF- κ B activation when BST-2 and HIV-1 Env were co-expressed with Vpu. These findings confirm that inhibition of NF- κ B activity by Vpu is dominant even in presence of BST-2 or/and HIV-1 Env.

Then we aimed to investigate the signaling cascade employed by BST-2, and HIV-1 and -2 Env to activate NF- κ B. For this purpose, we generated several knockdowns of proteins implicated in NF- κ B signaling in order to inhibit their expression. It has been reported that BST-2 could induce NF- κ B activity through activation of TRAF6 and subsequently TAK1 [28-30]. As expected, after knocking down TAK1 or TRAF6, NF- κ B activity decreases significantly compared to the activation induced by BST-2 alone (Figure 2A and B). According to the study conducted by Postler and Desrosiers, the cytoplasmic tail of HIV-1 Env stimulates NF- κ B activity through the recruitment of TAK1 [46]. In correlation with their results, we observe that inhibiting expression of TAK1 markedly reduces NF- κ B induction by HIV-1 Env (Figure 2A). Furthermore, we sought to identify the proteins utilized by HIV-2 Env for potent NF- κ B activation. According to our results, NF- κ B activation by HIV-2 Env was markedly reduced after inhibition of TRAF6 or MEKK3 expression (Figure 2B and C). This implies that HIV-2 Env activates NF- κ B through TRAF6 and MEKK3. The specificity and efficiency of the knockdown of these genes was verified by Western blot using the same whole cell lysates that those used for the luciferase assay.

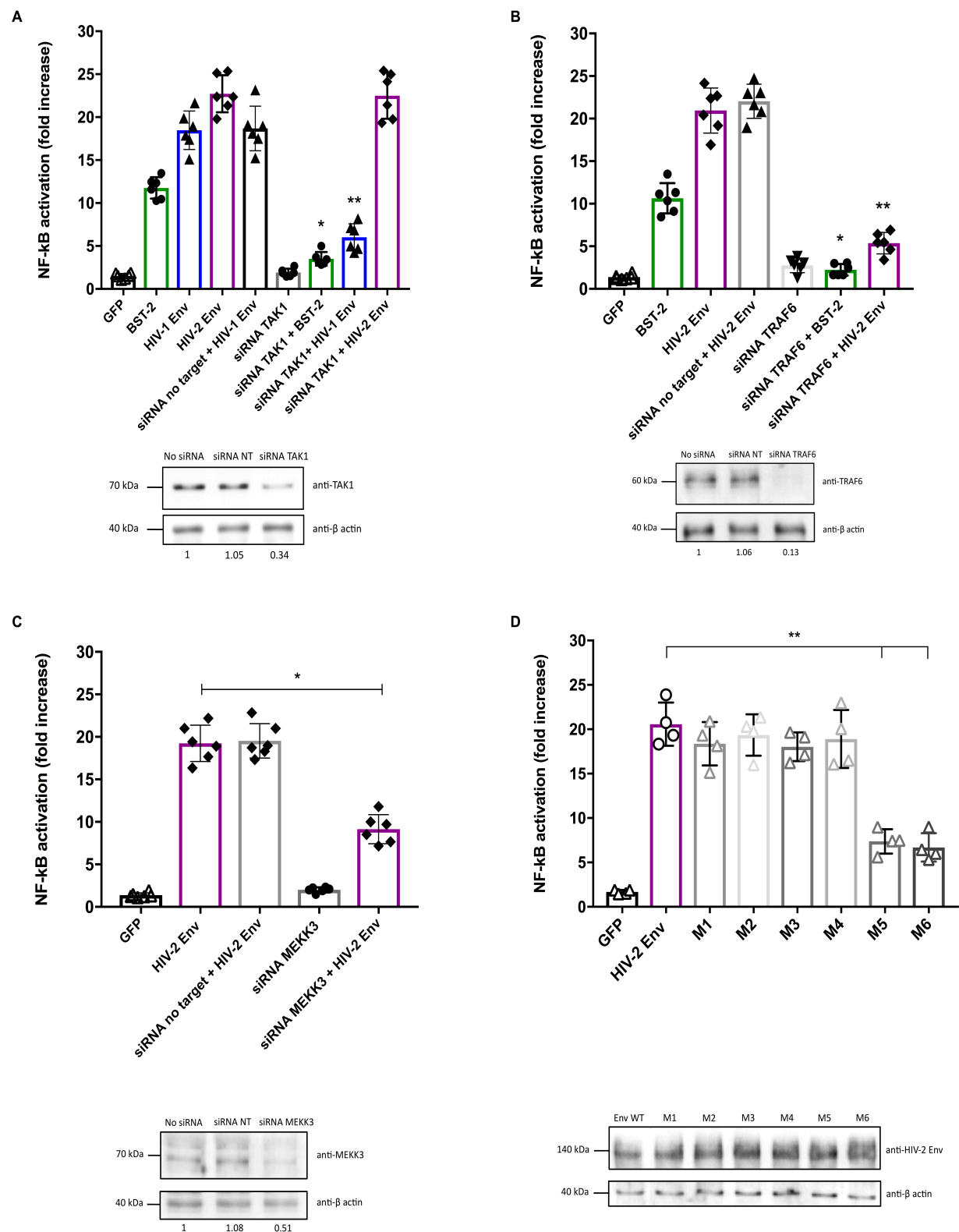


Figure 2: Implication of different proteins in the NF- κ B signaling pathway: HIV-2 Env activates NF- κ B through TRAF6 and MEKK3. Transduced HEK293T were either transfected with expression vectors encoding GFP (as a negative control), human BST-2 or viral Env protein of HIV-1 and HIV-2 or co-transfected with an siRNA targeting TAK1 (A), TRAF6 (B) or MEKK3 (C) mRNAs. A control siRNA (siRNA no target) not targeting any specific mRNA

was used as control. To check the specificity of the siRNA-based knockdown, one representative Western blot is depicted below each graph. (D) Plasmids (100 ng) encoding wild-type Env or one of the six different Env CT mutants were transfected into HEK293T cells and luciferase assays were performed 24 hours later. These results derive from six independent experiments ($n = 6$) in (A), (B) and (C), and four independent experiments in (D). Data were analyzed by one-way ANOVA statistical test followed by a multiple comparison (* p -value < 0.05 and ** p -value < 0.01). Error bars indicate mean $\pm$ standard deviation (SD).

Postler and Desrosiers also reported that the YHRL motif in position 766 within the HIV-1 Env cytoplasmic tail (CT) is largely involved in the NF- κ B activation [46]. Using site-directed mutagenesis experiments, we therefore built several plasmids encoding HIV-2 Env truncated in the CT domain. We created six different mutants (M1 to M6) of HIV-2 Env CT (see materials and methods section) and we tested their ability to activate NF- κ B in HEK293T cells (Figure 2D). Interestingly, while the mutant M4 was still able to activate NF- κ B, the mutant M5 has lost this ability, suggesting that the region comprises between the residue 759 and the residue 777 in the Env CT is essential in the NF- κ B activation.

CD4⁺ lymphocytes are the main targets of HIVs. In order to strengthen our previous results, we studied the modulation of NF- κ B activity in a CD4⁺ Jurkat cell line stably expressing the short-lived version of the firefly luciferase under the control of an NF- κ B promoter. First, we assessed if our model could reflect an activation and inhibition of NF- κ B activity over the time. Actually, treatment with TNF α induces NF- κ B activity and this activation was abolished when adding Parthenolide (PTL), a potent inhibitor of NF- κ B signaling that directly binds to and inhibits IKK β [52] (Figure 3A). We then aimed to evaluate the NF- κ B activation by HIV-1 or *vpu*-defective HIV-1 virions. As expected, we found that wild-type HIV-1 completely abolished NF- κ B activity after 36 hours post-infection, mainly through BST-2 antagonism and stabilization of I κ Bs [41]. In addition, HIV-1 lacking Vpu has lost the ability to inhibit NF- κ B activity compared to wild-type HIV-1. This confirms that Vpu is a potent inhibitor of the NF- κ B signaling pathway [28, 29, 41] (Figure 3B).

Interestingly, wild-type HIV-2 also induces NF- κ B activity after 24 hours post-infection. This activation is followed by a significant but not complete inhibition after 36 hours post-infection, in contrast to HIV-1 (compare Figure 3B and C). By using HIV-2 pseudotyped viruses (VSV-G or HIV-2 Env), we observed that Env trimers expressed at the surface of virions were not involved in the NF- κ B activation in the early stages of infection (Figure 3C). Nonetheless, by comparing wild-type and pseudotyped viruses we may confirm that the Env proteins are clearly involved in the NF- κ B activation at 24 hours post-infection (Figure 3C). Furthermore,

these results confirm that HIV-2 is unable to completely inhibit the NF- κ B signaling pathway during its replication cycle, in contrast to HIV-1.

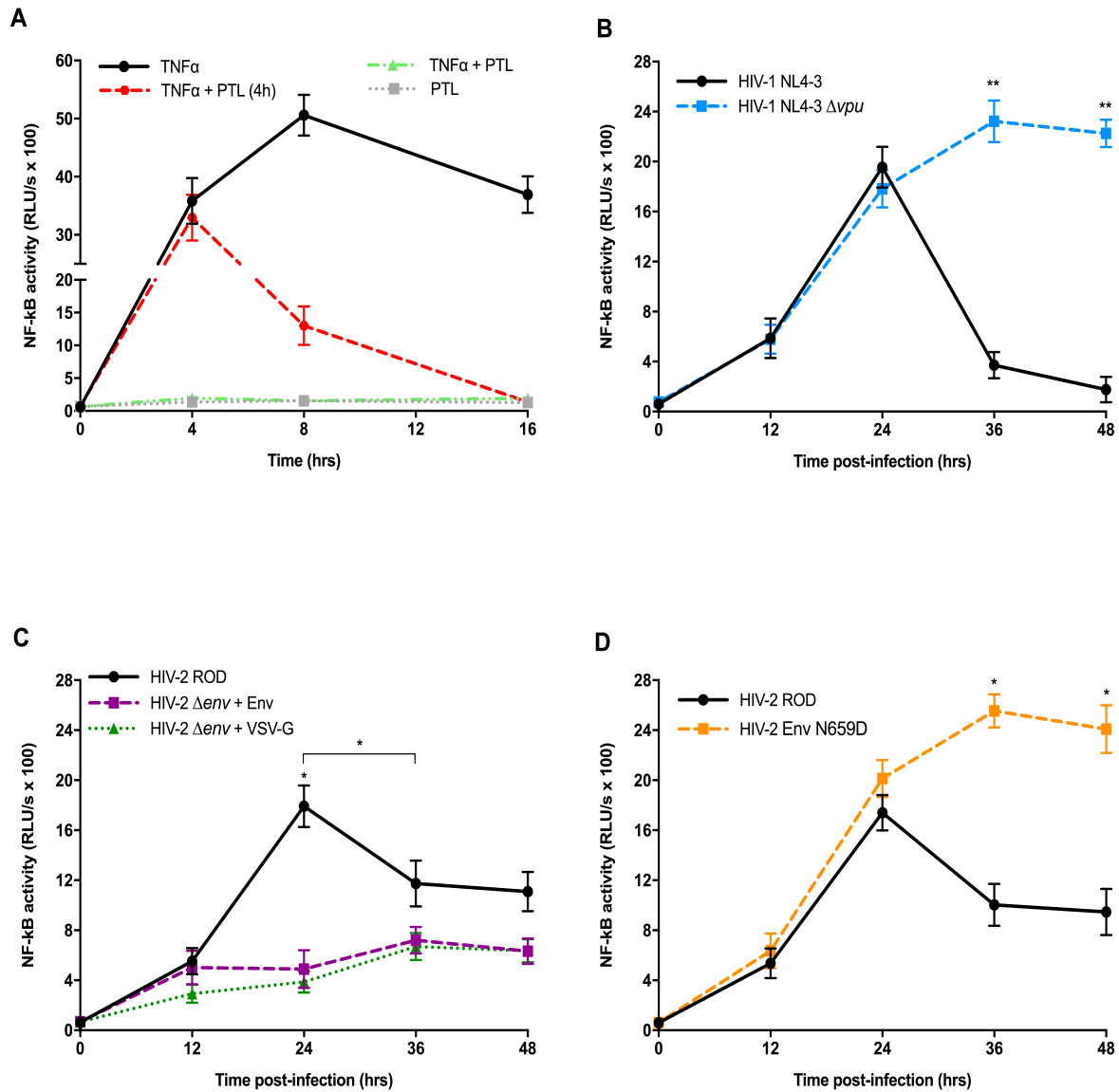


Figure 3: Modulation of the NF- κ B signaling pathway by HIVs in Jurkat cells (A) Jurkat-NF- κ B cells stably expressing a short-lived version of the firefly luciferase under the control of an NF- κ B promoter were either treated with TNF α or an inhibitor of NF- κ B (Parthenolide; PTL) alone or treated with TNF α and PTL (simultaneously or followed by PTL addition 4h later). Luciferase activity was determined at different time points after treatment, as indicated. Jurkat cells were infected with wild-type or *vpu*-defective HIV-1 in (B), with *env*-defective HIV-2 expressing either Env or VSV-G in (C) or with mutant HIV-2 incapable of interacting with BST-2 (HIV-2 Env N659D) in (D). Jurkat cells were also infected with wild-type HIV-2 (HIV-2 ROD) in (C) and (D). In the three last panels, cells were lysed, and luciferase activity was determined at different time points post-infection, as indicated. After 12 hours of infection, CCR5 and CXCR4 antagonists (inhibitors of HIV entry) were added to avoid multiple rounds of infection. All panels show NF- κ B activity expressed as RLU/s x 100. These results derive from four independent experiments (n = 4). Data were analyzed by ANOVA test (* p-value < 0.05 and ** p-value < 0.01). Error bars indicate mean $\pm$ standard deviation (SD).

Next, we sought to determine whether the Env-mediated BST-2 antagonism is involved in the incomplete inhibition of NF- κ B activity during the late stages of HIV-2 infection. To this end, we used the HIV-2 Env N659D mutant unable to bind and antagonize BST-2, as we reported previously [22]. Noticeably, these mutant viruses induced an increased NF- κ B activation at 36 and 48 hours post-infection, in striking contrast to HIV-2 wild-type viruses, highlighting the importance of overcoming BST-2 in the regulation of NF- κ B signaling pathway during HIV-2 infection (Figure 3D). In order to confirm this latter issue, we created a cellular clone of Jurkat-NF- κ B stably expressing specific shRNA against BST-2. The efficiency of the BST-2 knockdown was verified by Western blot (Figure 4 E). We used this cellular clone to assess whether wild-type HIV-2 or mutants viruses regulate the NF- κ B signaling pathway in the absence of BST-2, and we also used a Jurkat clone transduced with a shRNA no target as a control. As shown in the Figure 4, absence of BST-2 results in the inability of HIV-2 Env to (incompletely) downregulate the NF- κ B activity. Indeed, it is conceivable that the HIV-2 Env proteins expressed at the plasma membrane remain free and available to activate NF- κ B in the absence of BST-2 (Figure 4C and D). In the case of HIV-1, the absence of BST-2 facilitates the Vpu-mediated inhibition of NF- κ B activity (Figure 4A and B). Overall, these findings strongly suggest that the HIV-2 Env-mediated BST-2 antagonism may compensate (or neutralize) the NF- κ B activator effect of Env, although it results in an incomplete inhibition of this signaling pathway.

Finally, we reasoned that the inability of HIV-2 to inhibit the NF- κ B signaling pathway in the late stages of infection may promote the expression of proinflammatory molecules involved in the antiviral response. To test this hypothesis, we measured the mRNA levels of several NF- κ B-targeted genes at 24 and 48 hours post-infection by qPCR. We chose to follow the expression of four genes (*il-6*, *il-21*, *bcl-2* and *ifn- β*) and we observed that the inability of HIV-2 to downregulate NF- κ B engenders an upregulation of these genes at 48 hours of infection, in marked contrast with HIV-1 (Figure 5 A, C, E and G). We confirmed that the mRNA expression of these genes is specific of the NF- κ B activation by comparing the mRNA levels from infected Jurkat cells to those of infected Jurkat cells treated with Parthenolide (Figure 5 B, D, F and H).

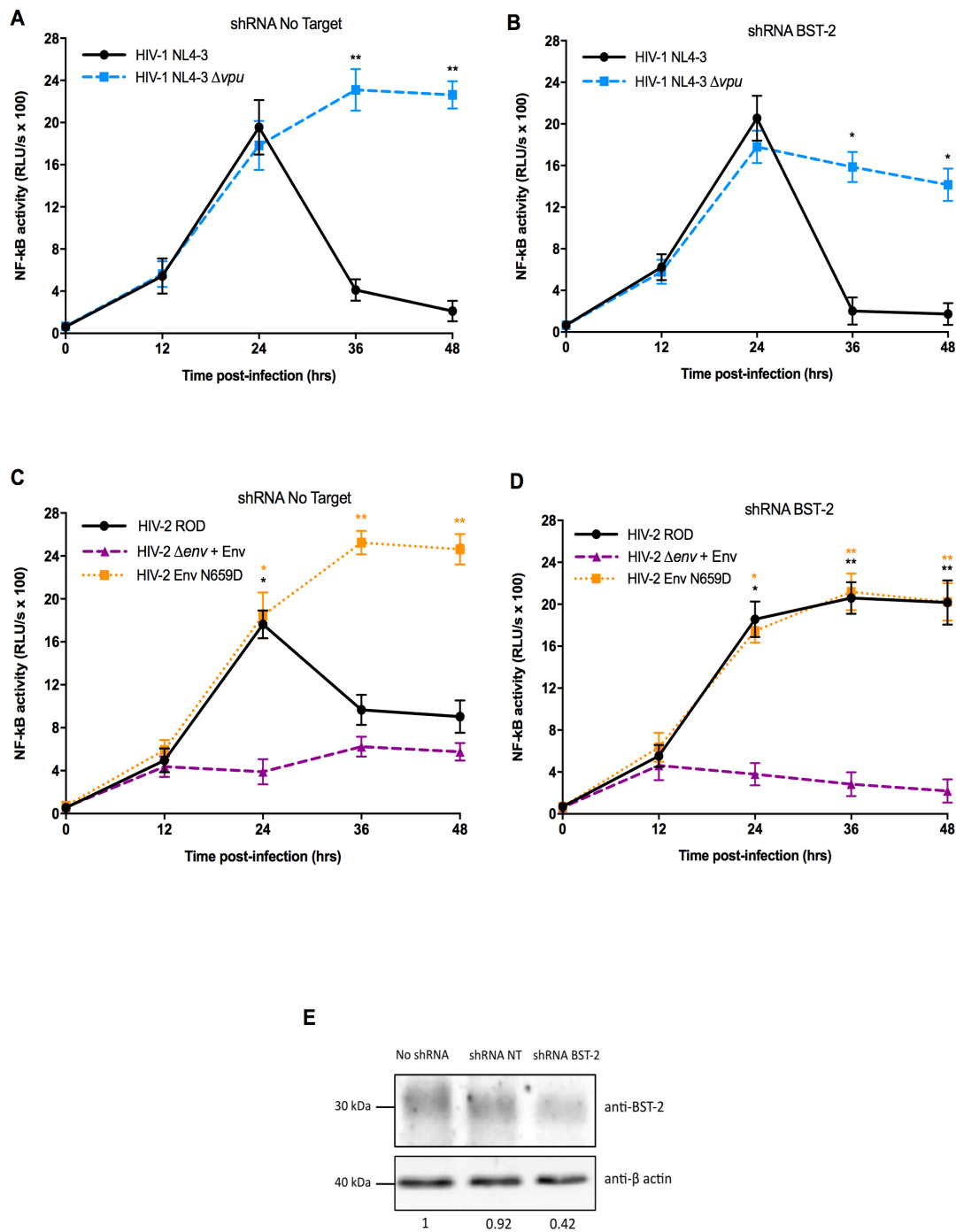


Figure 4 : Jurkat-NF- κ B clones were transduced with lentiviruses expressing shRNA against BST-2. Then, cells were infected with wild-type or *vpu*-defective HIV-1 in (A) and (B), with *env*-defective HIV-2 expressing either Env or VSV-G or with mutant HIV-2 incapable of interacting with BST-2 (HIV-2 Env N659D) in (C) and (D). Jurkat-NF- κ B cells were also infected with wild-type HIV-2 (HIV-2 ROD) in (C) and (D). Cells were lysed and luciferase activity was determined at different time points post-infection, as indicated. All panels show NF- κ B activity expressed as RLU/s x 100. All results derive from four independent experiments. Data were analyzed by ANOVA test (* p- value < 0.05 and ** p- value < 0.01). Error bars indicate mean $\pm$ standard deviation (SD). (E) One representative Western blot of the shRNA-based knockdown of BST-2 in Jurkat cells.

Discussion

Human BST-2 is an IFN-inducible host restriction factor that retains the virions at the cell surface and may promote the internalization and subsequent proteasomal degradation of the viral particles [2, 3, 12, 15]. Besides this virus retention ability, BST-2 acts as an immune sensor of the retroviral budding and release. Upon virus retention, the dityrosine motif in the cytoplasmic domain of BST-2 is phosphorylated and recruits a Syk kinase that can activate TRAF6. Ultimately, TRAF6 activates TAK1 which in turn activates the transcription factor NF- κ B. NF- κ B is translocated into the cell nucleus and binds the NF- κ B binding sites localized in numerous genes involved in the cell survival, cytokines and chemokines expression, as well as IFN- β and IFN-stimulated genes (ISGs) expression which are all able to shape an antiviral response against the viral replication and dissemination [31-34]. Yet, HIVs have evolved sophisticated means to avoid this antiviral response at the late stages of the viral replication cycle. HIV-1 Vpu proteins completely inhibit the NF- κ B activity by antagonizing BST-2 [41, 53]. A recent study demonstrated that the Vpu-mediated inhibition of NF- κ B largely downregulates the expression of a wide number of NF- κ B targeted genes, thereby facilitating the immune evasion of HIV-1 [48]. Compared to HIV-1, HIV-2 is less pathogenic and is strongly controlled by the host immune system, and it is thought that the early T cell-mediated antiviral response is a major contributor to this phenotype [54-59]. In this study, we demonstrate that the differences of pathogenesis between these two retroviruses may be partly explained by the differential regulation of the NF- κ B signaling pathway during the replication cycle of HIV-1 and HIV-2 in CD4⁺ T cell lines. The latter virus uses its envelope (Env) glycoprotein to antagonize BST-2 [19], but interestingly both HIV-1 and HIV-2 Env proteins can activate NF- κ B in HEK293 cells, as we revealed in a previous study [46, 49]. Therefore, we sought to determine whether/how HIV-2 regulates the NF- κ B signaling pathway in virally infected cells that are naturally targeted by HIVs. Interestingly, we observe in the present study that in contrast to HIV-1, HIV-2 appears to be bare of a strong inhibitor of the NF- κ B activity and that the Env-mediated antagonism of BST-2 is less effective in decreasing the NF- κ B-induced antiviral response. Indeed, in Jurkat cells infected with HIV-2, the NF- κ B activity in the late stages of infection is two to three-fold higher than in cells infected with HIV-1, mainly through the different mechanism used by these retroviruses to antagonize BST-2.

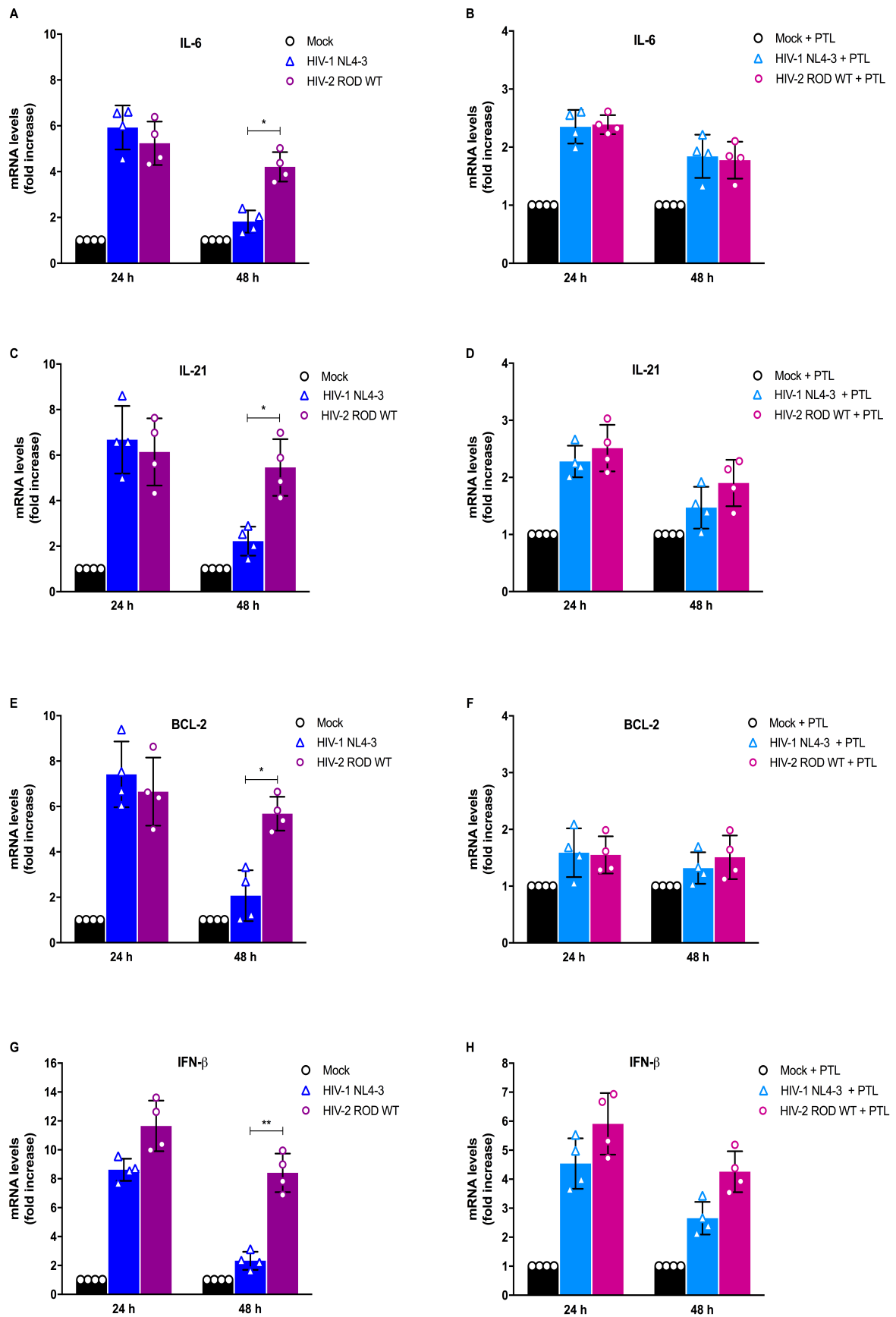


Figure 5: Levels of mRNA expression induced by NF- κ B signaling pathway during HIV-1 or HIV-2 infection. Jurkat-NF- κ B cells were mock-infected or infected with HIV-1 or HIV-2 and the expression levels of transcripts of *il-6*, *il-21*,

bcl-2 or *ifn- β* gene (panels A, C, E, G respectively) were measured by qPCR. The ratios of each mRNA transcripts at 24 and 48 were calculated relatively to those of *gapdh*. In panels B, D, F, H, the expression levels of mRNA of *il-6*, *il-21*, *bcl-2* or *ifn- β* gene were measured in infected Jurkat-NF- κ B cells treated with Parthenolide.

Interestingly, we observe a dual role of the HIV-2 Vpr accessory protein, in accordance with a recent study that determined such a role of Vpr from some SIV strains [60]. When expressed alone, HIV-2 Vpr slightly activates NF- κ B. However, when co-expressed with Env, a weak inhibition of NF- κ B can be observed. Therefore, it is quite probable that this Vpr inhibitory effect may differ among the HIV-2 isolates and further *in vivo* studies warrant to be performed.

We also describe that the Env N659D substitution, which disrupts the interaction with BST-2, markedly increases the NF- κ B activity in the late stages of infection. This suggests that the viral ability to antagonize BST-2 predominantly regulates the NF- κ B activity during the viral replication cycle. However, we used in this study an infectious molecular clone (IMC) of the HIV-2 ROD reference strain. It could be interesting to test other strains of HIV-2 (such as HIV-2 group B strains) or several HIV-2 isolates from infected patients in order to confront our findings. Furthermore, in this study we used a CD4⁺ T cell line (Jurkat cells) but not primary CD4⁺ T cells. We aim to recapitulate the experiments or assays by using PBMCs from healthy donors and monitor the NF- κ B activity in those cells infected with different IMCs or isolates of HIV-1 or HIV-2. HIV-1 Env is able to robustly activate the NF- κ B signaling pathway [46] but the inhibitory effect of Vpu is dominant [41]. It has been reported that the Y₇₆₆HRL (Genbank: AF324493.2) motif within the HIV-1 Env cytoplasmic tail (CT) is involved in the activation of NF- κ B [46]. By using site-directed mutagenesis experiments, we define the region in the HIV-2 Env CT that is thought to activate NF- κ B signaling pathway. Truncation of the CT may affect the expression, addressing or conformation of the Env glycoproteins in a cell-specific manner [61]. However, in HEK293T cells, these features of the Env glycoproteins with truncated CT are not modified (reviewed in [61, 62]), thus these cells are suitable to test different Env proteins and their ability to activate NF- κ B. Although we do not define the exact residue(s) involved in the NF- κ B activation, we depict the region within the HIV-2 Env CT, which corresponds to the residues localized between the position 759 and the position 777 (from HIV-2 ROD strain, GenBank: M15390.1; Uniprot: P04577). In this region, it is interesting to note that the HIV-2 Env CT also includes an Yxx Φ motif similar to HIV-1 Env CT that is involved in the NF- κ B activation. However, further experiments using HIV-2 Env proteins harboring point

region are needed to better define the precise residues involved in the NF- κ B activation. Moreover, it is still conceivable that a cellular protein may be recruited by the HIV-2 Env CT to prime the NF- κ B activation. Although we have been able to demonstrate that the HIV-2 Env protein activates NF- κ B via a TAK1-independent but TRAF6-dependent manner (and in a lesser extent MEKK3), the molecular mechanism underlying this NF- κ B activation remains to be fully elucidated.

Overall, this study provides new insights concerning the differential regulation of the NF- κ B signaling pathway during the HIV-1 and HIV-2 replication cycle. In contrast to HIV-1, HIV-2 fails to completely inhibit the NF- κ B activation in the late stages of infection in CD4⁺ T cells. As a consequence, the NF- κ B-mediated antiviral response during the HIV-2 infection is higher as compared with HIV-1 infection. This stronger NF- κ B-induced antiviral response may partly explain the better immune control toward HIV-2 and the differences between HIV-1 and HIV-2 pathogenesis. Moreover, this study also highlights the importance of an efficient viral antagonist of BST-2 in order to ensure both the viral replication and dissemination, and the immune evasion of these primate lentiviruses.

Materials and methods

Cell lines, plasmids, cloning, transfections, transductions and reagents

The HEK293T and Jurkat (clone E6-1) cells were obtained from the ATCC. The TZM-bl reporter cells were obtained from the NIH AIDS Reagent Program (catalog number: 8129). The HEK293T and TZM-bl cells were maintained in DMEM (Thermo Fisher Scientific) supplemented with 10% of FBS (GE Healthcare Life Sciences) and 1% of gentamicin (Thermo Fisher Scientific), while Jurkat cells were propagated in complete RPMI (Thermo Fisher Scientific). The infectious molecular clone for HIV-2 ROD (pKP59-HIV-2 ROD [63]), encoding a complete Env cytoplasmic tail, has been previously described [22]. The HIV-1 pNL4-3 and the *vpu*-deleted pNL4-3 infectious clones, as well as the plasmid encoding the codon-optimized *vpu* sequence, were all obtained from the NIH AIDS Reagent Program (catalogue number 114, 968 and 10076 respectively). HIV-2 *env*, *nef*, *vif*, *vpr*, and *vpx* coding sequences were amplified with specific primers from the pKP59-HIV-2 ROD plasmid and cloned into the pcDNA3.1 vector (Invitrogen). Plasmids encoding the six different mutants of HIV-2 Env cytoplasmic tail were created by introducing stop codon in different region of

the cytoplasmic tail by site-directed mutagenesis. It was as follows: M1: Env L₈₄₃-Stop, M2: Env L₈₂₄-Stop, M3: Env L₇₉₅-Stop, M4: Env L₇₇₇-Stop, M5: Env L₇₅₈-Stop and M6: Env G₇₃₉-Stop.

The expression plasmids of human BST-2, HIV-2 Env N659D, as well as the infectious clones of HIV-2 Δenv and Env N659D have been described in our previous studies [22, 49]. The pHAGE NF- κ B-TA-LUC-UBC-GFP-W lentivector containing the NF- κ B binding sites upstream of the *luciferase* gene has been previously described [64]. In order to allow an accurate monitoring of NF- κ B activity in Jurkat cells, we replaced the wild-type luciferase sequence by a short-lived version of luciferase (luc2P) harboring a PEST sequence. The *luc2p* gene were amplified from the pGL4.11(*luc2P*) vector (Promega) and inserted into the pHAGE NF- κ B-TA-LUC-UBC-GFP-W lentivector using the *NcoI/BamHI* restriction sites. All sequences were verified by nucleotide sequencing using the ABI 3500 platform (Applied Biosystems).

HEK293T and Jurkat cells were transduced with VSV-G pseudotyped viruses encoding the NF- κ B-Luc2P-GFP genomic cassette. The efficiently transduced cells were sorted following the GFP expression using a FACS Aria III cell sorter (BD Biosciences). We generated cellular clones of each cell type by serial dilutions. After several passages, we chose the cellular clones (HEK293T-NF- κ B and Jurkat-NF- κ B) that exhibited the minimal luciferase expression in absence of stimuli, and we verified the responsiveness of these clones upon stimulation with TNF α (Miltenyi Biotec).

The transfections of HEK293T (and HEK293T-NF- κ B) cells with the different plasmids were performed by using Lipofectamine 3000 or TurboFect reagents, following the manufacturer's instructions (Thermo Fisher Scientific). For the luciferase tests, the amounts of each plasmid transfected or co-transfected into the HEK293T-NF- κ B cells are provided in the figure legends. The total amount of transfected plasmid DNA was kept constant in all experiments by adding pcDNA-GFP plasmids.

The inhibitor of NF- κ B signaling pathway, Parthenolide, was purchased from Invivogen and was used at a final concentration of 5 μ g/mL when indicated in this study. Equivalent final concentrations of DMSO were added to controls. The HIV entry inhibitor bicyclam JM-2987 (used at 300 ng/mL; cat: 8128) as well as inhibitor TAK-779 (used at 100 ng/mL; cat: 4983) have been received from the NIH AIDS Reagent Program and were both added 12 hours after the infection of Jurkat-NF- κ B cells (see below).

Virus production and infections

HIV-1 wild-type or *vpu*-deleted viruses were generated by transfecting $3,5 \times 10^5$ HEK293T cells with the HIV-1 pNL4-3 or *vpu*-deleted pNL4-3 plasmids (1,5 μ g). HIV-2 wild-type or Env N659D viruses were also created following the same protocol. Twelve to fourteen hours post-transfections, the medium was removed, and fresh medium was added in the 12-well plate. Forty hours later, virus-containing supernatants were harvested and centrifuged at 1500 x g to discard cell debris. HIV-2 or HIV-1 viral particles were purified using the Lenti-X concentrator (Takara) and were resuspended in fresh DMEM medium supplemented with 5% of FBS. Viral titers were determined by infecting 4×10^4 TZM-bl reporter cells in serial dilutions in 48-well plates and we proceeded to a luciferase test to assess the replicative fitness of these viral particles. Then, 4×10^5 Jurkat-NF- κ B cells were infected with a quantity of HIV-1 (or variants) or HIV-2 (or variants) viruses. This quantity corresponds to the equal levels of luciferase expression observed in the infection assay using the TZM-bl mentioned above for both HIV-1 and HIV-2. The infections of Jurkat-NF- κ B cells have been performed in two technical and four biological replicates. After 12, 24, 36 and 48 hours post-infection, $\sim 3 \times 10^5$ infected Jurkat-NF- κ B cells were collected, washed with PBS and lysed with the passive lysis buffer. The luciferase expression levels, and thereby the NF- κ B activity, were determined as explained in the next section.

Reporter gene assays and monitoring of the NF- κ B activity

The transduced HEK293T-NF- κ B cells were used to assess the NF- κ B activation by expression of different HIV-1, HIV-2 or human proteins. Twenty-four hours (or 48h when indicated) post-transfection, cells were washed with PBS (Thermo Fisher Scientific) and lysed with 1x passive lysis buffer (Promega) for 15 min. The cell lysates were collected and centrifuged 5 min at 15000 x g and 20 μ l of cell-free lysates were added to 100 μ l of Luciferase Assay Reagent II (LAR II) containing luciferin. Bioluminescent signals were measured with a GloMax 20/20 luminometer from Promega.

For the monitoring of NF- κ B activity in the transduced Jurkat-NF- κ B cells infected with HIV-1 or HIV-2 (or variants), one of the replicates of each conditions was used to determine the luciferase expression levels after 12, 24, 36 and 48 hours post-infection. The infected cells were washed twice, counted using the Trypan Blue exclusion method, and $\sim 3 \times 10^5$ infected Jurkat-NF- κ B cells were lysed with 1x passive lysis buffer

and the NF- κ B activity was measured using the luciferase assay as explained above. The NF- κ B activity in mock-infected Jurkat-NF- κ B cells was also measured during the 48 hours of infections and remains at the basal level of luciferase expression (200-250 RLU/s).

SiRNA- and shRNA-based downregulation experiments, Western blots and antibodies

All siRNAs used to downregulate the expression of TAK1, TRAF6 or MEKK3 were purchased from Origene, as well as the siRNA No Target. The siRNAs were used at a final concentration of 10 nM. HEK293T-NF- κ B cells were seeded in 24-wel plate and were transfected the following day with the respective siRNAs using the siTran 1.0 transfection reagent (Origene). The next day, the medium was removed and replaced with complete medium. Eight hours after, cells were transfected with siRNAs alongside with plasmids encoding HIV-1, HIV-2 or BST-2 proteins (or variants). To perform the knockdown of BST-2 in the Jurkat-NF- κ B cells, a TRC lentiviral human BST-2 shRNA vector (pLKO.1 vector) was obtained through Dharmacon (Clone ID: TRCN0000107015). We generated pseudotyped viruses to transduce the Jurkat-NF- κ B cells (a shRNA No Target was also used as a control for further experiments). Cellular clones of both Jurkat-NF- κ B shRNA No Target and Jurkat-NF- κ B shRNA BST-2 were selected by serial dilutions in medium containing puromycin (2 μ g/mL).

Expression levels of HIV-2 Env proteins or human TAK1, TRAF6, MEKK3 proteins were determined from cell lysates from HEK293T-NF- κ B used in the NF- κ B activity experiments. The expression levels of human BST-2 were assessed in Jurkat-NF- κ B cells. Samples were added to 2x Laemmli sample buffer, boiled for 5 minutes, separated by electrophoresis on 12% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% BSA in TBS containing 0.1% Tween-20 for 1 hour and probed overnight at 4°C with the antiserum to HIV-2 ST gp120 (NIH AIDS Reagent Program, cat: 1410), or with anti-TAK1, anti-TRAF6 or anti-MEKK3 antibodies (all from Origene). A monoclonal anti-BST-2 antibody (Abcam, cat: ab134061) was used to detect BST-2. Endogenous β -actin was detected with the rabbit monoclonal anti- β -actin antibody (Cell Signaling, cat: 4970). Membranes were rinsed three times for 5 min in TBS 0.1% Tween-20 and the blots were probed with either HRP-conjugated anti-rabbit or anti-mouse secondary antibody (Cell Signaling, cat: 7074 and 7076, respectively). Membranes were then rinsed three

times and treated with the Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences), and blots were analyzed with the ChemiDoc XRS+ System (Bio-Rad).

Quantitative PCR (qPCR)

To determine the mRNA levels of four different genes targeted by the NF- κ B transcription factor, the Jurkat-NF- κ B cells from the remaining replicates of the infection assays (24h and 48h post-infection) were harvested, washed twice in PBS and counted by the Trypan Blue exclusion method. The RNAs of those cells were extracted by using the RNeasy Plus Kit (Qiagen) and were immediately subjected to reverse transcription with the Transcriptor First Strand cDNA Synthesis Kit (Roche). Each representative cDNAs of *il-6*, *il-21*, *ifn- β* and *bcl-2* genes were amplified and quantified by qPCR using a LightCycler 96 system (Roche). The mRNA levels of *il-6*, *il-21*, *ifn- β* and *bcl-2* were compared to those of *gapdh* at 24 and 48 hours post-infection of mock-infected and infected Jurkat-NF- κ B cells. The ratios were obtained using the LightCycler 96 software following the $\Delta\Delta C_t$ method.

Specific primers were as follows: IL-6: F 5'-GAACTCCTTCTCCACAAGCGC-3' and R 5'-GTGGTTATTGCATCTAGATTCTTTGCC-3'; IL-21: F 5'-GAGATCCAGTCCTGGCAACATG-3' and R 5'-CTTCACTTCCGTGTGTTCTAGAGG-3'; BCL-2: F 5'-CACGCTGGGAGAACAGGGTAC-3' and R 5'-GTTGACGCTCTCCACACACATG-3'; IFN- β : F 5'-GACCAACAAGTGTCTCCTCC-3' and R 5'-GGCCTTCAGGTAATGCAG-3' (Eurofins).

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4.4 Third article: “An insertion of seven amino acids in the envelope cytoplasmic tail of HIV-2 selected during disease progression enhances viral replication”

This third article has been published in *AIDS Research and Human Retroviruses* in February 2019 (<https://doi.org/10.1089/aid.2018.0129>). It is related to the third objective of the research project (see chapter III, section 3.3). This study highlights the contribution of the HIV-2 Env cytoplasmic tail in the viral replication and also demonstrates that sequence variability in this cytoplasmic domain may broadly influence the replication capacity of retroviruses [571].

Note that the references cited in this paper are shown at the end of the article and are not linked to the numbers of references cited in the text of the present thesis.

An insertion of seven amino acids in the envelope cytoplasmic tail of HIV-2 selected during disease progression enhances viral replication.

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Running head: Insertions in HIV-2 Env and viral replication

Keywords: HIV-2, Env, Cytoplasmic tail, Insertion, Viral replication, Disease progression

Abstract

The cytoplasmic tail (CT) of the HIV-2 envelope glycoprotein (Env) includes amino acid (aa) sequences similar to lentiviral lytic peptides (LLP) described in other lentiviruses. Within the putative LLP-2 region, we previously observed insertions of 3 or 7 aa in sequences deduced from plasma viral RNA of symptomatic HIV-2 infected individuals. Based on these observations, we reproduced the insertions in a molecular clone to assess their impact on replicative fitness and cell death *in vitro*.

Using a molecular clone of the HIV-2_{ROD} reference strain, site-directed mutagenesis experiments allowed the generation of plasmids with the insertion L₇₉₁TAI or L₇₉₁QRALTAI in the Env protein. The clone with 7 aa insertion enhanced viral release eight to eleven times in infected T cells and cell viability was impaired by more than 20%, compared to the wild-type HIV-2_{ROD} virus. The effect of the 3 aa insertion was milder, with a non-significant trend to enhance viral replication and cell death compared to the wild-type virus. Interestingly, the insertions in the Env proteins did not induce a significant increase of viral infectivity, as revealed by the infectivity assay using TZM-bl cells.

The insertions in the Env CT observed *in vivo* from disease progressors may therefore be involved in the higher viral load observed in these individuals. This study may open the way to the development of a prognostic marker related to the HIV-2 infection progression.

The envelope (Env) glycoproteins of primate lentiviruses play several key roles in the viral replication cycle. The Env proteins recognize and bind the cellular receptors and allow the fusion and virus entry processes in the early steps of infection. In the late stages of infection, envelope glycoproteins interact with cellular and viral proteins to stabilize the matrix formation, and these proteins are incorporated into the envelope of budding virions [1]. In Human Immunodeficiency Viruses type 1 and 2 (HIV-1 and -2), envelope glycoproteins are expressed from the *env* gene and consist of two subunits, the extracellular glycoprotein (gp120 or gp105) and the transmembrane glycoprotein (gp41 or gp36), non-covalently linked to constitute heterodimers. In turn, heterodimers form trimeric Env glycoprotein spikes as the functional and mature envelope glycoprotein on the surface of virions and infected cells [1, 2]. The transmembrane glycoprotein is divided in three domains: an extracellular, a transmembrane and an intracellular domain. The latter is also called the cytoplasmic tail (CT). Retroviral CTs diverge in size from 20 to 200 amino acids, and the CTs of HIV-1 and HIV-2 display approximatively a length variability of 160 amino acids. Immediately downstream the transmembrane domain, CT is structurally composed of an endocytic motif (YxxΦ), the Kennedy epitope, and three lentiviral lytic peptides (LLP-2, LLP-3 and LLP-1) involved in the induction of apoptosis in infected cells [1-3].

The CTs of HIVs are dispensable for binding and fusion activities but these domains are essential for antero- and retrograde trafficking, viral assembly, incorporation in newly synthesized virions and conformational structure of Env glycoprotein. Interestingly, Env glycoprotein has also been reported to impact viral replication and release via, for instance, activation of NF- κ B pathway and antagonism of BST-2/Tetherin in the case of HIV-2 [4, 5].

HIV-2 infection is characterized by lower transmission rates, lower plasma viral loads and better immune control compared to HIV-1 disease. In contrast to HIV-1 patients, the majority of HIV-2 patients do not progress to AIDS and 70–80% are called long-term non-progressors [6, 7]. In a previous study, our research group observed insertions of 3 or 7 amino acids (aa) in *env* sequences deduced from plasma viral

RNA of several HIV-2 infected individuals. A cohort of 72 HIV-2 patients from Belgium and Luxembourg was established and among these, 23 patients were selected because their detectable viral load (>100 copies/mL). Among these 23 progressors, the insertions of 7 (QRALTAI) or 3 (TAI) amino acids were observed in the Env CT from 4 different patients (17%). Noticeably, the patients infected with virus harboring the 7 aa insertion in the Env cytoplasmic tail displayed the highest viral load of this cohort (>35000 copies/mL) [8]. The only exception was a patient infected with a virus harboring an insertion of 7 aa LRALTAT in Env CT and showing a low viral load (405 copies/mL). Patient characteristics and available clinical information are summarized in table 1.

Table 1: Patient characteristics and clinical information for the 4 HIV-2 patients infected with viruses harboring an insertion in the Env CT.

Patient	Insertion	Viral load (copies/mL)	CD4 count (cell/mL)	ART	Gender	Age
1	QRALTAI	515000	100	Yes	M	33
2	QRALTAI	47435	N.A.	No	F	40
3	QRALTAI	35750	266	Yes	M	46
4	TAI	14800	N.A.	Yes	M	10

ART: Antiretroviral Therapy; N.A.: not available.

In order to confirm the impact of these insertions on viral replication *in vitro*, we reproduced the insertions by site-directed mutagenesis using a molecular clone of HIV-2_{ROD} encoding a complete envelope cytoplasmic domain (pKP59-HIV-2_{ROD} [9], GenBank: M15390.1). We generated two different mutants of HIV-2_{ROD}: a clone containing the insertion L₇₉₁TAI and another clone containing the insertion L₇₉₁QRALTAI in the envelope cytoplasmic tail (Fig. 1A). The sequences of primers used in inverse PCR were as follows: F Env 7aa 5'-cag aga gca cta *aca gca atc* aga gac tgg ctg aga ctt aga aca gcc ttc ttg-3' or F Env 3aa 5'- *aca gca atc* aga gac tgg ctg aga ctt aga aca gcc ttc ttg caa tat ggg tgc-3' (inserted nucleotides are in italic) and R Env : 5'-gag att ctg gta gat gag ttg gag ggt cag gaa gct cct gga tag-3'. Molecular clones were transfected in

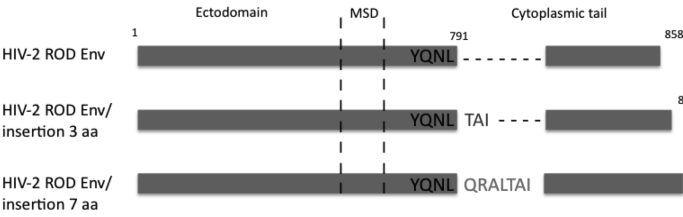
HEK293T cells and the produced viral particles were purified using a sucrose cushion three days post-transfection. We used our RT-qPCR assay to quantify the number of viral RNA copies from a fraction of viral stocks following a protocol previously described [9]. H9 lymphocyte T cells were infected with wild-type HIV-2_{ROD} (WT), HIV-2_{ROD} Env L₇₉₁*TAI* or HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses at a multiplicity of infection of three (MOI=3, based on the number of viruses calculated by the RT-qPCR assay). After 3 hours of infection, inocula were removed, infected cells were carefully washed three times with PBS and then incubated in complete RPMI culture medium. At three and six days post-infection, virus-containing supernatants were harvested and the number of viral copies released from infected cells was determined by RT-qPCR assay. We observed that viral replication and release ability of the HIV-2_{ROD} Env L₇₉₁*QRALTAI* virus were enhanced by 8- to 11-fold compared to that of wild-type HIV-2_{ROD} virus (Fig. 1B).

We also investigated the expression of viral proteins by western blot analysis to check the enhanced viral production in H9 cells. To this end, H9 cells were infected with the different viruses at a multiplicity of infection of three (MOI=3) and Maraviroc and bicyclam AMD-3100 (a CCR5 and a CXCR4 competitive antagonist, respectively) were added the following day. Two days post-infection, cells were harvested and lysed. The cell lysates were clarified by centrifugation at 20,000xg for 15 minutes at 4°C and protein sample loading buffer was added. Samples were boiled and resolved by electrophoresis on 12% polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in TBS supplemented with 0,1% of Tween-20 for one hour and probed with a rabbit polyclonal anti-HIV-2 gp120 antibody (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), a mouse monoclonal anti-HIV-2 gag p26 antibody (USBiological) or a rabbit monoclonal GAPDH antibody to detect endogenous proteins (Cell Signaling Technology). As shown in Fig. 1C, HIV-2 Env and p26 expression levels were increased in H9 cells infected with HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses compared to HIV-2_{ROD} WT.

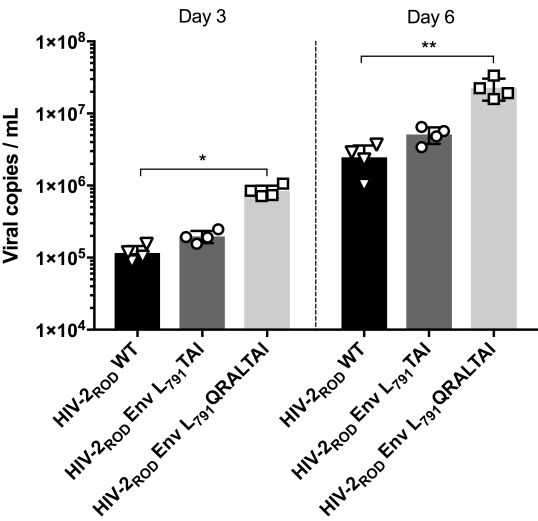
It is important to note that these insertions also modify the second exon of the HIV-2 *rev* gene. Since the Rev protein is implicated in viral RNAs exportation from the nucleus to the cytoplasm, the insertion of 7 aa (showing the most important effect on the HIV-2 replication) may conceivably enhance viral RNAs

transport and thereby improve virus production. To test this hypothesis, we constructed molecular clones for HIV-2_{ROD} Δrev and HIV-2_{ROD} Δrev Env L₇₉₁QRALTAI, and we obtained vectors encoding synthetic gene of wild-type *rev* or *rev* with the corresponding 7 aa insertion (Fig. 1D ; Eurofins Genomics, Germany). The different molecular clones for HIV-2_{ROD} Δrev were transfected alone in HEK293T cells or transcomplemented with plasmids encoding wild-type Rev or Rev with 7 aa insertion. Forty-eight hours later, accumulated viral particles in culture supernatants were quantified by RT-qPCR assay. Of note, HEK293T cells transfected with the molecular clone for HIV-2_{ROD} Δrev generated nearly no viral particles, given the absence of Rev proteins. Interestingly, the production of HIV-2_{ROD} Δrev (encoding wild-type Env protein) transcomplemented with Rev with the 7 aa insertion did not enhance viral production and release, whereas the production of HIV-2_{ROD} Δrev Env L₇₉₁QRALTAI viruses along with the expression in *trans* of wild-type Rev boosted viral production and release (Fig. 1E). These results demonstrate that the enhanced viral production is only due to the modification in the HIV-2 Env sequence, and not in the Rev sequence. Furthermore, these results corroborate previous observations that all the HIV-2 patients infected with HIV-2 virus harboring this 7 aa insertion QRALTAI in the Env cytoplasmic tail exhibit a high viral load and disease progression [8].

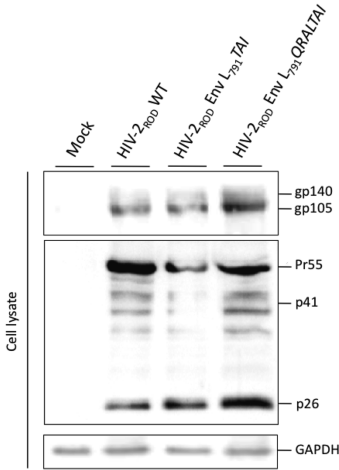
A



B



C



D

HIV-2 Rev

MNERADEEGLQRKRLIRLLHQTNPYPQGGPGTASQRRNRRRWKQRWRQILA

LADSIYTF PDPPADSPLDQTIQHLQGLTIQELPDPPTHLPESQRLAET

HIV-2 Rev 7aa

MNERADEEGLQRKRLIRLLHQTNPYPQGGPGTASQRRNRRRWKQRWRQILA

LADSIYTF PDPPADSPLDQTIQHLQGLTIQELPDPPTHLPESPESTNSNQRLAET

E

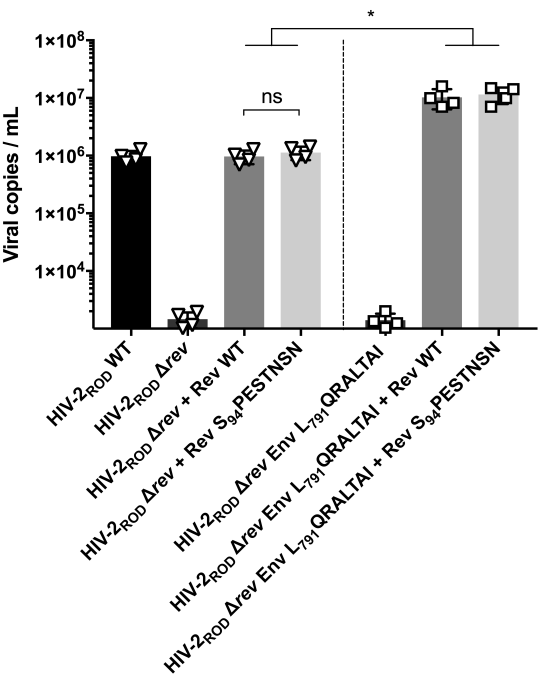


FIG. 1. Impact of 3 and 7 amino acid insertions in the HIV-2 envelope cytoplasmic tail on viral replication and release. (A) Schematic representation of the insertions (3 or 7 amino acids) in the HIV-2 envelope cytoplasmic tail. Site-directed mutagenesis using the HIV-2_{ROD} molecular clone (pKP59-HIV-2_{ROD}) was performed to introduce insertions of TAI or QRALTAI sequences in position 791 in the Env protein (MSD: membrane-spanning domain). (B) Absolute quantification of the number of viral particles released at three and six days post-infection. Amounts of wild-type HIV 2_{ROD} (WT), HIV-2_{ROD} Env L₇₉₁TAI or HIV-2_{ROD} Env L₇₉₁QRALTAI viruses released from H9 cells were evaluated using a RT-qPCR protocol. (C) Expression levels of viral proteins were investigated by western blot analysis. H9 cells were mock-infected or infected with HIV-2_{ROD} Env (WT), Env L₇₉₁TAI or Env L₇₉₁QRALTAI viruses and were lysed two days post-infection. Cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with antibodies to HIV-2 Env (gp105/gp140) or HIV-2 p26. Endogenous GAPDH proteins were detected as loading control. (D) Amino acid sequences of wild-type Rev and Rev with 7 aa insertion. This insertion (PESTNSN) is shown in italic. (E) Impact of the 7 aa insertion in HIV-2 Rev proteins on viral replication and release. HEK293T cells were transfected with full-length infectious molecular clones for HIV-2_{ROD} Δ rev or HIV-2_{ROD} Δ rev Env L₇₉₁QRALTAI (500 ng) alone, or transcomplemented with plasmids encoding wild-type Rev or Rev with 7 aa insertion (100 ng). Differences in the amount of plasmid DNA in each transfection were offset by the addition of empty pcDNA3.1 vector. Virus yield determined by RT-qPCR assay 48 hours post-transfection. Graph presents the number of viral copies on a logarithmic scale. Error bars indicate mean $\pm$ SD derived from four independent experiments for all data presented in this figure (n = 4; * *p-value* < 0.05, ** *p-value* < 0.01, ns= non significant; Kruskal-Wallis test for (B) and (E)).

Given that insertions are located in the putative Env LLP-2 region of HIV-2, we next sought to determine whether cell viability could be impacted following expression of the Env CT mutants. Indeed, it has been reported in HIV-1 that the LLPs, three conserved amphipathic α -helical segments in the Env CTs of HIVs, induce apoptosis or cytolysis of infected cells by disturbing cell membrane integrity [3, 10]. Therefore, at day four post-infection, we evaluated cell viability of the infected H9 T cells using a CASY cell counter able to discriminate living cells from dead ones. Whereas 77% of cells infected with HIV-2_{ROD} WT virus were still living, only 55% of cells infected with HIV-2_{ROD} Env L₇₉₁QRALTAI virus were living cells. In the case of HIV-2_{ROD} Env L₇₉₁TAI, there were no significant differences regarding cell viability compared to the infection with wild-type HIV-2_{ROD} virus (Fig. 2A). Thus, the 7 aa insertion in the HIV-2 Env CT causes a higher mortality possibly due to the presence of additional charged residues in the putative LLP-2 region or may be due to enhanced viral transmission followed by cell death.

To better define mechanisms involved in the enhanced viral replication and release observed with the HIV-2_{ROD} Env L₇₉₁*QRALTAI* virus, we hypothesized that the 7 aa insertion can promote fusogenicity and quantity of Env glycoproteins at the virion surface and, consequently, may favour binding and entry processes during early steps of infection. Therefore, we tested viral infectivity of HIV-2_{ROD} Env L₇₉₁*TAI* and HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses and we compared this ability with that of wild-type HIV-2_{ROD} virus. We used TZM-bl reporter cells to test viral infectivity of the three different viruses. These cells contain a *luciferase* reporter gene under the control of a HIV LTR promoter and can be used to assess viral infectivity. TZM-bl cells were seeded in a 24-well plate and infected with wild-type HIV-2_{ROD}, HIV-2_{ROD} Env L₇₉₁*TAI* or HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses at a multiplicity of infection of three (MOI=3) during three hours. After this step of infection, inocula were removed, adherent TZM-bl cells were gently washed three times with PBS and fresh culture medium was added to the wells. In order to avoid multiple cycles of infection and test only viral infectivity capacity, we added Maraviroc and bicyclam AMD-3100 in the culture medium, and we performed luciferase reporter assays 28 hours later. As presented in Fig. 2B, viral infectivity of the three viruses remained equal, suggesting that the insertions did not promote infectivity. However, insertions in the Env cytoplasmic tail may impede or promote the Env incorporation per virion, and thereby explain the improved viral fitness of the HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses. To observe and compare the Env incorporation ratio of these different viruses, we performed a western blot of the infection-derived viral fraction. Of note, we used equivalent amounts of virus to complete the western blot analysis. No real differences were observed in Env incorporation ratios although a slight decrease was seen with HIV-2_{ROD} Env L₇₉₁*QRALTAI* viruses (Fig. 2C). As fusogenicity and Env incorporation may compensate each other, it could still be that a lower Env incorporation may balance a higher fusogenicity in order to maintain a similar viral infectivity.

Our results suggest that the improvement in viral replication induced by the insertion of 7 aa in Env CT is due to a post-integration mechanism. Indeed, Env glycoproteins may impact and promote viral replication. In respect to this, we recently reported an important role of HIV-2 Env in activation of NF- κ B signaling pathway and this effect can induce LTR-driven proviral genes transcription [5]. Therefore, it is tempting to

speculate that insertions in Env CT could lead to a higher activation of NF- κ B, resulting in a higher viral production in infected cells. We used our HEK293T reporter cell line stably expressing the *luciferase* gene under the control of NF- κ B binding sequences to assess the NF- κ B activation ability of the different Env glycoproteins [5]. We cloned wild-type HIV-2_{ROD} Env, Env L₇₉₁*TAI* or Env L₇₉₁*QRALTAI* coding sequences into pcDNA3.1 expression vector and we transfected these plasmids in HEK293T-NF- κ B cells. Twenty-four hours later, we performed a luciferase reporter assay allowing to evaluate the effect of these insertions on NF- κ B activity. We observed no increased capability of the mutated Env glycoproteins in activation of NF- κ B (Fig. 2D).

Overall, our *in vitro* results validate *in vivo* observations that the 7 aa insertion in HIV-2 Env CT induces enhanced viral replication and cell mortality but the mechanisms underpinning this viral fitness and phenotype remain to be defined. We believe this study provides new insights into the role of the Env cytoplasmic tail in the HIV-2 replication and pathogenesis.

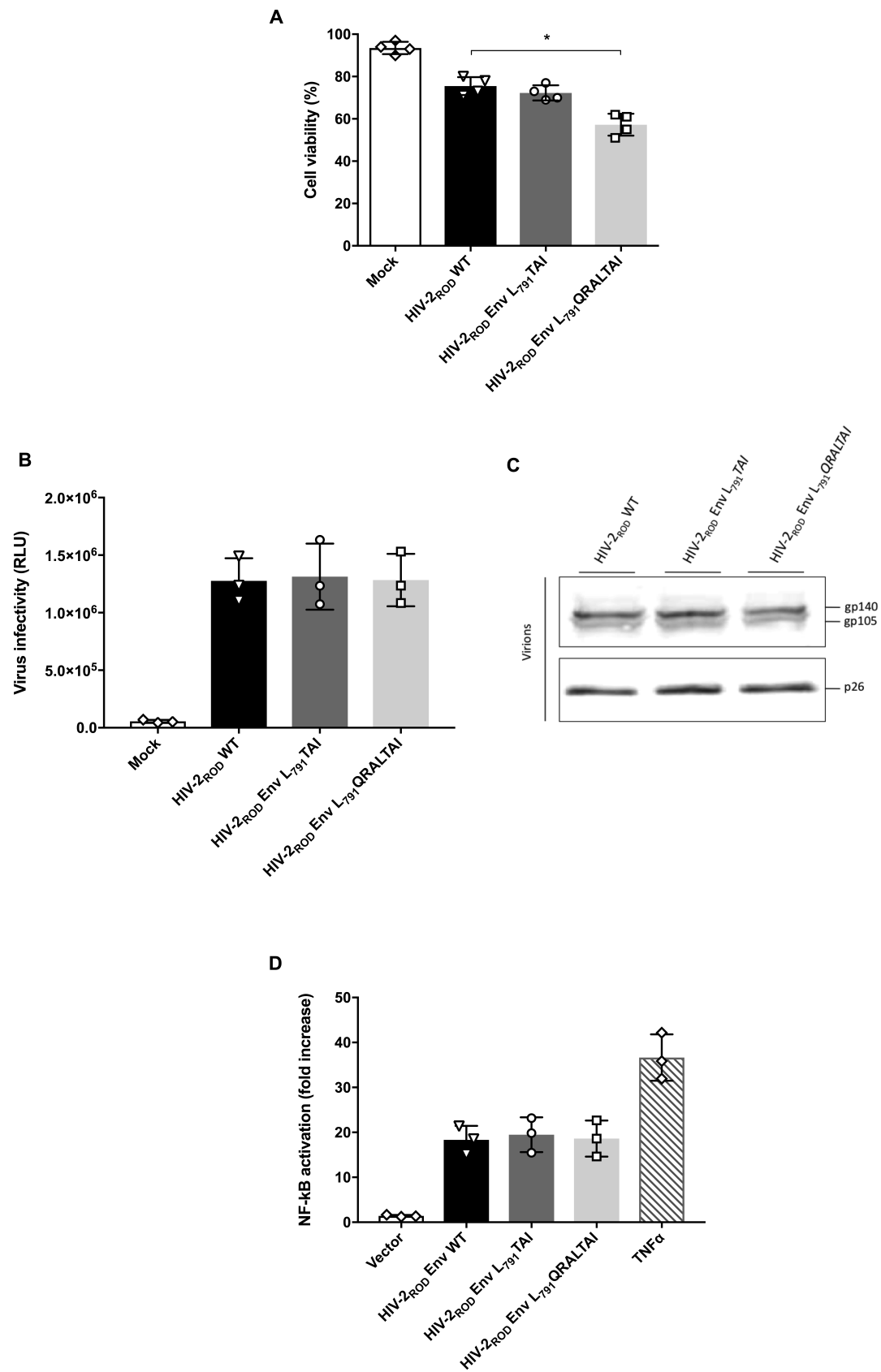


FIG. 2. Impact of 3 and 7 amino acid insertions in the HIV-2 envelope cytoplasmic tail on cell viability, viral infectivity, Env incorporation and NF- κ B activation. (A) Cell viability of H9 cells infected with wild-type HIV-2_{ROD} (WT), HIV-2_{ROD} Env L₇₉₁TAI or HIV-2_{ROD} Env L₇₉₁Q_RALTAI viruses at four days post-infection was monitored using a CASY cell counter. (B) Virus infectivity capacities for the three different viruses were assessed by infecting TZM-bl reporter cells at a multiplicity of infection of three (MOI=3) and luciferase assays were performed 28 hours later. (C) Envelope incorporation per virion of the wild-type HIV-2_{ROD} Env (WT), Env L₇₉₁TAI or Env L₇₉₁Q_RALTAI viruses were evaluated and compared by investigating the gp105/p26 ratios. (D) NF- κ B activation by the HIV-2_{ROD} Env (WT), Env L₇₉₁TAI or Env L₇₉₁Q_RALTAI proteins. 2.5×10^5 HEK293T-NF- κ B reporter cells were transfected with 100 ng of pcDNA3.1 (empty vector, used as a negative control), pcDNA3.1-HIV-2_{ROD} Env, pcDNA3.1-HIV-2_{ROD} Env L₇₉₁TAI or pcDNA3.1-HIV-2_{ROD} Env L₇₉₁Q_RALTAI. Luciferase tests were performed 24 hours post-transfection. As a positive control, we added TNF α (10 ng/ml) in the culture medium to validate this assay. Error bars indicate mean $\pm$ SD derived from four independent experiments for (A) and from three independent experiments for (B) and (D) (* *p-value* < 0.05, ANOVA test for (A), (B) and (D)).

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Chapter V: Discussion, conclusions and perspectives

5.1 The HIV-2 Env acts as a viral antagonist of the restriction factor BST-2

In the first part of this thesis, we have investigated the anti-tetherin function of the HIV-2 Env glycoprotein. In 2016, little was known about this function and in particular, the motifs within this large glycoprotein involved in the counteraction of human BST-2 were not described. Le Tortorec *et al.* have suggested a role of the gp36 ectodomain in this function by using several chimeric glycoproteins of HIV-2 – which are able to bind BST-2 – and SIVmac – which are not able to bind BST-2. The authors also demonstrated that the highly conserved endocytic motif (YxxΦ) in the HIV-2 Env CT is required to remove BST-2 from the cell surface. Moreover, they confirmed that the HIV-1 Env glycoprotein is unable to bind or antagonize this restriction factor [242]. Thus, both the gp36 ectodomain and the CT of the HIV-2 Env cooperate to achieve the antagonism of BST-2. A subsequent and interesting study reinforced the role of the CT in this anti-tetherin function. Most SIVs usually use Nef protein in order to antagonize simian tetherin. By using a Nef-deficient SIV strain, the authors have observed that after several passages in tetherin-expressing cells, some compensatory mutations occurring in the cytoplasmic tail of the SIV Env protein were selected, that could rescue the counteraction of tetherin, and thus the viral release. This highlights the exceptional plasticity of the retroviruses and the importance to overcome BST-2/Tetherin [551].

These findings prompted us to define the exact motif(s) in the gp36 ectodomain involved in the counteraction of BST-2 and also to determine the contribution of the CT in this function. We chose to use an infectious molecular clone (IMC) of HIV-2 ROD reference strain in our first study. This plasmid pKP59-HIV-2 ROD, unlike the other available IMCs such as pROD10 or pROD14 [572], encodes a complete Env cytoplasmic tail (158 aa) [549, 573]. Since the HIV-1 Env glycoproteins share conserved regions with HIV-2 Envs but are unable to bind or counteract human BST-2, we aligned the amino acid sequences of gp36/41 from HIV-1 and -2, and we selected the residues which were on the one hand highly conserved in all four HIV-2 reference strains and were in the other hand different in the HIV-1 counterparts (see our first article). According to this comparison, we identified 32 potential HIV-2 Env mutants that we have created by site-directed mutagenesis. Using different experimental approaches, we revealed that a conserved residue in position 659 (an asparagine which is not glycosylated) within the gp36 ectodomain is essential for the interaction with and antagonism of BST-2, at least concerning the HIV-2 ROD strain (HIV-2 group A). Indeed, although we have compared the

amino acid sequences of 4 HIV-2 reference strains of both group A and B, we were not able to test the importance of this asparagine residue using an IMC of HIV-2 group B (as EHO) since such an IMC is not currently available. It would be interesting to obtain a set of different HIV-2 IMCs or HIV-2 isolates to confront our findings, and test whether the sequence variability of Env from HIV-2 isolates may alter the anti-tetherin function.

A study, published several weeks after our study, has reported that another residue in position 568 of the HIV-2 gp36 ectodomain might be involved in the antagonism of BST-2 [574]. Although the authors did not observe that the substitution of this residue modifies the interaction between HIV-2 Env and BST-2, they suggested that the counteraction of BST-2 by the HIV-2 Env may rely on different residues within the gp36 ectodomain. We agree with this assumption and we further propose that the residue(s) involved in the counteraction of BST-2 may depend on the strains or isolates of HIV-2 because of the immune pressure imposed by antibodies directed to the HIV-2 Env proteins *in vivo* [575-577]. Indeed, a similar observation has been made with the viral antagonist of BST-2 from HIV-1. It has been reported that several (and different) residues in the transmembrane and/or the cytoplasmic domains of HIV-1 Vpu were involved in the antagonism of BST-2 [538, 565, 578, 579]. Thus, the fact that the viral antagonist of BST-2 is the HIV-2 Env protein, a structural protein which is immunogenic and highly recognized by neutralizing antibodies, imposes that the antagonism of BST-2 must be performed by several different residues or motifs within the HIV-2 Env. Indeed, to escape the immune recognition of the Env protein, viruses rapidly select mutations that confer immune evasion. However, this genetic variability in the Env proteins may represent a cost for HIV-2 concerning its viral fitness and counteraction of BST-2 [580, 581].

Interestingly, a recent study reported that this residue N659 - among others - within the ectodomain of Env was highly conserved in both HIV-2 and SIVsmm strains, and surprisingly that the Env proteins of some SIVsmm strains were capable of antagonizing human BST-2. These results explain how the SIVsmm has been transmitted to humans on at least eight independent cross-species transmissions and reveal a preadaptation of the SIVsmm for the Env-mediated antagonism of human BST-2 by HIV-2 [581]. Notably, this recent study indirectly corroborates the findings and observations of our first article, and we may conclude that this asparagine residue, in position 659 of the HIV-2 Env from ROD strain, is clearly involved in the counteraction of the BST-2 restriction.

The virus evolutionary rate plays an important role in the disease progression and in the HIV pathogenesis as it may be used to distinguish progressors from nonprogressors, or to predict the disease progression. A high viral evolutionary rate, resulting in extensive genetic variability, is positively correlated to a faster disease progression in HIV-1-infected patients [582, 583]. Although this issue has been extensively investigated in HIV-1 infection, little is known about the potential correlation between the virus evolutionary rate and the disease progression in HIV-2 infection. Palm *et al.* have recently observed that the slow disease progression commonly seen in HIV-2-infected individuals was associated with higher levels of positive selection on several residues that are conserved in the gp105 ectodomain of Env from HIV-2 or SIVsmm isolates [409]. It would be interesting to investigate whether the conserved residues involved in the antagonism of BST-2 are under positive or negative selection in progressor versus in nonprogressor individuals in order to determine if the intra-host HIV-2 evolutionary rates may alter or improve the Env-mediated counteraction of BST-2 *in vivo*.

Conversely, it could be interesting to observe the presence of SNPs (single nucleotide polymorphism) in the promoter or in the coding sequence of *bst-2* gene from HIV-2 progressors or nonprogressor and determine if particular SNP(s) can be found in one of the two groups of HIV-2 patients. Indeed, Laplana *et al.* have pointed several indels (insertion/deletion) or SNPs in the promoter of *bst-2* in HIV-1-infected patients [584]. Moreover, a recent study demonstrated that several polymorphisms in the *tetherin* gene of rhesus macaque are involved in differences in the acute viremia in animals infected with a *nef*-depleted SIV [585].

In our study, we have also demonstrated that the Env cytoplasmic tail participates to the efficient BST-2 antagonism. This is in line with the previous study using the *nef*-deleted SIVmac that selects compensatory changes in its Env CT to overcome BST-2, although HIV-2 and SIVmac are not the same viruses but are closely related [2, 50, 58]. Actually, it would be thought-provoking to test if similar compensatory changes may also occur in the CT while the N659D substitution within the HIV-2 Env extracellular part is present. For instance, an elegant and recent study demonstrated such compensatory mutations in both the Env gp41 ectodomain and CT (in HIV-1) that rescue the antagonism of the restriction factors APOBEC3 in a Vif-deficient virus, underscoring the importance of the Env gp41 and CT as well as the plasticity and adaptation of retroviruses in overcoming the restriction factors [586]. We have created a HIV-2 Env mutant harboring only the first 48 aa in the CT

and we observed that it slightly impedes the anti-tetherin function of HIV-2 ROD envelope glycoprotein. We propose that this truncation of the HIV-2 Env CT may remove some important residues and thereby may hamper the antagonism of BST-2. Thus, we may conclude that the HIV-2 Env CT cooperates with the extracellular domain of gp36 to overcome human BST-2. In line with this, the *env* genes from HIV-2 strains circulating in the human population invariably harbor a complete cytoplasmic tail (150-160 aa) [569].

Even though the molecular mechanism by which HIV-2 Env CT contributes to the anti-tetherin function is not fully elucidated, it is quite possible that a yet-to-be-defined intracellular partner interacts with the Env cytoplasmic tail and thereby takes part in the antagonism of BST-2. This warrants further experiments or assays to test this hypothesis and it would be also interesting to determine whether the HIV-2 Env CT interacts directly or indirectly with the cytoplasmic domain of BST-2.

5.2 Roles of the NF- κ B signaling pathway in the differences between HIV-1 and HIV-2 pathogenesis

Upon HIV entry and replication in the cell, a large number of cellular proteins can recognize the pathogen-associated molecular patterns (PAMPs) expressed by the invading viruses. A set of cellular proteins, the host restriction factors, are able to inhibit virtually each step of the viral replication cycle and to shape a strong and immediate immune response against the retroviruses by inducing the expression of proinflammatory cytokines and chemokines, as well as ISGs and interferons [489, 491, 502]. HIVs have evolved several means to circumvent and bypass this antiviral response to replicate and disseminate in the host. HIV-2-infected individuals display a slowly progressing disease, lower viral loads and a stronger immunological control by the host, as compared with HIV-1 [392, 396, 587]. The leading hypothesis that could explain the difference in disease phenotype between HIV-1 and HIV-2 implies a more efficient T cell-mediated immunity in the immune control of HIV-2 infection [425].

In this research project, we have focused on a host restriction factor, BST-2/Tetherin, which presents a robust restriction activity towards the viral budding and release, but also acts as an immune sensor that can trigger an early NF- κ B-mediated immune response [512, 526]. Thus, to ensure the viral replication and dissemination while allowing the immune evasion, retroviruses must efficiently antagonize BST-2 and as a result, the NF- κ B signaling pathway [248]. Indeed, it has been reported that the efficient counteraction of BST-2 is a prerequisite for the virus replication and for successful inter- and intra-species transmissions, as well as the IFN resistance [531, 536, 567, 568]. HIV-1 and HIV-2 antagonize BST-2 by using different viral proteins (i.e. Vpu and Env respectively) with contrasting efficiencies [242, 552]. Therefore, we aimed to define whether and how these different efficiencies of viral counteraction of BST-2 could result in a differential regulation of the NF- κ B-mediated antiviral response, and we hypothesized that such a difference may account for the better immune control and the slow disease progression of HIV-2 infections.

In our second article, we first identify an unknown role of the HIV-2 Env in the activation of NF- κ B in HEK293T cells, similar to HIV-1 and SIVs Env proteins [243]. However, and importantly, we also reveal an inability of the HIV-2 Env anti-tetherin function to completely inhibit the NF- κ B

activity, whereas the HIV-1 Vpu anti-tetherin function does. As a consequence, the NF- κ B activity levels - and the associated expression of several antiviral genes - during HIV-2 infection of CD4⁺ T cells remain higher than those of HIV-1 infection, as we reveal in our article “in preparation”. In this article, we also test the impact of the N659D substitution within the gp36 ectodomain of HIV-2 Env, that impairs the BST-2 antagonism, on NF- κ B activity during the viral replication cycle. We observe that the activity levels of NF- κ B generated by this mutant virus are even greater than those observed during infections with the wild-type virus, suggesting that the ability to antagonize BST-2 largely governs the regulation of this signaling pathway. Interestingly, a recent study demonstrated that the Ebola virus matrix protein (VP40) and the envelope glycoprotein (GP) cooperate with human BST-2 to induce NF- κ B activity [588]. Although the GP glycoprotein from Ebola virus is, like the HIV-2 Env, the viral antagonist of BST-2, the mechanism of antagonism is unclear. It does not remove BST-2 from the cell surface and there is no evidence of degradation of BST-2, like is the case of the HIV-2 Env-mediated BST-2 antagonism [589, 590]. Therefore, the lack of degradation of BST-2 by the HIV-2 Env protein may result in the incomplete inhibition of NF- κ B.

Given these data, we can conclude that the HIV-2 Env glycoprotein acts as a potent activator of NF- κ B and that its inability to efficiently overcome and degrade BST-2 results in an elevated NF- κ B-mediated antiviral gene expression. This is not observed in HIV-1 infection since Vpu is a robust antagonist of both BST-2 and NF- κ B signaling pathway, as revealed in a recent study [562]. The fact that HIV-1 employs several means, via its Vpu protein, to dampen the NF- κ B signaling pathway underscore the importance for the viruses to inhibit the antiviral gene expression in order to ensure the viral replication and dissemination [440, 567, 568, 591]. The dual role of the structural HIV-2 Env protein (i.e. incomplete antagonism of BST-2 in addition with the activation of NF- κ B) and the absence of a viral accessory protein able to inhibit NF- κ B activity could be seen as a lack of adaptation of HIV-2 to the human host [6, 581]. These insights are the most interesting presented in this thesis. Indeed, since NF- κ B is a key regulator of the innate and adaptive immune responses against pathogens, our findings prompt us to conclude that this differential regulation of NF- κ B by HIV-1 and HIV-2 may be a major contributor of the better immune control of HIV-2 infections, as compared with HIV-1 infections.

In our study concerning the NF- κ B signaling pathway during HIV infections, we have described that the *il-21* gene was markedly upregulated at 48h post-infection in CD4⁺ T cells infected with HIV-2, but not with HIV-1, and that was largely dependent to the NF- κ B activity [592]. In the introduction

of the present thesis, we have underscored the beneficial role of interleukin-21 in the cooperation between CD4⁺ and CD8⁺ T cell responses and the fact that HIV-specific CD4⁺ T cells expressing IL-21 are enriched in the acute phase of infection in HIV controllers. IL-21 also contributes to control of HIV replication through both enhancing specific CD8⁺ T cell response and managing the regulatory CD4⁺ T cell (Treg) functions (see also Figure 17, section 1.5) [297-301]. We therefore may conclude that the upregulation of *il-21* gene expression during the HIV-2 infection that we have observed in our study is mainly due to NF- κ B activation and may probably contribute to the better immunological control of HIV-2 infections.

In the same way, the upregulation of *bcl-2* gene expression in CD4⁺ T cells infected with HIV-2 may explain, at least partly, the higher CD4⁺ T cell counts commonly seen in HIV-2-infected individuals, since this gene produces an anti-apoptotic protein [593]. Interestingly, we also revealed an upregulation of *ifn- β* gene expression upon HIV-2 infection (but not HIV-1) at 48h post-infection. As we have mentioned in the introduction, the relationship between the IFN/ISG responses and the immunopathogenesis of HIV and SIV infections is still debated until now since both deleterious and beneficial effects of IFN/ISG responses in HIV and SIV infections have been reported. However, accumulating evidences suggest that high IFN-I production during the acute phase of HIV (or SIV) infections is beneficial to prevent viral replication and dissemination, to maintain healthy CD4⁺ T cell counts and to slow the progression of the disease (reviewed in [464, 465, 468]). Notably, an important study conducted with SIV revealed that the blockade of the IFN-I signaling in rhesus macaques prior to infection or during the acute phase of infection worsens the disease progression and clinical outcome (reduced antiviral gene expression, higher viral loads, accelerated decline of CD4⁺ T cells and a more rapid progression to AIDS) [469]. Moreover, in humans, it has been observed that the immune cells from HIV-1 elite controllers have an increased ability to produce IFN-I compared to those of HIV-1 non-controllers [471, 472, 594] and studies have reported that the plasma levels of IFN-I in HIV-2 individuals are significantly higher than those of HIV-1, without resulting in higher levels of immune activation [418, 473]. In the introduction, we have postulated that the early and strong IFN/ISG responses during the HIV-2 infection may be a major determinant of the lower levels of viral load, the stronger immune control, and may be involved in the delayed disease progression, as compared with HIV-1 infection. Remarkably, our study corroborates these previous observations or hypothesis, at least with the experimental design used in this study. Indeed, we described that HIV-2

can activate NF- κ B via the Env protein in the early phases of infection but contrary to HIV-1, HIV-2 is unable to inhibit this signaling pathway in the late stages of infection, mainly because of an inefficient BST-2 antagonism.

Concerning our studies in the activation of NF- κ B induced by HIV-2, we may point out several caveats or weaknesses. First, we have tested the ability of HIV-2 Env to activate the NF- κ B signaling pathway in HEK293T cells or in a CD4⁺ T cell line (Jurkat). Although we obtained similar effects of the HIV-2 Env protein (and its incomplete BST-2 antagonism) on this signaling pathway in both cellular models, it could be interesting to recapitulate these experiments by infecting PBMCs or purified dendritic cells from healthy donors with HIV-1 or HIV-2 and to perform a monitoring of the NF- κ B activity by immunoblotting the I κ Bs (inhibitors of κ B) during the infection course. In these studies, we used the *env* gene from the HIV-2 ROD reference strain. Thus, we could consider amplifying sequences of the *env* gene from viruses infecting several progressors and nonprogressors HIV-2 patients and testing their ability to activate NF- κ B in our cellular models or in PBMCs. This will allow to determine whether the potential for activation of this transcription factor by the Env protein is found only in a particular group of patients and could support that the enhanced NF- κ B activation contributes to the better immune control of HIV-2 infections. Moreover, this could determine whether the genetic variability of the *env* gene in the circulating strains of HIV-2 may alter or modify the NF- κ B activation *in vitro* and *in vivo*.

Second, although we demonstrated that the interaction between HIV-2 Env and BST-2 results in a (moderate) decrease of NF- κ B activity, we did not provide the mechanism(s) that could explain the sustained and persistent activation of NF- κ B. We may hypothesize that the Env cytoplasmic tail and the cytoplasmic domain of BST-2, each activating NF- κ B, remain “free” or “available” to activate NF- κ B even if the interaction between these two proteins occurs. Further studies are needed to elucidate this issue.

Third, a recent and noteworthy study described that the restriction factor SAMHD1 can suppress the innate immune response induced by HIV-1 infection by inhibiting NF- κ B [595]. Notably, HIV-2 encodes Vpx that is able to degrade SAMHD1 in myeloid cells, whereas HIV-1 lacks Vpx [495, 496, 596]. Therefore, elucidating the impact of the Vpx-mediated degradation of SAMHD1 on the NF- κ B activation could bring new knowledge regarding the regulation of NF- κ B signaling pathway during the HIV-2 infections in myeloid cells in which SAMHD1 is functional (reviewed in [597]). By doing so, it

will be interesting to observe whether the counteraction of SAMHD1 by Vpx represents an advantage or a drawback for both the viral replication and the immune evasion of HIV-2.

Finally, it has been suggested that the Nef-mediated downregulation of the TCR-CD3 receptor complex could induce an indirect decrease of NF- κ B activity, mainly by reducing the responsiveness of the infected CD4⁺ T cells to further antigenic stimulation (reviewed in [248]). However, this point has never been addressed and warrants to be further studied. For instance, infection assays using wild-type or *nef*-deleted HIV-2 viruses in primary CD4⁺ T cells stimulated or mock-stimulated with a CD3 antibody while monitoring the NF- κ B activation could represent a suitable model to address this issue.

5.3 Contributions of the HIV-2 Env cytoplasmic tail in the viral replication cycle

In this thesis, we have unveiled three essential roles of the HIV-2 Env cytoplasmic tail (CT) in the antagonism of a host restriction factor (BST-2), in the activation of a transcription factor (NF- κ B), and in the enhancement of the viral replication (our third article). For the role in NF- κ B activation, we were based on a previous study that revealed a role of the HIV-1 Env CT in this activation [243]. The authors of this study demonstrated the involvement of the Y₇₆₆HRL (GenBank accession no. AF324493.2) domain in the Env CT and interestingly, we observed that the HIV-2 Env CT displays an analogous motif (Y₇₇₀SIC; Genbank accession no. M15390.1) that appears to be involved in the NF- κ B activation. However, we did not describe whether this YSIC motif is specifically involved in the NF- κ B activation since we only used truncated Env CT proteins. Therefore, site-directed mutagenesis introducing point mutation in this motif should be performed. Nevertheless, we may conclude that the HIV-2 Env CT, like those of HIV-1 and SIVmac, can activate the NF- κ B signaling pathway. Intriguingly, we also determined that, unlike HIV-1, HIV-2 Env activates NF- κ B in a TRAF6-dependent but TAK1-independent manner. The fact that TAK1 was not associated in this activation came as a surprise because this kinase is commonly activated by TRAF6 in the signaling cascade [598]. However, an alternative route of NF- κ B activation via TRAF6 exists and uses MEKK3 (and not TAK1) [599, 600], and we have demonstrated by using siRNA-based downregulation of these proteins that HIV-2 Env preferentially exploits this alternative pathway. The rationale or reasons for this are currently unknown and it necessitates to be studied, but still, we provide new insights concerning the molecular functions of the HIV-2 Env proteins during the replication cycle.

The Env CT of retroviruses is acknowledged to fulfill numerous functions, including the Env trafficking to the PM, the Env incorporation into virions by interacting with the matrix protein (MA), the viral infectivity and the stabilization of Env's structure in a cell type-dependent manner (reviewed in [175, 191, 201]) (see also section 1.4). However, the cytoplasmic domain remains the least studied part of the Env protein [211]. So, it is quite likely that the cytoplasmic tail may play more functions still unknown to date. At the beginning of the thesis, we noticed with interest the presence of identical amino acid (aa) insertions in the same region of the cytoplasmic tail from HIV-2 viruses infecting individuals monitored in our laboratory who displayed the highest viral loads of the cohort (>35000

copies/mL) [569]. We therefore aimed to reproduce these insertions into the HIV-2 ROD infectious molecular clone by site-directed mutagenesis and we tested the impact of these insertions in the viral replication and viral infectivity. Remarkably, the production and release of HIV-2 viruses harboring the 7 aa insertion in the Env CT were increased 8 to 11-fold, without modification of viral infectivity or Env incorporation, as compared with the wild-type viruses. Yet, the cytolytic effect of this insertion was moderately increased and was probably due to the high virus production into the infected cells (see our third article). These preliminary results corroborated that the 7 aa insertion induces an enhanced viral replication, and consequently the high viral loads that were observed *in vivo*.

Since this insertion of 7 aa (QRALTAI) is always localized in position 791 within the Env CT, we asked whether the Env-induced NF- κ B activation could be impacted by this insertion. Of note, this insertion is not located in the Y₇₇₀SIC motif that is thought to activate NF- κ B. It turned out that the activation of NF- κ B by the HIV-2 Env wild-type and HIV-2 Env bearing this insertion remained equal. Given these data, we may (only) conclude that this insertion of 7 aa markedly enhances the viral replication and release. Unfortunately, we are currently unable to provide an explanation for this phenotype or to explain the mechanisms underlying this increased viral replication. However, and considering previous results, a hypothesis should be tested: would this insertion promote the contribution of the HIV-2 Env CT in the antagonism of BST-2? Indeed, it could be appealing to test whether the Env-mediated antagonism of BST-2 is boosted in presence of the insertions. It is quite possible that the Env glycoproteins harboring this insertion are more stabilized at the cell surface, inducing an increased binding capacity to BST-2. A more efficient interaction between the Env cytoplasmic tail and the cytoplasmic domain of BST-2 is also conceivable. Finally, this insertion may potentially reinforce the sequestration of BST-2 in the perinuclear compartment by HIV-2 Env proteins, thereby facilitating the release of nascent virions from the infected cells.

Although we have observed these insertions from a small number of HIV-2-infected patients, it remains to be determined if these insertions are selected during the infection course and whether they represent a cause or a consequence of the faster disease progression seen in those patients. In order to study this topic, we can consider performing a longitudinal study with a HIV-2 cohort in which several patients are infected with viruses bearing this insertion and following the intra-host evolution of the *env* gene.

Strikingly, these insertions of 7 aa in the Env CT are not only observed in HIV-2 isolates. Indeed, similar insertions of 7 aa can also be detected in some HIV-1 subtype C strains [601]. This insertion, SSLRGLQ between the R₇₈₇ and the R₇₈₈ within the LLP-2/3 domain of the Env CT, is not involved in an increase or decrease of viral replication as compared with other HIV-1 subtype C strains. It has been reported that the HIV-1 subtype C isolates generally display a slower viral replication *in vitro* compared to the HIV-1 subtype B counterparts [602]. However, this is a comparison between different HIV-1 subtypes, which all are invariably and constantly more replicative and infectious than all HIV-2 strains. Therefore, it is tempting to speculate that the presence of the insertion (QRALTAI) in the HIV-2 Env CT may result in a replicative fitness that comes close to that of HIV-1 subtype C. However, this assumption needs to be confirmed. We can also propose that since the insertion adds two α helix turns and an arginine residue in the LLP-2/3 domain of Env, the interaction with the cell membrane as well as the Env's multimerization can be increased [192, 603]. Finally, it is quite possible that the 7 aa insertions may interact with an unknown cellular partner or may antagonize an unknown restriction factor, thereby promoting the viral replication and/or viral release. The latter point strongly warrants further investigations since, for instance, the impacts of polymorphisms in the CT of the HIV-1 subtype C on the viral replication depend on the cell type (i.e. CD4+ T cells or macrophages) [191, 601]. Overall, our third article provides a gripping issue concerning the importance and the contribution of the retroviral Env cytoplasmic tail in the viral cycle and paves the way to further interesting investigations.

Chapter VI: References

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