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HIV Infection «HIV Tat Protein, a Key Factor in Pathogenesis and Immune System Dysregulation: Implication of IL-10»

Ben Haij N., Planès R., Mzoughi O. and Bahraoui E.
Université Paul Sabatier, Unité mixte INSERM/CNRS/UPS 1043, Toulouse France

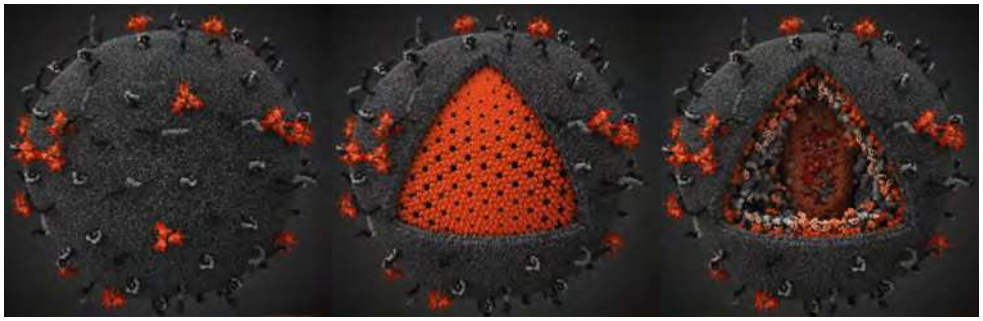
1. Introduction

Human Immunodeficiency Virus (HIV) is the causative agent of AIDS (Acquired Immune Deficiency Syndrome). Currently, the infection by HIV is considered as one of the major problem of public health and social stability. The scale of HIV/AIDS epidemic has exceeded all expectations since its identification 30 years ago. Currently, according to the report on HIV/AIDS by UNAIDS and World Health Organization (WHO) in 2009, we estimated approximately 33 million people are currently living with HIV-1 and about 25 millions have already died (Piot, Feachem et al. 2004). HIV infection leads to a decline in the number of T helper CD4 cells leading to a progressive dysfunction of the immune system. This weakening of the immune system results in the development of opportunistic infections leading to death of the patient. Despite prevention policies and anti-retroviral therapies, AIDS still remains one of the most serious humanitarian crises that we have never known.

1.1 HIV taxonomy, structure and genome organization

Identified in 1983, HIV belongs to lentivirus genus of Retroviridaea (Barre-Sinoussi, Chermann et al. 1983). Lentiviruses are host-specific viruses which cause slowly progressive diseases in their hosts and are characterized by selective T CD4 depletion (Fauci 1988) associated with a severe immunological and neurological disorders. These disorders are amplified by the fact that the virus infects the key cells of the immune system. As consequences, immunological disorders in HIV infected patients are observed since the asymptomatic stage. Two major subtypes of HIV have been identified: HIV-1 and HIV-2 (Clavel, Guetard et al. 1986). The first, HIV-1, is world wide, virulent and responsible of HIV infection globally. However, the second, HIV-2, is less pathogenic and is largely confined to West Africa. These two types of virus share a 40% homology in their sequences. HIV-1 strain is subdivided into three genetically groups: M (Major), N (New or non M non O) and O (Outlier) (Clavel, Guetard et al. 1986). The M strain is the most spread worldwide, it represents more than 95% of the global virus isolates. The variability within the sequence of *env* gene of HIV-1, allowed it to be subdivided into 10 clades A to L (McCutchan 2000). At least 15 additional circulating recombinant forms (CRF) has been identified in HIV-1 patients living in different geographic regions of the world (Peeters and Sharp 2000). However, the presence of these CRF seems to be more present in Africa, Asia and South of America. Despite the high sequence homology between HIV-1 and HIV-2 and the

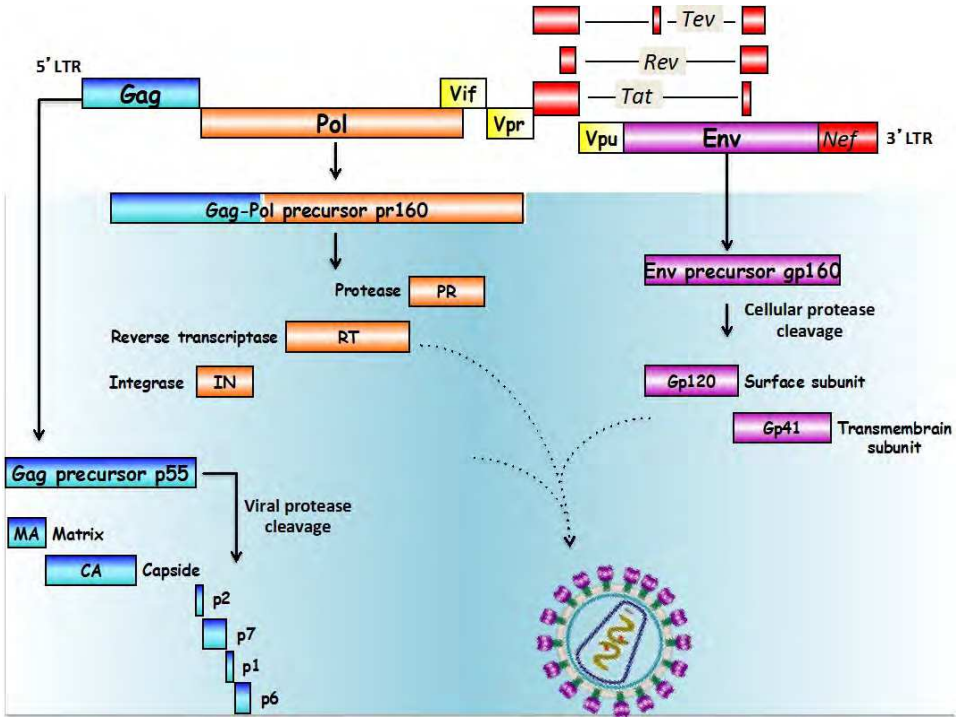
existence of co-infection *in vivo* with the two viruses, no recombination between HIV-1 and -2 have been reported. About the question of the origin of HIV-1 and HIV-2 transmission to human, it is admitted that it comes respectively from a cross-species of SIVcpz (Simian Immunodeficiency Virus which infects chimpanzees) in Central Africa and SIVsm (Simian Immunodeficiency Virus which infects sooty mangabey) in West Africa (Hirsch, Olmsted et al. 1989; Gao, Gorelick et al. 2003).
HIV-1 is an enveloped virus. The viral particles are produced as immature virions by budding from infected cells. After maturation by the viral protease, mature particles adopt spherical forms with a diameter of 90-100 nm (figure 1).



This spatial representation takes into account and summarizes the results from more than 100 latest scientific publications in the fields of virology, X-ray analysis and NMR spectroscopy. The depicted spatial configurations of 17 different viral and cellular proteins found in HIV particle are in strict accordance with known 3D-structures. This model of HIV virion is put on the cover of the special issue of Nature Medicine (September 8, 2010) prepared by the Global HIV Vaccine Enterprise.

Fig. 1. Model reconstitution of spatial structure of HIV-1.

Those mature particles have a cone shaped core composed of the p24 viral protein. It was estimated that each viral particle contains 1500-2000 Gag and 100-200 Pol molecules. The envelope glycoproteins are present as trimers of gp120/gp41 and were estimated to ten trimers per particle. The HIV capsid contains the viral genome composed of two identical single stranded RNA molecules allowing the establishment of frequent genetic changes by recombination. The RNA viral genome is capped at its 5'-end and polyadenylated at its 3'-end. The viral reverse transcriptase, integrase and nucleoproteins were found associated with the viral genome. The HIV genome of approximately 10kb encodes for 16 distinct proteins (Sodroski, Rosen et al. 1985). A schematic representation of HIV genome and proteins is illustrated in figure 2. As in all retroviruses, those proteins are derived from three essential genes *gag, pol* and *env*. The first gene *gag* encodes for Gag precursor p55, which is cleaved by viral protease to give capside, matrice and nucleocapside proteins : p24, p17, p7 and p6. Enzymatic proteins are encoded by *pol* gene and give three viral enzymes : protease p11, transcriptase reverse p65/51 and integrase p32. The *env* gene encodes for the precursor gp160 which is later proteotically cleaved by a cellular protease into the two envelope proteins gp120 and gp41. These steps of maturation by the viral or the cellular proteases are crucial for the production of infectious viral particles. In addition to these structural and enzymatic gene products, HIV genome encodes for two regulatory proteins Tat (transcriptional transactivator) and Rev (regulator of virion gene expression) and also for four accessory proteins : Vif (viral infectivity factor), Vpr (viral protein r), Vpu (viral protein



Gag (group specific antigen) and Gag-Pol (polymerase) are polyprotein precursors that are processed by viral protease. The 9 mature subunits are protease (PR), reverse transcriptase (RT), integrase (IN), matrix (MA), capsid (CA), p2, nucleocapside (NC), p1 and p6. Envelope is also cleaved, however by a host cellular protease into surface (SU) gp120 and transmembrane gp41 moieties. HIV genome encodes also for accessory and regulatory proteins: transcriptional transactivator (Tat), regulator of virion gene expression (Said, Dupuy et al.), viral infectivity factor (Vif), viral proteins u (Vpu), viral proteins r (Vpr) and negative factor (Nef). Tev contains Tat, Env and Rev sequences and functions like Tat and Rev.

Fig. 2. HIV genome organization and proteins.

u) and Nef (negatif factor) (Emerman and Malim 1998). The gene products of *rev* and *tat* are produced early in the viral cycle and their expression is essential for the viral replication. Deletion of one of them leads to an abortive viral cycle. The Tat protein is implicated in the initiation and elongation/stabilisation of transcribed viral mRNA. Tat binds to a specific site called TAR (Tat responsive region) located in the LTR. Tat is the major protein involved in the upregulation of HIV-1 replication. In parallel, the Rev protein interacts with a structure called RRE (Rev responsive element) located in the *env* gene (Emerman and Malim 1998). This interaction permits mono and unspliced mRNA to cross the nuclear membrane in order to enter in the cytoplasm, where, they will be translated to proteins or encapsidated in the nascent viral particles. As Tat and Rev, Nef is also an early produced protein. This protein has at least three distinct activities including down regulation of cell surface CD4 and MHC class I molecules and enhancement of virion infectivity. It has been reported that this protein is essential for proviral DNA synthesis. It is also important for cell-cell transmission of the virus. In fact, the Vif protein seems to be involved in the final stages of the nucleoprotein

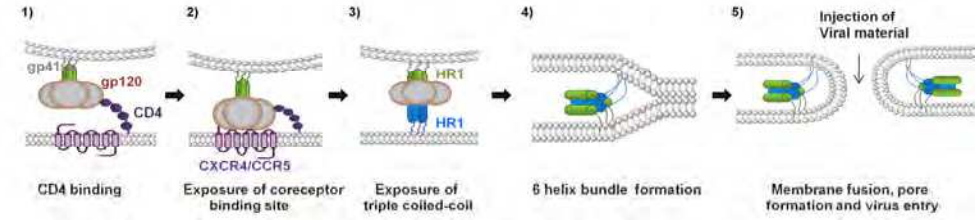
core packaging (Peterlin and Trono 2003). The Vpr protein affects the nuclear localization of viral nucleic acids in non dividing cells and induces cell differentiation (Peterlin and Trono 2003), while the Vpu protein enhances virion release and degrades CD4 in the endoplasmic reticulum (Hoglund, Ohagen et al. 1994). Many studies have put forward that Vpr, Vif, Nef and perhaps Tat are found associated in the viral particles.

1.2 HIV-1 tropism and replication cycle

At least 11 distinct steps can be identified in HIV-1 viral cycle including: viral attachment, entry by fusion and uncoating, reverse transcription of the RNA genome by reverse transcriptase to double stranded DNA (dsDNA), nuclear translocation of the pre-integration complex, integration of the proviral dsDNA, transcription of the proviral genome after cellular activation, splicing and nuclear transport of mono and unspliced RNA, translation of the viral RNA, assembly and packaging of the genomic RNA, budding to give immature viral particles and maturation to give infectious viral particles. Since the infection are mediated through the bodily fluids, HIV infects specially CD4 positive cells and essentially T-CD4 helper cells, monocyte/macrophage and dendritic cells (DC) (Dalgleish, Beverley et al. 1984). The virus enters cells by fusion and endocytosis (Chambers, Pringle et al. 1990; Bernstein, Tucker et al. 1995). Productive infection of target cells is initiated by the binding of the external envelope glycoprotein gp120 to CD4 receptor on the cell surface membrane (Bour, Geleziunas et al. 1995). At the surface of the virus the trimer of gp120 is associated with three molecules of the transmembrane gp41 by non-covalent interactions. CD4-gp120 interaction is mediated by conserved domains of gp120 and the first domain of CD4 receptor. Different reports have shown that gp120 interacts with CD4 with high affinity with dissociation constant of nanomolar level (Fenouillet, Clerget-Raslain et al. 1989). It is interesting to note that the conserved domains of gp120 appear to be few or non-immunogenic in HIV-1 infected patients. Generation antibodies against the conserved domains of gp120 are of great interest. In fact, despite the great variability observed in HIV-1 sequence, all viruses recognize the same receptor CD4. Thus, the production of such antibodies will probably exhibit a large spectrum of HIV neutralization by blocking gp120-CD4 interaction with all HIV-1 subtypes despite their variability. A great number of studies are working to design potential vaccine candidates based on these conserved domains of HIV-1 envelope glycoproteins. Entry of HIV requires also an interaction of gp120 with a coreceptor: CCR5 (R5 tropic virus) and/or CXCR4 (X4 tropic virus) (Maddon, Dalgleish et al. 1986; Kozak, Platt et al. 1997; Platt, Wehrly et al. 1998). At the time of primo-infection, HIV recognizes and infects principally cells that express CCR5 proteins (monocytes/macrophages) (Dragic, Litwin et al. 1996). With time the virus switch from R5 to X4 tropic to infects T cells (Grivel and Margolis 1999; Shankarappa, Margolick et al. 1999). This tropic evolution correlates with late stage of HIV infection : AIDS stage. Thus, interaction of gp120 to CD4 receptor and coreceptors induces HIV entry by allowing viral and host cell membranes fusion. This mechanism continues to be largely investigated at a molecular level, by different groups. We will briefly summarize here the key steps.

Subsequently to gp120-CD4 interaction, conformational changes occurs firstly in the viral gp120 and then, in the transmembrane gp41. Modifications in gp120 structure contribute to the exposure of V3 region, which contains the binding site of the viral chemokine receptors CCR5 or CXCR4. This latter interaction mediates further modifications in the gp41 structure

leading to the exposition of the hydrophobic N-terminal gp41 fusion domain. Then the insertion of this fusion domain into the membrane of the host cell allows its fusion with the viral membrane (Chan, Fass et al. 1997) (figure 3).



The mechanism of HIV-1 entry can be summarized in the following model. After binding of gp120 to receptor and coreceptor (step 1-2), gp41 undergoes conformational changes leading to the exposure of the fusion peptide and the accessibility of trimeric HR1 and the hydrophobic N-terminal domain, which contains the fusogenic region, and the trimeric HR2 domains (step 3). Then, HR1 and HR2 interacts together forming a 6-helix bundle leading to the membrane fusion and virus entry (step 4-5). The triple stranded coiled-coil structure (step 3), which probably explains its low immunogenicity in human patients infected with HIV-1.

Fig. 3. Representation of the different steps from the viral-host interaction and the viral entry after membranes fusion.

The chemokine coreceptors CCR5 and CXCR4 of HIV are preferentially found in lipid rafts (Kozak, Heard et al. 2002). These cholesterol and sphingolipid-enriched microdomains in the plasma membrane mirror the optimal lipid bilayer of the virus and provide likely a better environment for membrane fusion (Chambers, Pringle et al. 1990; Chazal and Gerlier 2003). Several inhibitors including T20 (enfuvirtide) have been developed. T20 is a synthetic peptide of 36 amino acids which blocks HIV-1 entry by interfering with the formation of the six helix bundle formed following the interaction between the trimeric HR1 and HR2 domains of the transmembrane envelope glycoprotein gp41 (Kilby, Hopkins et al. 1998). More recently a selective CCR5 antagonist, named maraviroc or vicriviroc has been developed. By blocking gp120-CCR5 interaction this antagonist present a potent anti-HIV-1 activity (Kromdijk, Huitema et al.; Perry). As T20, maraviroc is also active at nanomolar concentrations. It is postulated that CCR5 antagonists may be of great interest, if we consider that HIV-1 infection is essentially mediated by CCR5 tropic viruses (Kromdijk, Huitema et al.). The use of these type of entry inhibitors has been found to effectively inhibit infection of cells *in vitro* and *in vivo* by R5 strains tropic HIV-1 that have developed resistance to the other major classes of anti-retroviral drugs (anti reverse transcriptase and anti protease inhibitors). It is interesting to note that this is the first anti-HIV drug that targets the host cells rather than the virus directly. However, one potential limitation of such inhibitors is the possible selection and rapid emergence of CXCR4 tropic viruses. In fact, the isolation of HIV-1 from patients, who have never received an anti-retroviral therapy, naïve for HAART, showed the presence, of R5 tropic viruses in 85% of the cases. These results are in agreement with the natural resistance of persons harbouring CCR5delta32 deletion (Dean, Carrington et al. 1996; Huang, Paxton et al. 1996; Liu, Paxton et al. 1996; Samson, Libert et al. 1996). While the early stage of infection was characterized by the predominance of R5 tropic viruses, the emergence of viruses with CXCR4 tropism were associated with the late stage of

infection (Scarlatti, Tresoldi et al. 1997). The molecular basis of this R5 to CXCR4 switch remains to be clarified. CXCR4 strains seem to emerge only in 50% patients who develop AIDS (Connor, Sheridan et al. 1997; Scarlatti, Tresoldi et al. 1997). In addition to these two selective HIV-1 strains, an intermediate strain named dual tropic HIV-1 strain which use indifferently R5 and CXCR4 was also reported in some HIV-1 infected patients.

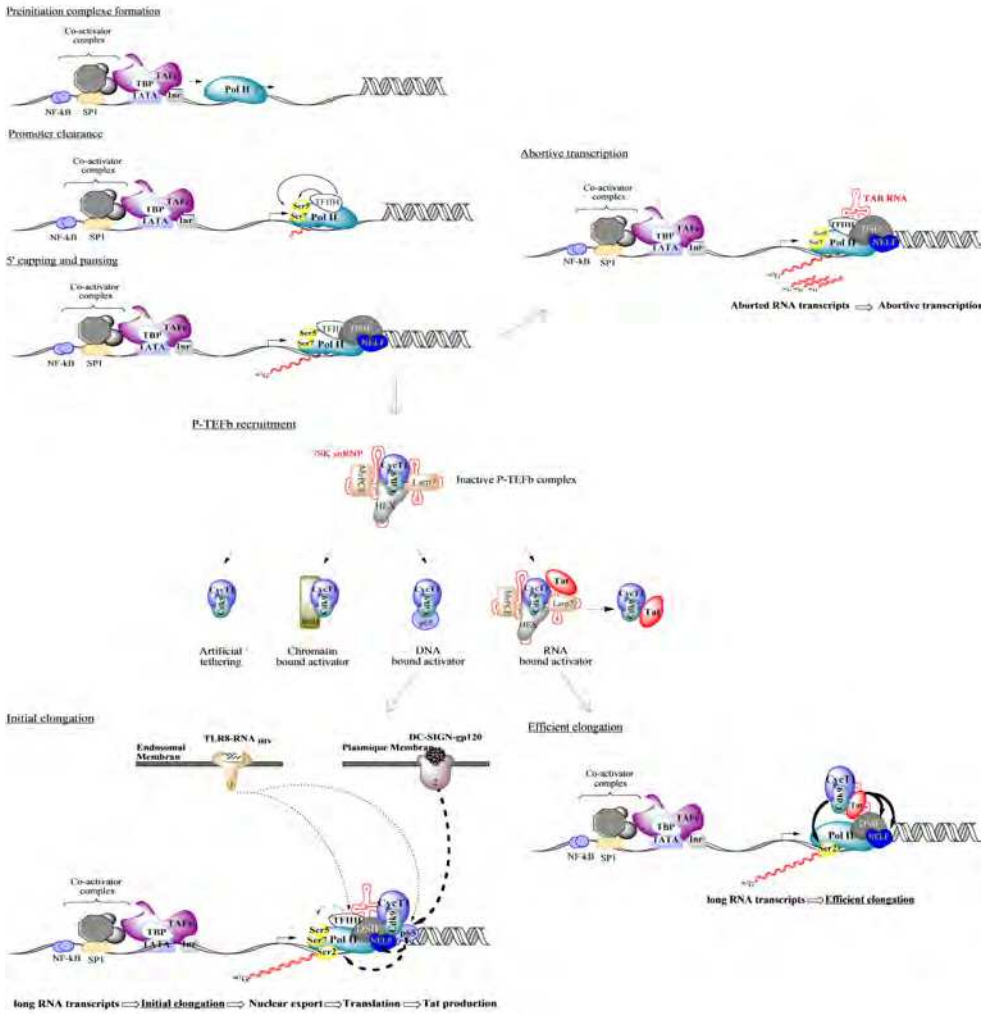
The external envelope gp120 is a highly glycosylated protein. Carbohydrates represent about 50% of its molecular weight (Fenouillet, Clerget-Raslain et al. 1989). The majority of these carbohydrates are of N-type glycosylation. O-glycosylation seems to be rarely present in HIV-1 envelope glycoproteins. Viruses use glycosylation as tool to escape immune responses, resist to protease degradation, and adopt structural native conformation or to bind cellular receptors. By its carbohydrate parts, envelope glycoprotein gp120 recognizes and interacts with high affinity (Kd of nM level) with DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule–3-Grabbing Non integrin) receptor. DC-SIGN is a C-type lectin present on the surface of membranes of dendritic and monocyte/macrophage cells (Geijtenbeek, Kwon et al. 2000; Geijtenbeek, Torensma et al. 2000).

Submucosal DC, which normally process and present antigens to immune cells, bind HIV-1 using C-type lectin essentially at the vaginal mucosa. However this interaction does not trigger the conformational changes necessary for the fusion of the virus and DC membranes. But virus bound on DC, or internalized by endocytosis, can migrate to regional lymph nodes where its target T-helper cells are found. Thus, dendritic cells expressing DC-SIGN appear to act as a "Trojan horse" facilitating the spread of the infection from mucosal surfaces to lymphatic organs (Geijtenbeek, Kwon et al. 2000; Geijtenbeek, Torensma et al. 2000; Kwon, Gregorio et al. 2002).

After entry and uncoating, the viral genome is released into the cytoplasm. This step is concomitant with the start of reverse transcription. The resulted dsDNA migrates into the nucleus where it is integrated by the viral integrase into host genome. The integration site is preferentially located into active genes locus because HIV like many other viruses cannot replicate into quiescent/latent cells. This DNA is flanked by two repeated sequence named LTRs (Long Terminal Repeats). Following cell activation, the viral DNA is transcribed by cellular RNA polymerase II (RNA pol II). Viral transcription is initiated when cellular RNA pol II is recruited at the 5'end of LTR provirus. For efficient transcription elongation a 5'end TAR-hairpin structure named TAR (Tat associated region) element have to be present and bind HIV Tat protein. When Tat protein is absent, the initiation can begin but elongation is abrogated and/or inefficient. After translation, of viral protein and assembly in cholesterol rich lipid rafts, immature virions are released by budding from plasma membrane infected cells.

1.3 HIV-1 genome transactivation: an essential role of HIV-1 Tat protein

Once integrated, HIV genome behaves like the host genes and can be transcribed using the cellular RNA pol II and host cell machineries in addition to the viral Tat protein. The HIV LTR contains proximal core sequence and a distal promoter. The core element is composed by the initiator (Inr) and TATA box (TATA). The distal promoter carries enhancer sequences that contain two NF-κB and three SP1 binding sites that position RNA pol II at the correct site for transcription initiation (Peterlin and Trono 2003; Peterlin and Price 2006). Downstream the core element, HIV LTR contains a short nascent stem bulged loop leader RNA named TAR (Transcription associated Region) (Berkhout, Silverman et al. 1989; Jeang, Xiao et al. 1999). HIV Tat protein is known to be the transcriptional activator that binds to this TAR RNA structure and HIV transcription mediated by Tat is illustrated in figure 4.



The first step of HIV transcription consists of the formation of the pre-initiation complex by RNA pol II recruitment to the promoter. The second step consists to clear the promoter by phosphorylation of the RNA pol II at serine position 5 of the CTD by cyclin dependent kinase : CDK7 of the TFIIH protein. This phosphorylation increases the binding and affinity of human enzyme capping (Brenchley, Price et al. 2006) that leads to the 5'end capping of nascent RNA transcripts. At this step a pausing of RNA pol II occurs by the recruitment of two negatives elongation factors named: DSIF (DRB sensitivity inducing factor) and NELF. Finally, productive elongation occurs by the recruitment of P-TEFb. P-TEFb activity is tightly regulated by its association with a complex composed by 7SK snRNA and heterodimer HEXIM1/2. Tat recruitment of P-TEFb leads to efficient elongation and the obtaining of totally RNA transcripts production.

Fig. 4. Transcription of the HIV genome and implication of its Tat protein.

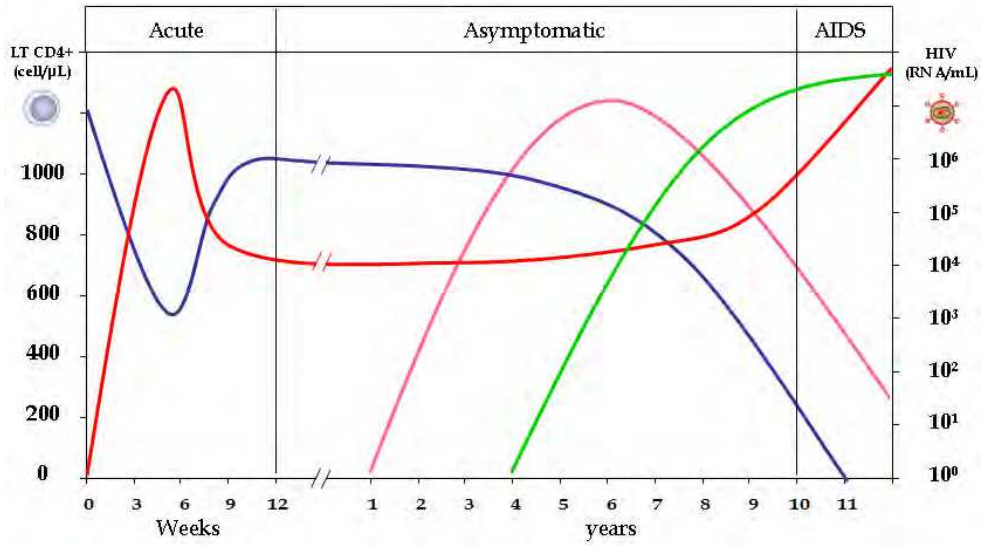
For summary, after PIC formation and clearance of the promoter a pause of RNA pol II occurs. Without recruitment of P-TEFb (positive transcription elongation factor b), only short RNA transcripts are formed and subsequently aborted. In fact, in absence of Tat, transcription is initiated but elongation do not occurs and only short RNA abortive transcripts are produced (Peterlin and Price 2006). At a molecular level, P-TEFb can be recruited by several mechanisms (figure 4) and notably by HIV Tat protein. Indeed, transcription from the HIV-1 LTR is several hundred folds higher in presence of Tat than in its absence. So, the question that follows is how can HIV initiate the synthesis of the first viral proteins, including Tat protein, in the absence of the latter viral factor essential for an efficient elongation ? Or how the first Tat molecules are synthetized ?
Several studies have demonstrated that, in HIV, LTR transcription can be separated in two steps, the first is Tat independent whereas the second is Tat dependent. Recent studies from Greijtenbeek et al. and Gringhuis, van der Vlist et al.(Greijtenbeek et al. 2000; Gringhuis, Van der vlist et al. 2010) have shown that HIV can activate initiation and elongation by two separate mechanisms. The first, transcription initiation, is activated in endosomal structure following the HIV RNA-Toll Like Receptor 8 (TLR8) interaction. More precisely, HIV ssRNA-TLR8 interaction induces activation of NF-κB p65 and RNA pol II phosphorylation at serine 5 residue leading to the recruitment of CDK7 to the transcription units. In parallel, they have also shown that elongation depends upon gp120-DC-SIGN interaction. This membrane interaction activates elongation by phosphorylation of serine 276 and serine 5 of NF-κB p65 and RNA pol II respectively and their recruitments with CDK9 to the HIV LTR. This mechanism can explain how the HIV initiates the production of the first HIV-Tat protein. Then, Tat proteins take over and amplify viral transcription by its capacity to bind the 7SK small nuclear RNA (7SK nRNA) complex and recruits P-TEFb to the paused RNA pol II, by interacting cooperatively with TAR element. This interaction is mediated by Tat arginine rich motif. Once recruited to the transcription units, P-TEFb phosphorylates NELF (negative elongation factor) and the CTD of RNA pol II at serine 2 residue. Tat has also been reported to interact with many factor/co-factor implicated in transcription as: Sp1, TATA box binding protein, CDK2, CBP/p300, TFIIH and RNA pol II. Overall, Tat permits an efficient elongation and co-transcriptional processing by splicing and polyadenylation machineries. When levels of the regulatory Tat protein fall to below threshold levels, HIV becomes latent.

1.4 HIV-1 infection and its effects on immune and the central nervous system

1.4.1 Natural history of HIV-1 infection

The course of HIV infection could be divided into three distinct clinical phases: acute infection, asymptomatic stage or chronic infection and symptomatic stage characterized by AIDS development (Furrer, Opravil et al. 2001) (figure 5).
The first step called acute phase corresponds to the contamination, the primary viral replication and the beginning of immune sensitization. The most common routes of HIV infection are mediated by sexual transmission of the virus, transfusion by contaminated blood products, the use of unsafe needles by drug users, and perinatal transmission from infected mother to infant. This latter case of HIV transmission can occur either in utero or during breast-feeding (Coovadia, Rollins et al. 2007). Cases of contamination during delivery are also reported. Early after exposure, the virus reaches some strategic organs such as the secondary lymphoid organs (mucosa-associated lymphoid tissue and gut-associated lymphoid tissues), which content most of the cells targeted by HIV. At this stage,

the virus replicates actively during a period of 6 to 12 weeks called the acute phase. Viral load reaches several millions copies of viral RNA per millilitre (ml) of plasma. T cells are depleted in blood and the gastro-intestinal tract, but the pool of T cells present in the blood compartment was restored by the central lymphoid organs. In general this step remains asymptomatic, but some patients could develop signs of of routine infection including, fever, lymphadenopathy and rash (Quinn 1997). These symptoms may last in general two to three weeks. At this stage of infection, the innate and adaptive immune systems react strongly to control HIV replication to lower the viral load to a set point often below 20000 RNA copies per ml of plasma (figure 5).



A generalized graph that represent the relationship between T cells number by blood microliter (blue line) and HIV viral RNA copies by blood ml (red line) over the time course of untreated HIV infection. Two major mechanisms involved in T cells dysfunction and progression toward AIDS are represented (arbitrary units) such as increase in pro-inflammatory factors TNF-α, IL-6, IFN-α (pink line), and upregulation of immunosuppressive factors including IL-10, PD-1, PD-L1 and IDO (green line).

Fig. 5. Evolution of T cell count and viral load over the different stages of untreated HIV infection.

The second stage is an asymptomatic phase where the virus persists and the infection becomes chronic. During this period, that varies from 6 months to several decades depending on the patients, the virus replicates continuously mainly in the reservoir cell such as macrophages, the nervous system and regulatory T cells (Treg) (Tran, de Goer de Herve et al. 2008). During this phase, most of the patients maintain a normal CD4 T cell count with apparent normal health. The immune system is still continuously activated against viral replication but HIV will escape to this immune pressure by different mechanisms including the emergence of new variants. One of the most dramatic viral emergence is the appearance of X4 and dual tropic R5/X4 viruses, which replicate more rapidly, produces high amount of viruses progeny and are more cytopathic (Schuitemaker,

Koot et al. 1992; Connor, Sheridan et al. 1997; Singh and Collman 2000). In fact, a loss of $CD4^+$ cells of about 30 to 60 cells per microlitre and per year was observed in HIV-1 infected patients (figure 5). This asymptomatic phase has duration of 6 to 10 years and may be greatly modulated by highly anti-retroviral therapy (HAART). However in less than 1% of HIV-1 infected patients this asymptomatic phase is highly reduced (1 to 2 years), and can last longer, more than 25 years, in patients under HAART (Markowitz, Mohri et al. 2005). In addition to the direct lyses of infected cells this phase is accompanied with several immune disorders leading to immune exhaustion.

One hallmark of this immune disorder is chronic immune activation characterized by increase production of pro-inflammatory cytokines such as TNF-α and IL-6. TNF-α, thus, lead to the activation of HIV replication, loss of CD4 and CD8 through apoptosis (Said, Dupuy et al. 2010) and to neuronal disorders including HIV associated dementia (Nixon and Landay 2010). Increase of IL-6 production has been associated with immunological disorders including B cell lymphoma and hypergammaglobulinemie. It is interesting to note that Tat protein is able to induce TNF-α and IL-6. Production of these two cytokines has also been correlated with a systemic increase of bacterial products such as LPS. Indeed Brencley et al., (Brenchley, Price et al. 2006) have shown that chronic phase of HIV infection is characterized by an increase in circulating bacterial products related to the damage of the intestinal epithelium by the HIV-1 cytopathic effect. Released viral antigens and bacterial products contribute to the constant persistence of a chronic immune activation and T cells death. Other mechanism involved in immune activation is mediated by IFN-α produced by plasmacytoïd DC (pDC), a particular DC population specialized in antiviral immunity. It was reported that HIV induce IFN-α production by pDC through the activation of TLR7 by viral ssRNA. This cytokine acts on CD4+ and CD8+ lymphocytes to induce an activated phenotype (CD69+, CD38+) and will in turn lead to T cell apoptosis (Martinson, Montoya et al. 2010). This great and continual immune activation fails, however, to contain HIV infection. This phenomenon could be explained by the upregulation of several immunosuppressive factors that impaired T cell responses. Among those, it has been reported an a) increase in the production of the immunosuppressive cytokine IL-10, principally by monocytes cells; b) increase expression of programmed death 1 (PD-1) on monocytes (Said, Dupuy et al. 2010), LT CD4+ and LT CD8+, and its ligand PD-L1 on monocytes, macrophages, mDC and pDC. Subsequently, PD-1/PD-L1 interaction stimulates IL-10 production, induces negative signals for TCR activation and leads to T cell death; c) enhancement in the activation of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) in mDC and pDC which acts by depleting the essential amino acid tryptophan and inhibits T cell proliferation and d) increase in the generation and activation of T reg, a subset of T lymphocytes with immunosuppressive activity. All of these immunosuppressive factors will contribute to immune inactivation and have been associated with disease progression to AIDS. As the CD4+ cell count drops below 200 cells/mm3 the risk of developing opportunistic infections including *pneumocystis carini, CMV, JC* or malignancies increases Kaposi's sarcoma (Clifford and Demierre 2005; Bonnet, Balestre et al. 2006). Advanced HIV-disease is characterized by a strong T CD4 depletion to less than 50 cell/mm^3, high viral load and multiple opportunistic infections and malignancies.

AIDS represents the final stage of HIV-1 infection and is characterized by a profound depletion of CD4 positive T cells. In fact, after several years of unresolved chronic infection,

the rate of CD4+ T cells drops dramatically in the blood to a critical rate of 200 T CD4 cells microliter. This phase is characterized by the occurrence of several rare opportunistic infections. The first opportunists that are taking advantage of immunocompromised patients are oral candidiasis, pneumococcal infections, mycobacterial infections and reactivation of herpes simplex and varicella virus. AIDS is also associated with tumor proliferations such as Kaposi's sarcoma, due to Human Herpes Virus 8 (HHV8), lymphoma associated with Epstein-Barr virus and cervical and anal carcinoma associated with human papilloma virus. In fact, this stage results in an extreme immunosuppression with diverse clinical features including opportunistic infections, malignancies and neurological disorders (So, Holtzman et al. 1988; Coopman, Johnson et al. 1993; Simpson and Tagliati 1994) and without treatment will irremediably lead to death of the patient within a few months.

1.4.2 Neuropathogenesis of HIV-1 infection

In addition to its tropism for the cells of the immune system, HIV is also known for its neurotropism. The virus is early found in the CNS carried by infected cells essentially macrophages. The virus can be directly isolated and cultured from the cerebrospinal fluids (CSF) from HIV-1 infected patients. In the CNS the virus replicates strongly in macrophages and microglia and moderately in astrocytes. Although HIV-1 is qualified as a neurotropic virus, it does not infect neurons. Consequently to CNS infection, about 60% of HIV-1 infected patients showed HIV associated neurological, which includes dementia and neuropsychiatric and cognitive motor impairments (Ozdener 2005; Giunta, Obregon et al. 2006). Infiltration of the virus in the brain is concomitant with the development of astrocytosis and microgliosis. While HIV-1 productively replicates in macrophages and microglia, infection of astrocytes allow only, the expression of early proteins Rev, Tat and Nef (Tornatore, Nath et al. 1991; Lipton and Gendelman 1995; Kutsch, Oh et al. 2000). It is interesting to note that astrocytes function also as an reservoir for HIV-1. Neuronal injury observed in the CNS of HIV-1 infected patients results from the activation direct by the virus or indirectly by inflammatory neurotoxins. For example it has been shown that the infection of astrocytes allowed to the impairment of the uptake and secretion of glutamate and to the modulation of glutamate receptor expression (Bezzi, Carmignoto et al. 1998). It was also demonstrated that, by its Tat protein, HIV stimulates the production of various chemokines including MCP-1, IL-8 and IP-10 which participate in the recruitment of macrophages and various type of leukocytes (Conant, Garzino-Demo et al. 1998; Kutsch, Oh et al. 2000). Production by HIV-1 infected macrophages of neurotoxins including quinolate, glutamate and L-cysteine, and inflammatory mediators including, IFN-α, IFN-β, IL-6, IL-1α, IL-1β, TNF-α, and TNF-β play an essential role in HAND. The loss of neurons in HAND by apoptosis and other mechanisms is directly related to these release of toxic mediators by activated and infected cells (Zink, Zheng et al. 1999). Tat, Nef, Vpr, gp120 and gp41 have been described as directly implicated as neuropathogenic factors. By acting with CXCR4, gp120 induces apoptosis of neurons. Also by acting on voltage gated calcium channels and N-methyl-D-aspartate receptor (NMDAR) gp120 induces calcium mobilization which mediates the production of reactive oxygen species (ROS), apoptosis and various neurotoxin mediators (Medina, Ghose et al. 1999). As gp120, HIV-1 Tat protein has been reported to be implicated in neuron injury directly via apoptosis (Sabatier, Clerget-Raslain et al. 1989; Shi, Raina et al. 1998; Nath, Haughey et al. 2000) and indirectly via the induction of inflammatory cytokines including TNF-α and IL-1β.

1.4.3 HIV and Kaposi's sarcoma: Effect of Tat protein

The high incidence of Kaposi's sarcoma (KS) in patients with HIV-1 infection has been related to the co-infection with HHV-8 also named KSHV (Kaposi sarcoma-associated herpes virus) (Schulz, Arbusow et al. 1998; Sirianni, Vincenzi et al. 1998; Aoki and Tosato 2004). It was shown that the presence of KSHV alone is not sufficient for KS development. The presence of HIV-1 seems to be essential for the development of KS lesions. This observation is in agreement with the drastic decrease of KS in patients under HAART. The analysis of the HIV-1 viral factor implicated underlined the essential role of Tat protein which acts as a transactivator factor for KSHV lytic cycle and activates proliferation, MAP-kinase activation, angiogenesis and tumoregenesis. It was shown that Tat promotes the tumorogenesis of endothelial cells both by acting at different ways by: stimulating the production of β-FGF, recruiting VEGF-2 receptor, both on KS and on endothelial cells, whose stimulation is essential for VEGF angiogenic effects (Albini, Benelli et al. 1996; Albini, Soldi et al. 1996; Ganju, Munshi et al. 1998), blocking apoptosis, and stimulating KSHV replication (Pyakurel, Pak et al. 2007). In addition Tat is able to stimulate the production of inflammatory cytokines following its interaction with chemokine receptors.

1.4.4 HIV and immunological disorders

From the asymptomatic stage and before T CD4 lymphocyte depletion, a generalized immune depression is observed in HIV-1 infected patients including disorders of both in the specific/acquired and innate immunities. This immunodepression in HIV-1 infected patients is associated with a profound alteration of the cytokine production (McMichael, Borrow et al. 2010; Clerici and Shearer 1993; Fauci 1996). These alterations have effects on target cells and influence also the virus replication. Increased production of pro-inflammatory cytokines stimulates HIV-1 replication *via* the activation of NF-κB pathway. In fact the LTR promoter of HIV-1 contains NF-κB binding sites. HIV-1 infection is associated, since the asymptomatic stage with a chronic immune activation and dysfunctional cytokine production. The acute host response to primary HIV-1 infection is characterized by Th0 cytokine profile, including the pro-inflammatory cytokines IL-1, IL-2, IL-6, TNF-α, IFN–α/β and IFN-γ as well as the anti-inflammatory cytokines IL-4, IL-10 and IL-13 (Harrich, Garcia et al. 1989; Hatada, Saito et al. 1999). At later stages of infection with disease progression, the pattern of cytokine production shifts toward a strongly based Th2 response mediated by IL-4, IL-6 and IL-10 (Rinaldo, Armstrong et al. 1990; Graziosi, Gantt et al. 1996; Takizawa, Ohashi et al. 1996). This profile was revealed inefficient to eradicate the virus. The mechanism of HIV induced cytokine production has been largely studied. There are many HIV-1 proteins that are capable of inducing the production of several cytokines. These proteins include HIV-1 glycoprotein gp120 that induce the secretion of many pro-inflammatory cytokines including IL-1, IL-6, IL-8, TNF-α, and IFN-γ (Francis and Meltzer 1993; Ameglio, Capobianchi et al. 1994; Ankel, Capobianchi et al. 1994; Capobianchi, Barresi et al. 1997). Interestingly, gp120 is also able to induce the secretion of IL-4 and IL-13 in basophils and IL-10 in mononuclear cells. Nef protein is able to induce the production of IL-1β, IL-6, IL-10, IL-15, TNF-α and IFN-γ in various human leukocyte populations (Schols and De Clercq 1996; Patella, Florio et al. 2000). There is also evidence that Vpr induces the expression of IL-6, IL-8, IL-10, TNF-α and IFN-γ in a variety of cell types (Brigino, Haraguchi et al. 1997).

Another HIV-1 protein, Tat, is known to stimulate the production of many cytokines and chemokines including IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α and MCP-1 (Clerici and Shearer 1993; Moore, O'Garra et al. 1993; Badou, Bennasser et al. 2000; Nath, Haughey et al. 2000;

Bennasser, Badou et al. 2002; Bennasser and Bahraoui 2002; Contreras, Bennasser et al. 2005). Our laboratory has shown that HIV-1 Tat protein induces the production of IL-10, a highly immunosuppressive cytokine by human peripheral blood monocytes. Since IL-10 levels have been shown to progressively increase as the disease progresses toward AIDS, one can hypothesize that this event can play a crucial role in the immune deregulation observed during HIV-1 infection. In line with this hypothesis, Shearer's group in a study including more than 1000 patients identified four patient classes depending on the capacity of their CD4 T lymphocytes to respond to different stimuli (mitogen, alloantigen, influenza virus, and HIV antigens). The progressive loss of the response of the immune system to these stimuli was found to be associated with a course leading to AIDS. Considerable production of IL-10 by peripheral blood mononuclear cells was observed in these patients and paralleled the alteration in CD4 T cell proliferative function. In addition, the immunosuppressive effect of IL-10 also correlated with the restoration of the capacity of isolated mononuclear cells of patients infected by HIV and immunodepressed to proliferate *in vitro* after stimulation by peptide antigens of HIV-1 envelope glycoprotein in the presence of a neutralizing anti-IL-10 antibody (Clerici, Wynn et al. 1994). In another study, Stylianou et al. evaluated the level of IL-10 in the sera of HIV-1 infected patients at different stages of infection. In agreement with Shearer's conclusion, they showed an increase in IL-10 level with disease progression. They also showed a decrease in IL-10 level in patients under highly active retroviral therapy (HAART). Inversely, it was reported an increase of IL-10 levels in patients with failure in tri-therapy treatment. Similarly low level of IL-10 was observed in HIV-1 long term non progressors or elite controllers (Clerici, Wynn et al. 1994). Inversely, it was showed that the persistence of a great level of IL-10 in the sera correlates with the failure in HAART (Stylianou, Aukrust et al. 1999).

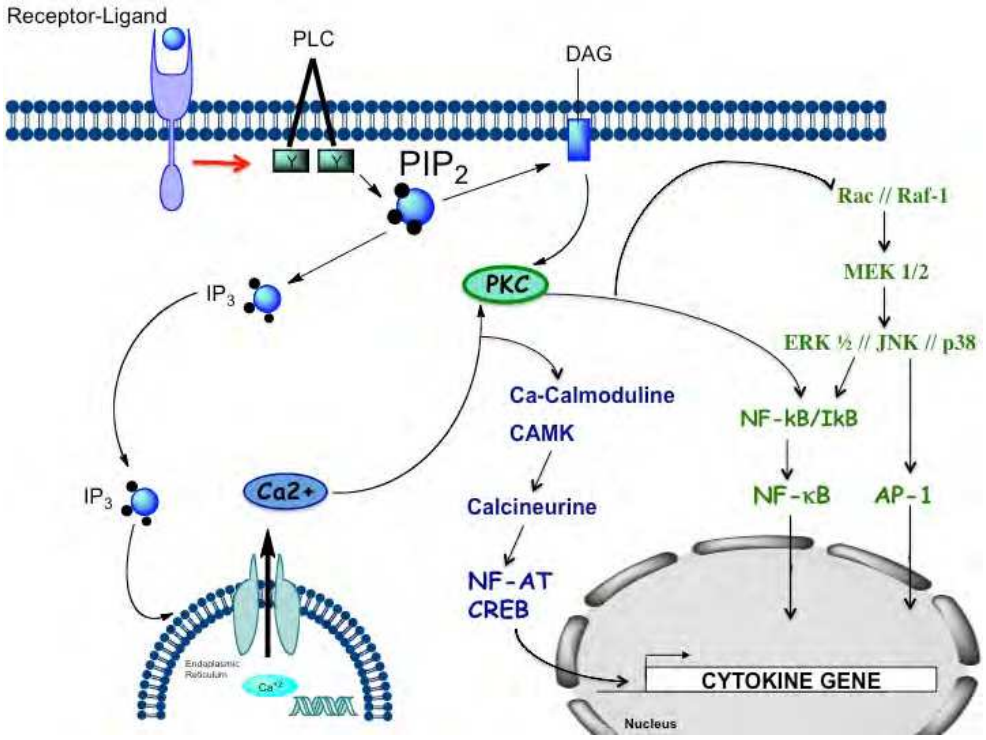
HIV-1 infection affects cellular signaling. The two most important signaling pathways involved in cytokine production include the PKC and calcium pathway (figure 6). These pathways are activated after ligand-receptor interaction. Subsequently, this interaction activates phospholipase C which then cleaves its substrate PIP2 (phosphoinositol biphosphate) in IP3 (inositol 1,4,5-triphospate), responsible of the mobilization of intracellular calcium and to DAG (diacylglycerol), which initiates PKC activation. These two pathways lead to the phosphorylation and activation of cellular proteins mitogen-activated protein (MAP) kinase and of transcriptional factors including NF-AT, NF-κB, CREB and AP-1 responsible for the induction of cytokine genes (Spitaler et Cantrell 2004). In our laboratory we have accumulated several data showing the mechanism recruited by Tat protein to stimulate the production of the highly immunosuppressive cytokine IL-10. In the following part we will describe the role of Tat protein at different levels including its structure, secretion and uptake, mode of action for the transactivation of HIV-1 transcription, activation of signaling pathways and in the conclusion we will discuss Tat as potential target for drug or vaccine development.

2. HIV Tat protein

2.1 Background

HIV Tat protein is one of HIV conserved protein. It is early produced after HIV infection. Tat is a 9-14 kDa protein encoded by two exons separated by a non coding region. The first exon encodes the 72 first amino acids (figure 7). The length of HIV-1 Tat protein ranges from 86 to 101 amino acids and consists of 130 amino acids for HIV-2 (Johri, Mishra et al. 2011).

The 101 amino acids form of Tat is predominant in clinical isolates. Another form that comprises 86 amino acids is also found in some subtype B isolates and in the laboratory strain HXB2. The Tat sequence can be subdivided into six functional domains including N-terminal a proline and cysteine rich region, an hydrophobic core, a basic region followed by a glutamine rich region and a C terminal domain that contain a tripeptide RGD (Arginine, glycine, aspartatic acid) (figure 7). In addition, a domain containing the basic region is also called protein transduction domain (PTD), which renders Tat to have the ability to cross cell membranes and to be used as vector for gene transfer. Thus, viral Tat protein can be either intracellular or extracellular. The main function of HIV Tat protein is to transactivate the HIV long term repeat for retroviral transcription.



External stimulus activates a G protein coupled receptor, which activates a stimulating G protein. This G protein activates phospholipase C (PLC), which cleaves phosphoinositol-4,5-biphosphate (PIP2) into 1,2 diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP3). The IP3 interacts with calcium channel in the endoplasmic reticulum (ER), releasing calcium (Ca2+) into the cytoplasm. Intracytosolic calcium can interact with calmoduline proteins. This, leads to the activation of CAM kinases and calcineurine. Activation of CAMK and calcineurine induce activation and nuclear translocation of CREB and NF-AT respectively. In other hand, the increase in calcium levels activates PKC, which translocates to the membrane, anchoring to DAG and phosphatidylserine. Active catalytic PKC then, phosphorylate targets such as Rac, Raf that lead to downstream activation of MAP kinases and NF-□B and AP-1 transcription factor.

Fig. 6. The Protein Kinase C and calcium pathways.

Tat is encoded by 2 exons in the HIV genome and it is composed by several regions. N-ter region 1-21 is a rich proline region. A region implicated in HIV transactivation is the 21-37 amino acids cysteine rich region and the core region 38-48 implicated in the Tat binding of HIV RNA. The basic rich region 49-59 is involved in Tat internalization whereas the glutamine rich region 60-72 in HIV replication. Finally the last region with the RGD sequence is encoded by the second exon.

Fig. 7. Organization of HIV-1 Tat protein: primary amino acids sequence and different domain of Tat.

2.1.1 HIV-1 Tat structure

Few studies have been devoted to studying secondary and tertiary structure of Tat protein. It seems clear that Tat belongs to the family of intrinsically unstructured proteins (Foucault, Mayol et al. 2010; Bayer, Kraft et al. 1995). Circular dichroïsme studies of Tat (analyze of secondary structure) showed a majority of β-turns structure and the absence of α-helical and β-sheets structures when the spectra were performed in aqueous solutions. However about 20 to 50% of alpha helical structures were detected when the protein was analyzed in organic solvant. Tertiary studies with using NMR confirmed that HIV Tat was generally an unfolded protein (Bayer, Kraft et al. 1995; Shojania and O'Neil 2006). They also demonstrated that the cysteine and basic regions are highly flexible and the N-terminal region is localized between the hydrophobic core and the glutamic rich domains (Watkins, Campbell et al. 2008). The X-ray cristallographic determination of Tat structure is not available. In summury all the available data indicated that Tat contains no ordered α-helical or β-sheet structures. Thus the biological active Tat protein seems to be conformation independent and perhaps only a limited gain of structure is necessary for its function. All these studies suggest that Tat protein does not have a defined secondary or tertiary structures and that biological active Tat protein is natively unfolded and only a limited gain of structure is necessary for its function.

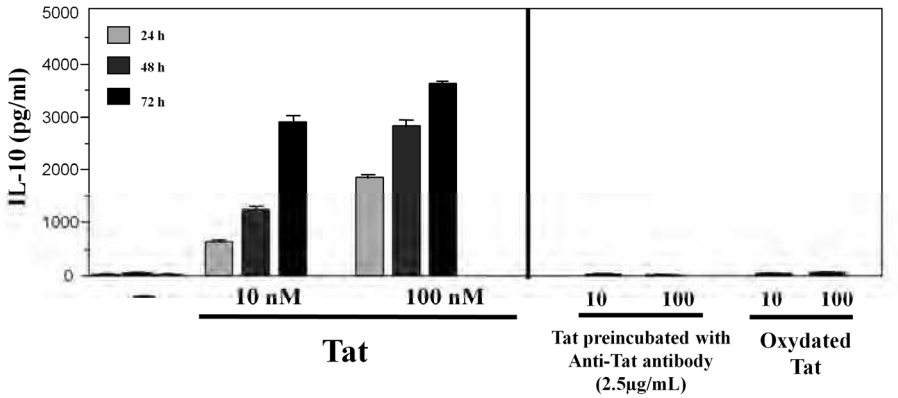
2.1.2 HIV-1 Tat secretion and internalization

Despite the importance of extracellular Tat, few studies have focused on its mechanism of secretion from infected cells and its entry into uninfected cells (Rayne and Debaisieux et al. 2010; Vendeville and Rayne et al. 2004) (Westendorp, Frank et al. 1995). In fact, HIV Tat protein does not have a signal sequence and seems to be secreted by infected cells by an non conventionnal pathway. It is found present in the sera of HIV-1 infected patients at the nanomolar level (Ensoli, Buonaguro et al. 1993; Xiao, Neuveut et al. 2000) a value which may be underestimated since Tat may be trapped by its potential receptors, and particularly the heparan sulfates which are largely expressed on cell surfaces. Thus, the concentration of Tat, *in vivo*, is probably higher in the vicinity of the active HIV replication sites. After its secretion, Tat protein is taken up by neighboring cells infected or not. Despite the previous data reporting a particular mechanism for Tat uptake, it is clear now, that Tat protein enter

viable cells essentially by endocytosis. This entry is mediated by the basic domain of Tat, also named PTD (protein transduction domain) and used for the development of peptidic vectors (Futaki and Suzuki 2001).

2.2 Tat activates the production of IL-10 in human monocytes

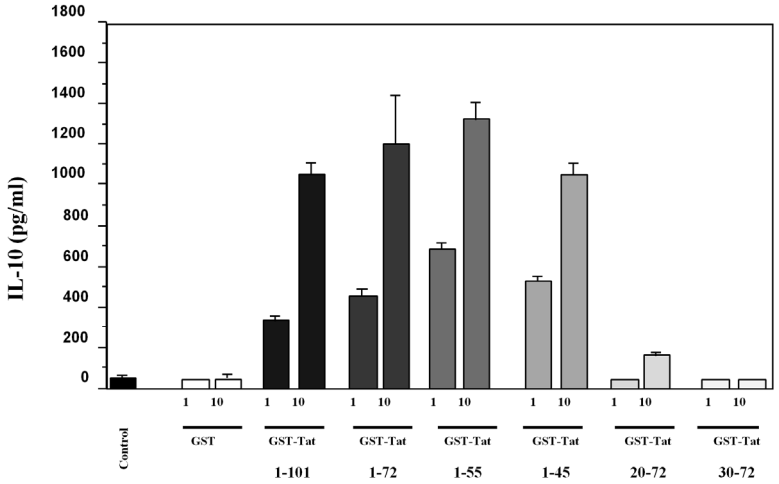
Our group has shown that HIV-1 Tat protein, by acting at cell membrane level, induces the production of IL-10, by non infected human monocytes (Badou and Bennasser 2000; Bennasser and Bahraoui 2002). To this end monocytes from healthy donors were purified and stimulated during 24 hours with increasing concentrations of recombinant Tat protein. Results showed that Tat protein induced strong and dose dependent IL-10 production. In contrast in the absence of Tat no IL-10 production was observed. This Tat induced IL-10 production persists after 48 and 72 hours of Tat stimulation. The amount of IL-10 produced by monocytes in response to Tat is dose and stimulation time dependent. Specifity of Tat effect was demonstrated by showing that chemically modified Tat, after H_2O_2 oxidation, becomes unable to stimulate IL-10 production. The specificity of Tat effect was further demonstrated by showing that when the stimulation of monocytes was performed in the presence of anti-Tat antibodies, IL-10 production by monocyte was totally inhibited (figure 8).



Tat 1-86 at 10 and 100 nM were incubated with or without anti-Tat antibody 2.5 □g/mL or were oxidated by H2O2 treatment. After 1 hour treatment, Tat treated or not, was incubated with human primary monocytes for 24h. IL-10 production was measured by ELISA.

Fig. 8. HIV-1 Tat induces specifically IL-10 production by human monocytes.

To determine the active domain of Tat that is responsible for the activity of Tat, monocytes were stimulated with GST-Tat deleted mutants including GST-Tat 1-72 (deleted form RGD domain), GST-Tat 1-55 (deleted from RGD and glutamate domains), GST-Tat 1-54 (deleted from RGD and basic domains), GST-Tat 20-72 (deleted from the N-Terminal domain), and GST-Tat 30-72 (deleted from de N-Terminal domain). Only GST-Tat deleted mutants but containing the N-terminal domain continues to stimulate IL-10 production. While the deleted mutants GST-Tat 20-72 and GST-Tat 30-72 as GST alone were unable to stimulate IL-10 production (figure 9). These data underlined the importance of the N-Terminal domain in IL-10 Tat induced activity.



Monocytes were treated with 1 and 10 nM of wild type GST-Tat 1-101 or deleted mutant of Tat or GST alone as negative control. After 24 h of stimulation IL-10 production was measured by ELISA.

Fig. 9. The N-terminal domain of Tat : 1-45 is sufficient to induce IL-10 production by human monocytes.

As previously demonstrated in different report Tat protein is able to enter cells and to localize in the nucleus. So Tat protein is able to activate IL-10 production by acting at the cell membrane level or inside the cell. To understand its mechanism of action during the first steps of the activation, Tat protein was immobilized by coating in wells before adding purified human monocytes. In this condition, immobilized Tat leads to a dose dependent production of IL-10 indicating that Tat mediates its action by acting at the cell membrane. These results are in line with the capacity of the Tat deleted mutant, GST-Tat 1-45, to stimulate IL-10 production. In contrast to native Tat protein, the deleted mutant GST-Tat 1-45 is unable to penetrate into cells because it lacks the basic domain, responsible for penetration and nuclear localization of Tat.

The specific receptor implicated in this interaction remains to be characterized, despite the fact that different regions of Tat have been implicated in interaction with various receptors: the N-terminal region with CD26 receptor (Gutheil, Subramanyam et al. 1994), the tripeptide RGD with integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ of DC, and the basic region with membrane lipids and the VEGFR of endothelial cells (Rubartelli, Poggi et al. 1998). Other reports, demonstrated the interaction of the cysteine rich region of Tat 24-51 with CCR2, CCR3 and CCR5 chemokine receptors and the heparan sulfate proteoglycans (Johri, Mishra et al. 2010; Ensoli, Buonaguro et al. 1993). It has been proposed that Tat could interact with the coreceptor CXCR4 and thus competitively inhibit infection by X4-tropic HIV-1 strains (Xiao, Neuveut et al. 2000). In this way, Tat contributes to immune system disorders as it induces apoptosis of T-lymphocytes, inhibits MHC class I expression, alters cell activity by blocking L-type channels (Li, Li et al. 2009).

Using chemical inhibitors and molecular analysis, we showed that Tat-cell interaction lead to activation of different signaling pathways including PKC. In fact, the PKC pathway, one of the major pathway involved in the production of cytokines, plays an essential role in this

induction. Downstream PKC, activation of MAP kinases and NF-κB pathways by Tat is crucial for Tat induced IL-10 (Bennasser and Bahraoui 2002; Leghmari and Bennasser 2008; Leghmari and Bennasser 2008; Contreras 2008).

2.3 Analysis of the activated signaling pathways

Calcium and PKC pathways are activated following the action of the phospholipase C (PLC). Implication of PLC signaling in the production of IL-10 following Tat activation was evaluated by using U73122, an inhibitor of PLC. In the presence of this inhibitor a strong inhibition, more than 50% of the production of IL-10 by Tat was observed. Thus, the activation of PKC signaling pathways known to induce the expression of cytokine genes was further investigated.

2.3.1 Tat activates PKC pathway

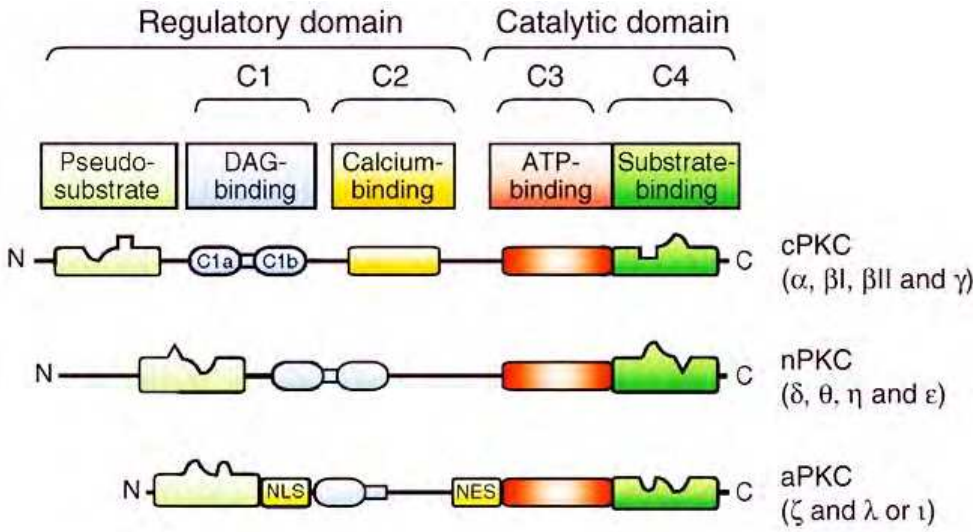
2.3.1.1 The PKC pathway

The PKC belongs to the serine/threonine kinase family. It represents a key element recruited to regulate cell responses to external stimuli. Eleven PKC isoforms have been identified and classified into three groups based on their ability to be activated by Ca^{2+} and DAG (Spitaler and Cantrell 2004). The classical PKC-α,–βI,–βII and -γ, isoforms are activated by Ca^{2+} and DAG, the novel PKC-θ, -η, -δ and -ε, are Ca^{2+} independent but DAG dependent, finally the atypical PKC–ζ, –μ and -λ (also named -α in murine cells) are Ca^{2+} and DAG independent (Lee, Duan et al. 2008) (figure 10). PKC are single polypeptide chains of heterogeneous size, ranging from 67.2 kDa for PKC–τ to 83.5 kDa for, PKC–ε. Each PKC isoform is the product of a separate gene, except for PKC-βI and PKC-βII which are alternative spliced variants of the same gene. At the structural level, PKC contained four conserved domains (C1 to C4) (figure 10) and five variable regions (V1 to V5) that encode isoform-specific properties. The conserved region mediates binding to the activating cofactors: C1 for DAG or to PMA when used as pharmacological tool, and C2 for Ca^{2+}.

2.3.1.2 Effects of HIV-1 Tat on PKC pathway

The implication of PKC pathway in the control of IL-10 production mediated by Tat was analyzed by complementary approaches. Pre-treatment of cells with Ro31-8220, an inhibitor of all PKC isozymes inhibits totally Tat induced IL-10, thus demonstrating the crucial role of PKC pathway. In agreement with the essential role of PKC pathway, Tat becomes unable to stimulate IL-10 production in monocyte previously treated during 48h with PMA. In order to understand the nature of PKC isoforms activated by Tat and essential for IL-10 production, monocytes isolated from healthy donors were cultured in the presence of Tat at 10 or 100nM and cytoplasmic and membrane proteins were isolated after 30 min or after 1 or 2 hours. Whereas PKC is localized in the cytoplasm in unstimulated human monocytes, Tat stimulation induces in a dose dependent manner, PKC translocation to the membrane indicating PKC activation. This activation observed after 30min of Tat stimulation reaches a peak in 1h and decreases after 2 hours of stimulation. Thus HIV-1 Tat induces PKC activation in a dose and time dependent manner in human monocytes. To investigate the involvement of PKC activation in Tat-induced IL-10 production, monocytes were incubated with different PKC inhibitors. Ro318220 as well as Gö6983 which inhibits several PKC isoforms including PKC–α, –β (IC50= 7 nM), PKC-γ (IC50 = 6 nM), PKC-δ (IC50 = 10 nM), PKC-ζ (IC50= 60 nM) and PKC-μ (IC50 = 20 μM) totally inhibit IL-10 production (Bennasser

and Bahraoui 2002; Leghmari and Bennasser 2008; Contreras 2008). Gö6976 which inhibits classical PKC isoforms -α and -βI has no effect on the capacity of Tat to induce IL-10 production by monocytes. Altogether, these results suggest that PKC isoforms -α, -βI and –μ are not primarily involved in the signaling pathways implicated in IL-10 production. To evaluate the implication of PKC-βII and δ two other inhibitors were used. Hispidin, PKC-βI and βII inhibitor (IC50 = 20 μM), and rottlerin, an inhibitor of PKC-δ (IC50 = 3-6 μM). Hispidin used at 2-20 μM inhibits IL-10 production by Tat in a dose dependent manner, an inhibition of 60% is reached in the presence of 20 μM of Hispidin. The implication of PKC-δ in IL-10 production was further evaluated by using rottlerin 5-30 μM. In these conditions an inhibition reaching 90% was observed. Interestingly, PKC-βII and PKC–δ inhibitors used separately at 2 and 5 μM inhibited IL-10 production induced by Tat by 15 and 45% respectively, but when used in combination, they inhibited IL-10 production by 85% thus suggesting a possible synergistic effect between these two PKC isoforms (Bennasser and Bahraoui 2002).

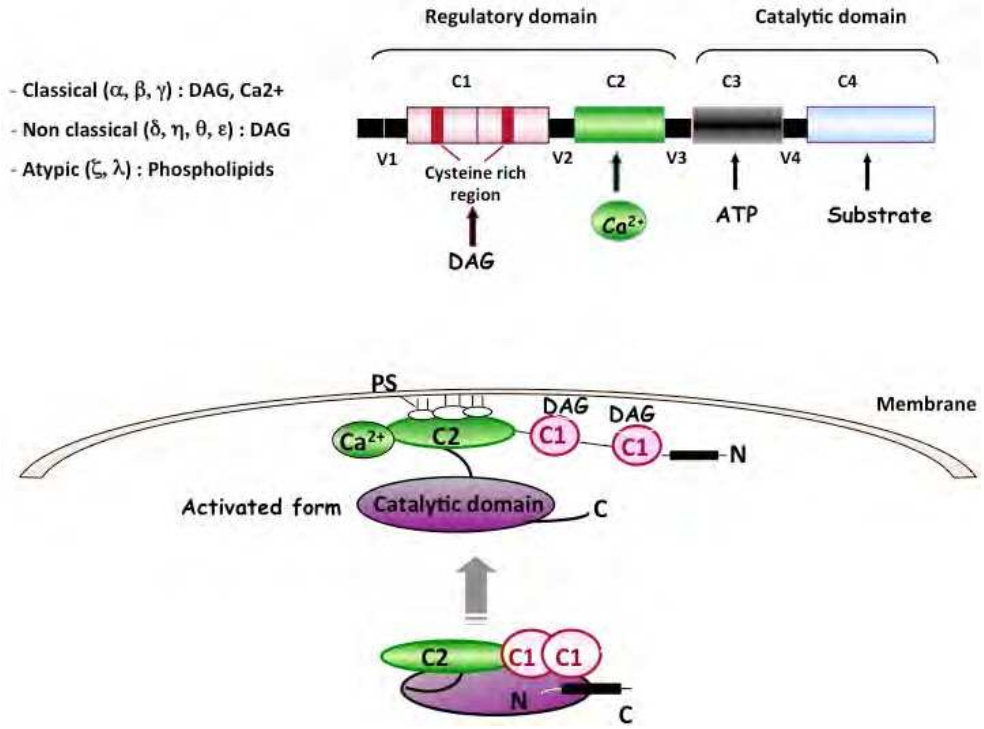


The three subgroups of PKC have been identified and their regulatory domains identified. The classical PKC isoforms (cPKC) share all typical regulatory features: the autoinhibitory pseudosubstrate motif, two DAG-binding C1 domains (C1a and C1b) and the calcium-binding C2 domain. Novel PKC isoforms (nPKC) lack a calcium-binding motif but contain an extended N-ter domain that can receive regulatory signals. They are regulated by DAG. The catalytic activity of atypical PKC isoforms (aPKC) is independent of DAG and calcium, and they seem to be regulated by regulatory proteins in association with the nuclear localization signal (NLS) and nuclear export signal (NES) in their regulatory domain

Fig. 10. Primary structure and identification of the different PKC family and their functional domains adapted from Spitaler et Cantrell 2004.

After ligand-receptor interaction on the cell surface, phospolipids are hydrolyzed by PLC, producing DAG and IP3 both acting as second messengers. Subsequently, DAG activates PKC, which in turn phosphorylates a range of cellular proteins. More precisely activation of PKC is mediated by phospatidylserine (PS) binding domain that includes C2, the basic

pseudosubstrate peptide, and the cysteine rich region that contributes to the increased affinity of PKC to PS by interacting with DAG. These PS binding domains have also been reported to be involved in affinity, PKC localization, membrane translocation and binding to potential receptors. In resting cells, PKC is localized in the cytoplasm in an inactive form where the pseudosubstrate sequence upstream from C1 binds to the catalytic domain, but cannot be phosphorylated since it lacks a phospho-receptor amino acid. Upon activation, Ca^{2+} and or phosphoinositides induce a conformational change leading to the release of the pseudosubstrate. Thus the catalytic site becomes accessible to anchoring proteins called RACK (receptor for activated kinase) or substrates STICK (substrate that interacts with C-kinase). This PKC activation is accompanied by a translocation from the cytoplasm to the membrane. Subsequent to membrane translocation and PKC activation, a second messenger seems to be stimulated and leads to the phosphorylation of PKC binding proteins thus, reducing their affinity for PKC and PS.

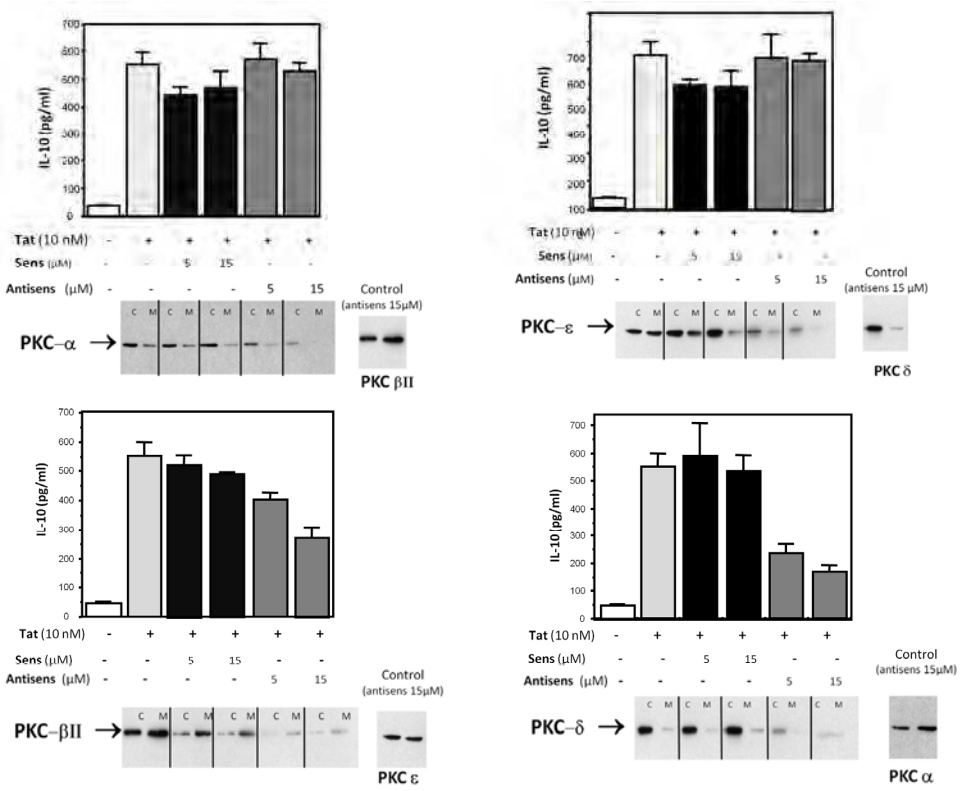


In the cytoplasm, PKC is present as an inactive form. After its activation by phosphorylation, it undergoes conformational modifications leading to its translocation from the cytoplasmic to the membrane compartment. In membrane compartment, PKC associates with a second messenger DAG (and in the case of classical PKC isoforms, calcium) produced after receptor stimulation. Binding to the second messengers is also required for the release of the pseudosubstrate motif from the active site to give an active enzyme.

Fig. 11. Activation of PKC pathway

We next investigated which isoforms of PKC are activated by Tat protein. Monocytes were incubated with Tat at 10 nM for 15, 30 min or 1 hour and PKC localization was analyzed by

western blot using specific antibodies. Results showed that as soon as 15 min Tat induces translocation to the membrane of four PKC isoforms: PKC-α, PKC-βII, PKC-δ, and PKC-ε. To further analyze the implicated PKC isoforms in Tat induced IL-10 production, these PKC isoforms activated by Tat were targeted with isoform specific antisense oligonucleotides. Monocytes were pre-incubated during 24 hours with PKC-α, PKC-βII, PKC-δ, and PKC-ε or with the corresponding sense sequences as negative controls. After 24h monocytes were stimulated during 1h by Tat at 10 nM and then the level of each isoform in the cytoplasmic and membrane compartments were determined by western blot (Bennasser and Bahraoui 2002). Treatment with 5 μM of PKC-α antisense oligonucleotide down regulates PKC-α expression and the use of 15 μM oligonucleotide totally inhibits PKC-α expression. The use of antisense PKC-α inhibits specifically PKC-α, since PKC-βII is still detected in the same monocytes and is activated by Tat at 10 nM (figure 12). The same specificity of inhibition



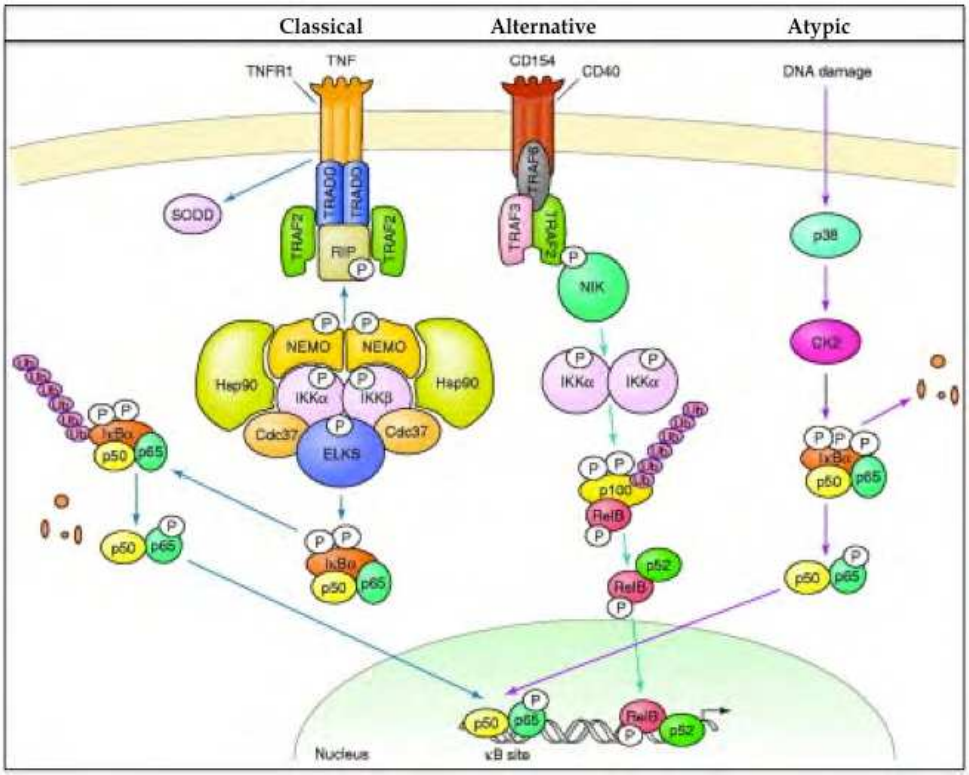
Monocytes were treated with sense or antisense oligonucleotides (5, 15 μM) specific for PKC-α, PKC-βII, PKC-δ, PKC-ε. After washing, cells were stimulated with HIV-1 Tat protein at 10 nM for 1 hour. Isoform specific PKC inhibition was assessed by western blot (bottom). IL-10 production was measured by ELISA 24h later (Sirianni, Vincenzi et al. 1998). In each case, specific inhibition of each isoform was verified by visualizing in the same cell extracts another PKC isoform activation by Tat.

Fig. 12. Effects of isoform specific PKC inhibition with antisense oligonucleotides on Tat induced IL-10 production.

was observed with the other isoforms: PKC-βII, δ, and ε antisense oligonucleotides caused a specific down regulation in the level of their corresponding isoforms (figure 12). In agreement with the data obtained with chemical inhibitors, an inhibition of Tat induced IL-10 was observed in the presence of antisense oligonucleotides directed against PKC-βII and δ. In contrast no significant inhibition was observed with antisense oligonucleotides directed against PKC-α and -ε (figure 12) (Bennasser and Bahraoui 2002).
Overall, it was shown that HIV-1 Tat, activates four PKC isoforms in human monocytes: PKC-α, PKC-βII, PKC-δ, and PKC-ε. These data introduce a way to explore the role of PKC in signaling pathways in Tat activated monocytes and on search for the partners of PKC-βII and δ involved in the production of IL-10, an immunosuppressive cytokine that participates in the deregulation of the immune system as early as the asymptomatic stage of HIV-1 infection.
Monocytes were treated with sense or antisense oligonucleotides (5, 15 μM) specific for PKC-α, PKC-βII, PKC-δ, PKC-ε. After washing, cells were stimulated with HIV-1 Tat protein at 10 nM for 1 hour. Isoform specific PKC inhibition was assessed by western blot (bottom). IL-10 production was measured by ELISA 24h later (Sirianni, Vincenzi et al. 1998). In each case, specific inhibition of each isoform was verified by visualizing in the same cell extracts another PKC isoform activation by Tat.

2.3.2 Tat activate NF-κB pathway

In this part, we will describe the NF-κB signaling pathways implicated in the IL-10 production induced by Tat protein in the human monocytes, and the mechanism of NF-κB regulation exploited by this viral protein.
NF-κB is a major transcriptional regulator for the expression of cytokines that are involved in the control of the immune and inflammatory response (Baeuerle and Baltimore 1996; Baldwin 1996). NF-κB is a dimeric transcription factor that consists of REL family members, including RelA/p65, c-Rel, RelB, p50 and p52 (Li and Verma 2002). p50 and p52 are derived from the larger precursors p105 and p100, respectively, through proteolytic processing by the proteasome. All NF-κB proteins contain a highly conserved REL-homology domain (RHD) that is responsible for DNA binding, dimerization, nuclear translocation and interaction with the inhibitory proteins IκB within the cytoplasm. The IκB proteins, bind to NF-κB and block its nuclear import and, thereby, its transcriptional activity. The p105 and p100 precursors also contain the IκB-like repeats that must be degraded to generate the mature p50 and p52 subunits, respectively. In contrast to the other NF-κB family members, p50 lacks a transactivation domain and therefore, usually forms heterodimers with p65 to bind to NF-κB sites in the nucleus. Homodimers p50:p50 can also be formed but they act as a suppressor of inflammatory cytokine gene expression (Ghosh, May et al. 1998). Three distinct NF-κB activating pathways have emerged (Viatour, Merville et al. 2005) (figure 13).
Most of our knowledge concerns the "canonical" pathway, which mostly targets ubiquitous heterodimers p65:p50 and p50:c-Rel. The critical event in initiating this pathway is activation of an IκB-phosphorylating protein kinase, IKKβ/IKK2, which occurs within the "IKK signalosome", in association with a structurally homologous kinase, IKKα/IKK1, and an adaptor protein, IKKγ/NEMO (Yamaoka, Courtois et al. 1998). IKKβ-mediated phosphorylation of IκBα and leads to its proteasomal degradation and, hence, activation of its associated NF-κB dimers that translocate in the nucleus. This pathway is normally triggered in response to microbial and viral infections or exposure to pro-inflammatory

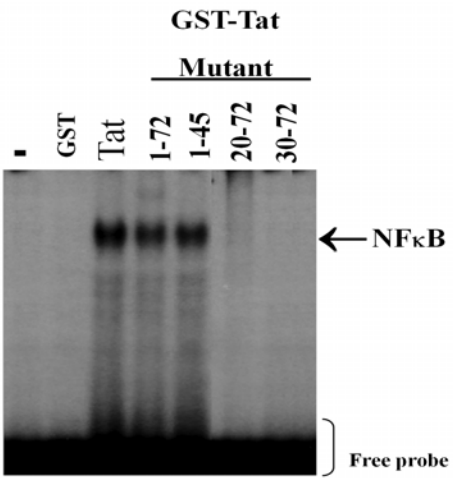


The classical, alternative and atypical NF-κB-activating pathways as illustrated by the TNF-α-mediated, CD40-mediated and DNA-damage-mediated NF-κB activation pathways, respectively. In the classical NF-κB-activating pathway, upon binding of TNF-α to TNFR1, SODD is released from the receptor and triggers the sequential recruitment of the adaptors TRADD, RIP and TRAF2 to the membrane. Then, TRAF2 mediates the recruitment of the IKK complex composed of IKKα, IKKβ and NEMO. Hsp90 and Cdc37 are also part of the IKK complex and are required for- IKK activation. Activation of the IKK complex leads to the phosphorylation of IκBα at specific residues, ubiquitination and its degradation via the proteasome pathway. Then, the heterodimer p50–p65 is released and migrates to the nucleus where it binds to specific κB sites and activates a variety of NF-κB target genes. The alternative pathway is triggered by binding of the CD40 ligand to its receptor, leading to recruitment of TRAF proteins and the sequential activation of NIK and IKKα, which induces the processing of the inhibitory protein p100. p100 proteolysis releases p52 which forms heterodimers with RelB. This pathway is NEMO-independent and relies on IKKα homodimers. The atypical pathway, which is triggered by DNA damage relies on sequential p38 and CK2 activations, and involves phosphorylation and degradation of subsequent IκBα via an IKK-independent pathway. Abbreviations: CK2, casein kinase 2; ELKS, Glu-Leu-Lys-Ser; Hsp90, heat shock protein 90; IκB, inhibitor of NF-κB; IKK, IκB kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; RSK1, ribosomal S6 kinase 1; SODD, silencer of death domains; TNF-α, tumour necrosis factor α; TNFR1, TNF receptor 1; TRADD, TNF-receptor-associated death domain protein; TRAF, TNF-receptor-associated factor; Ub, ubiquitin.

Fig. 13. Three pathways of NF-κB modified by Viatour & al. 2005.

cytokines such as tumour necrosis factor TNF–α. By contrast, the "alternative" pathway, occurs independently of IKKβ or NEMO, but is dependent on NF-κB -inducing kinase and IKKα. Activation of this pathway leads to a limited proteasomal processing of the NF-κB precursor protein, allowing the resulting p52 fragment to translocate to the nucleus in association with some NF-κB proteins (mainly RelB) (Xiao, Harhaj et al. 2001). This pathway is triggered by cytokines such as lymphotoxin B (Dejardin, Droin et al. 2002), or CD40 ligand (Coope, Atkinson et al. 2002), and by viruses such as the Epstein-Barr virus (Eliopoulos, Caamano et al. 2003). The third signaling pathway is classified as "atypical" because it is independent of IKK proteins but it still requires the proteasome and is triggered by DNA damage such as UV oxidative stress (Imbert, Rupec et al. 1996; Tergaonkar, Bottero et al. 2003).
However, recent data suggest another role for IKKα in NF-κB dependent gene expression in response to cytokine treatment (Anest, Hanson et al. 2003; Yamamoto, Verma et al. 2003). Independently of its previously described cytoplasmic role, IKKα functions in the nucleus by activating the expression of NF-κB-responsive genes after TNF-α stimulation. IKKα recruited to NF-κB-responsive promoters, interacts with the histone acetyltransferase CBP/p300 (CREB-binding protein). Then it mediates phosphorylation and subsequent acetylation of specific residues of histone H3 leading to the NF-κB binding sites accessibility.
In inactivated cells, NF-κB is sequestreted in the cytoplasm by the inhibitory protein IκB, which masks its nuclear localization sequence. In order to be active, NF-κB. The involvement of NF-κB was first tested by the capacity of Tat to activate the nuclear translocation of this factor by using the mobility shift technic assay. These experiments were performed with an oligonucleotide containing an NF-κB site and showed the formation of a complex with nuclear extracts of monocytes stimulated with Tat (figure 14). The observed interaction between NF-κB and the probe seems to be specific, since no complex was observed when the protein extract was incubated in the same conditions with the mutated NF-κB site (Badou and Bennasser 2000).
In agreement with the inability of this mutant to stimulate the production of IL-10, no complex was detected in these conditions. These results showed that Tat induces NF-κB activation specifically in monocytes. Then the region of Tat involved in NF-κB activation was investigated by stimulating monocytes with different GST-Tat deleted mutants. EMSA analysis showed that only, the C-terminally deleted mutants GST-Tat 1-72 and GST-Tat 1-45 activate NF-κB as the wild type GST-Tat 1-101. In contrast, no activation was observed with GST-Tat 30-72 or GST alone (figure 14). These results showed that NF-κB activation correlates with the ability of Tat and Tat mutant to mediate IL-10 production. In addition the role of NF-κB activation in the production of IL-10 was evaluated. Monocytes were treated with non toxic doses of TLCK (an inhibitor of NF-κB pathways), and then stimulated with Tat at 10 nM. Then, NF-κB activation and IL-10 production were analyzed by EMSA and ELISA respectively. In this conditions no NF-κB activation nor IL-10 production were obtained. Thus, Tat activates transcriptional factor NF-κB, one of the substrates of PKC, thereby causing induction of IL-10 gene.
Three distinct NF-κB activating pathways have emerged. Interestingly by using the same approache we showed that Tat protein is also able to stimulate the nuclear translocation of p52 in addition of p65 (figure 15). This results suggest that Tat protein activates both classical and alternative NF-κB pathways, while TNF-α activates only the classical pathway.
The critical event in initiating this pathway is activation of an IκB-phosphorylating protein kinase, IKKβ/IKK2, which occurs within the "IKK signalosome", in association with a

structurally homologous kinase, IKKα/IKK1, and an adaptor protein, IKKγ/NEMO (Yamaoka, Courtois et al. 1998). IKKβ-mediated phosphorylation of IκBα leads to its proteasomal degradation and, hence, activation of its associated NF-κB dimers that translocate in the nucleus. This pathway is normally triggered in response to microbial and viral infections or exposure to pro-inflammatory cytokines such as tumour necrosis factor TNF-α. By contrast, the "alternative" pathway, occurs independently of IKKβ or NEMO, but is dependent on NF-κB inducing kinase and IKKα. Activation of this pathway leads to a limited proteasomal processing of the NF-κB 2/p100 precursor protein, allowing the resulting p52 fragment to translocate to the nucleus in association with some NF-κB proteins (mainly RelB).

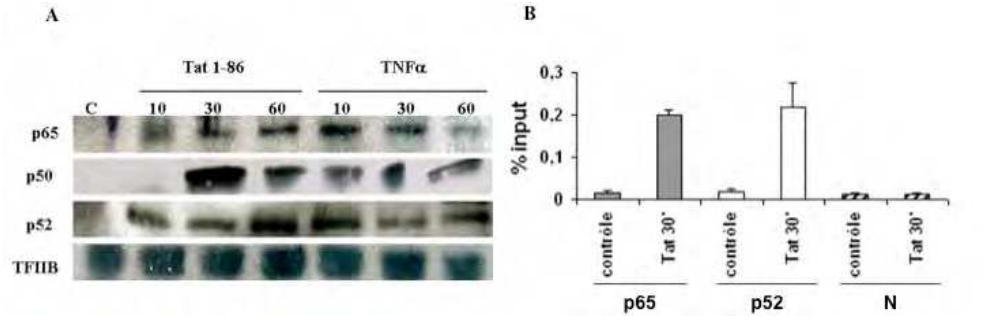


Nuclear protein extracts of human monocytes treated with wild type GST-Tat 1-101 or Tat deleted mutants or negatif control GST at 10 nM for 16 h were incubated with a 32P-labeled NF-κB probe sequence containing the wild type NF-□B site. To verify that NF-κB activation was specifically mediated by Tat, monocytes were treated with chemically mutated Tat (oxidized Tat), and nuclear extracts were analyzed by EMSA.

Fig. 14. Activation of NF-κB by HIV-1 Tat 1-45 in human monocytes.

The analysis of signaling pathways allowed to demonstarte the crucial role of NF-κB transctivation factor as showed by the capacity of Tat to activate the nuclear translocation of the transcription factor NF-κB which is shown to be required for the IL-10 production (Badou and Bennasser 2000). At the light of the known mechanisms of NF-κB activation pathways, the molecular mechanisms recruited by Tat to activate NF-κB was further investigated. To this end, and in order to use transfection approach, we have developed and validated the U937 promonocytic cells as model, in addition to primary human monocytes.
Treated in the same conditions as primary human monocytes, U937 monocytic cells produced IL-10 (Leghmari and Bennasser 2008; Contreras 2008). U937 promonocytic cells previously cotransfected with a NF-κB reporter plasmid, pNF-κBLuc, expressing the luciferase gene under the control of four NF-κB sites and the pCMV-βGal, expressing the β-galactosidase gene under the control of the CMV promoter were treated by different concentrations of Tat.The obtained results showed that Tat protein is able to activate NF-κB

in a dose dependent manner. In agreement with the data obtained in primary human monocytic cells, only, the totally Tat protein or its N-terminal fragment Tat 1-45 are able to activate NF-κB. In contrast no activation was observed with Tat 30-72 or with GST alone (Figure 13, 14).



Tat induces recruitment of p65 and p52 to the IL-10 promoter. (A) Primary monocytes (107 cells) were stimulated or not with Tat 1-86 (10 nM) or TNF-α (20 ng/ml). Nuclear extracts were prepared and analyzed by Western blot. Anti-TFIIB was used as a loading control. (B) Monocytes were treated or not with 10nM Tat 1-86 for 30 min, and ChIP assays were performed with anti-p65, anti-p52 or without (N) antibodies. The proportion of co-immunoprecipitated IL-10 promoter was analyzed by quantitative realtime PCR.

Fig. 15. Tat activates classical and alternative NF-κB pathways.

Using negative transdominant mutants of NIK, IKKα and IKKβ, we showed that their expression inhibit strongly NF-κB activation induced by Tat. Interestingly when both IKKα and IKKβ are inhibited simultaneously by their corresponding negative transdominants, NF-κB activation was totally inhibited. In summury our data indicate that Tat induced NF-κB activation requires NIK, IKKα and IKKβ kinases.

To adress the question whether Tat was also able to induce nuclear translocation of IKKα, nuclear extracts from Tat treated cells was analysed by SDS-PAGE and western blot using antibodies specific to IKKα. This analysis clearly showed that Tat protein is able to stimulate IKKα translocation from the cytoplasm to the nucleus. This activation is also obtained with wild type Tat protein and its N-terminal fragment but not with Tat 30-72. However, recent data suggest another role for IKKα in NF-κB dependent gene expression in response to cytokine treatment. Independently of its previously described cytoplasmic role, IKKα functions in the nucleus by activating the expression of NF-kB-responsive genes after TNF-α stimulation. IKKα recruited to NF-κB-responsive promoters, interacts with the histone acetyltransferase CBP/p300 (CREB-binding protein). Then it mediates phosphorylation and subsequent acetylation of specific residues of histone H3 leading to the NF-κB binding sites accessibility.

In summury, Tat protein secreted by infected cells will be taken up by neighboring cells to activate HIV-1 replication in HIV-1 infected latent cells, and also to activate non infected cells to produce different chemokines and cytokines including IL-10 a highly immunosuppressive cytokine greatly implicated in the dysregulation of the immune system in HIV-1 infected patients. The analysis of the signaling pathway activated by Tat underlined the crucial role of PKC and NF-κB pathways.

3. Conclusion

HIV-1 infection hijacks the cellular machinery and uses cellular signaling pathways to its own advantage. Early secreted Tat protein, as gp120 via its interaction with CD4 receptor and CCR5 coreceptor, trigger a signaling cascade which activates calcium and protein kinase C pathways (Contreras 2008; Sieczkarski S et al. 2003). All these signaling pathways regulate cell activation by acting on the state of cell differentiation, transcription activation, cell survival or cytoskeleton dynamics which are required for viral replication (Lamph et al 1988; Burnette et al 1993; Llewellyn et al 2006).

Among the activated pathways, PKC pathway plays a critical role for HIV-1 replication. Indeed, PKCs stimulate NF-κB (Meichle et al 1990; Junttila et al. 2003; Taunton et al. 2001) via the phosphorylation of IκB. NF-κB binds to the HIV promoter and is involved in initiation and elongation of transcription. In addition, PKCs activate other transcription factors like AP-1 and NF-AT, which have specific binding sites on the HIV-1 promoter. Thus, activation of PKCs can reverse HIV-1 latency in infected T cells and in cell lines (Guy et al. 1987). Moreover, PKCs have been suggested to phosphorylate a number of viral proteins such as p17 Gag (Yang et al. 1999), Nef (Popik et al. 1998; Ghosh et al. 1990; Hamamoto et al. 1990) and Rev (Fantuzzi et al. 2000). The PKC pathway is also critical for the step of entry of a number of enveloped viruses like rhabdoviruses, alphaviruses and herpesviruses (Yang et al 1999) and for type 2 adenovirus (Owen et al. 1996). Thus, the understanding of the signaling pathways implicated in the stimulation of virus replication or in the production of immunopathogenic cytokines may suggest possible targeted therapeutic approaches to neutralize such key steps.

Taking into account the role of Tat protein in viral replication and induction of immunological and neurological disorders, this Tat protein represents a potential vaccine candidate (Caputo, Gavioli et al. 2009). Tat protein appears to be an effective candidate to include for an HIV vaccine, since its gene product is produced early during the viral life cycle. Targeting the immune responses against Tat which plays an important role in viral infectivity and pathogenicity could aid in lowering the viral load through the destruction of HIV-1 replicating cells by CTL or antibody dependent cell-cytotoxicity. It is important to underline the critical role of HIV-1 Tat in the dysregulation of the immune system by the the induction of the higly immunosuprressive cytokine: IL-10 (Badou and Bennasser 2000). Interestingly several reports showed that humoral or cellular responses against Tat are associated with the control of HIV-1 infection as showed by the low viral load and the protection against AIDS progression (Wieland et al 1990; Krone et al. 1988; Reiss 1990; Re 1996; Rezza et al. 2005; Zagury et al. 1998). In agreement with these observations the group of Ensoli showed that vaccination of macaque with native Tat protein allowed protection against viral infection and disease development (Cafaro et al. 1999; Maggiorella et al. 2004). However, experiments performed by other groups showed no protection, or only a partial protection in rhesus macaques immunized with native or denatured Tat protein (Allen et al. 2002; Silvera et al. 2002; Goldstein et al. 2000; Pauza et al. 2000). This apparent discrepancy may be related to the nature of animal used cynomolgus versus rhesus and the nature of the virus used for the challenge, SHIV89.6P, SIVmac239 or SHIV33.

It is interesting to note that a therapeutic phase I clinical trial in human, using recombinant Tat protein as vaccine candidate is under investigation by the group of Ensoli.

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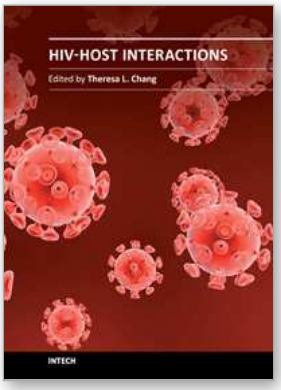

HIV-Host Interactions

Edited by Dr. Theresa Li-Yun Chang

ISBN 978-953-307-442-9
Hard cover, 364 pages
Publisher InTech
Published online 02, November, 2011
Published in print edition November, 2011

HIV remains the major global health threat, and neither vaccine nor cure is available. Increasing our knowledge on HIV infection will help overcome the challenge of HIV/AIDS. This book covers several aspects of HIV-host interactions in vitro and in vivo. The first section covers the interaction between cellular components and HIV proteins, Integrase, Tat, and Nef. It also discusses the clinical relevance of HIV superinfection. The next two chapters focus on the role of innate immunity including dendritic cells and defensins in HIV infection followed by the section on the impact of host factors on HIV pathogenesis. The section of co-infection includes the impact of Human herpesvirus 6 and Trichomonas vaginalis on HIV infection. The final section focuses on generation of HIV molecular clones that can be used in macaques and the potential use of cotton rats for HIV studies.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821