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FUNCTIONAL INTERACTIONS OF THE TAX
AND p13 PROTEINS OF HUMAN T-CELL
LEUKEMIA VIRUS TYPE I

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ABSTRACT

Human T-cell leukemia virus type 1 (HTLV-1) establishes a lifelong persistent infection in humans. Approximately 3% of the infected individuals will develop adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells. The viral protein Tax plays a major role in HTLV-1 pathogenicity by activating the NF- κ B pathway. Tax activates both the canonical and non-canonical NF- κ B pathways, promoting NF- κ B translocation to the nucleus and transcription of genes that favour T-cell proliferation and survival. Our previous studies showed that the p13 protein of HTLV-1 enhances mitochondrial ROS production, resulting in activation of normal T-cells. ROS constitute a homeostatic rheostat that controls the activity of several key pathways, including the NF- κ B pathway. Thus, we hypothesized that the effects of p13 on ROS production could affect the activation of the NF- κ B pathway by Tax in primary T-cells.

The work described in the present thesis was aimed at testing the hypothesis that Tax and p13 might act in concert to activate the NF- κ B signal transduction pathway in primary T-cells. To this end, we optimized a transfection protocol for primary T-cells using an innovative approach based on the electroporation of *in vitro*-transcribed RNA. Activation of the NF- κ B pathway was then analysed by measuring expression of the NF- κ B target genes CD25 and 4-1BB.

Results showed that the co-transfection of Tax and p13 resulted in a synergistic activation of the NF- κ B pathway in primary T-cells measured as an increase in the expression levels of both CD25 and 4-1BB. In addition to being a transcriptional target of NF- κ B, CD25 is also an early marker of T-cell activation. To further test the effects of Tax and p13 on cell activation, we measured CD38 expression by flow cytometry. Jurkat T-cells, which exhibit a constitutively activated CD38 positive phenotype, were used as a control. Results of this analysis confirmed the synergy of Tax and p13, although the effect was not so prominent as that observed for the CD25 marker, suggesting that, within the time frame of our experiments, Tax and p13 drove T-cells to an early-intermediate stage of activation.

Taken together, these findings suggest that, in contrast to the well-established role of Tax as an activator of the NF- κ B pathway in tumor cell lines, in the context of normal T-cells, the induction of NF- κ B target genes requires the concerted action of Tax and p13.

Current studies are aimed at verifying the ROS-dependence of this effect and testing the functional interaction of Tax and p13 in the context of the complete HTLV-1 genome using wild type HTLV-1 and a p13-knock-out HTLV-1 molecular clone. These experiments will be carried out in primary T-cells as well as in dendritic cells, which have recently emerged as an important target of the virus *in vivo*.

RIASSUNTO

Il virus T-linfotropico umano di tipo 1 (HTLV-1) stabilisce un'infezione persistente negli uomini. Circa il 3% degli individui infettati sviluppa la leucemia/linfoma a cellule T dell'adulto (ATLL), un'aggressiva neoplasia a carico dei linfociti T CD4+ maturi. L'attivazione della via di segnale di NF- κ B mediata dalla proteina virale Tax è un evento cruciale nella patogenesi dell'infezione da HTLV-1. Tax attiva entrambe le vie di segnale di NF- κ B, canonica e non-canonica, promuovendo la traslocazione nucleare di NF- κ B e la trascrizione di geni che favoriscono la proliferazione e la sopravvivenza delle cellule T. I nostri studi precedenti hanno rivelato che la proteina virale p13 favorisce la produzione di specie reattive dell'ossigeno (ROS) a livello mitocondriale, causando l'attivazione di cellule T normali. I ROS possono essere paragonati ad un reostato che controlla l'attività di diverse vie di trasduzione del segnale, inclusa la via di NF- κ B.

Lo scopo primario di questa tesi è stato quindi di verificare l'ipotesi che Tax e p13 potessero attivare sinergicamente la via di trasduzione del segnale di NF- κ B in cellule T normali. A tal fine, è stato ottimizzato un protocollo di trasfezione di cellule T primarie utilizzando un approccio innovativo basato sull'elettroporazione di RNA trascritto *in vitro*. L'attivazione della via di NF- κ B è stata analizzata misurando l'espressione dei geni target di NF- κ B CD25, mediante analisi citofluorimetrica, e 4-1BB, mediante RT-PCR quantitativa.

I risultati ottenuti hanno mostrato che in cellule T normali, la co-trasfezione di Tax e p13 causa l'attivazione sinergica della via di NF- κ B misurata come incremento dei livelli di espressione di entrambi i geni target. Oltre ad essere un target trascrizionale di NF- κ B, il CD25 è anche un marcatore precoce di attivazione di cellule T. Per verificare il possibile effetto di Tax e p13 sull'attivazione cellulare, abbiamo misurato mediante analisi citofluorimetrica l'espressione del CD38, un marcatore intermedio-tardivo di attivazione. La linea T-cellulare leucemica Jurkat, caratterizzata da un fenotipo costitutivamente CD38 positivo, è stata utilizzata come controllo. I risultati di questa analisi hanno confermato la sinergia di Tax e p13, nonostante l'effetto sull'espressione del CD38 non fosse così prominente come quello osservato per il

CD25, suggerendo che, nel nostro contesto sperimentale, Tax e p13 spingano le cellule T in uno stadio precoce-intermedio di attivazione.

In complesso questi risultati suggeriscono che, in contrasto con il ruolo ben stabilito di Tax nell'attivazione della via di NF- κ B in linee cellulari tumorali, nel contesto delle cellule T normali, l'induzione dei geni target di NF- κ B necessita l'azione sinergica di Tax e p13.

Gli studi attualmente in corso sono volti a verificare la ROS-dipendenza dell'effetto sinergico di Tax e p13 sulla via di segnale di NF- κ B. Inoltre, verificheremo la validità dell'interazione funzionale di Tax e p13 nel contesto del intero genoma di HTLV-1. A tal fine, paragoneremo l'attivazione della via di NF- κ B indotta da un clone molecolare di HTLV-1 *wild type*, con quella indotta da un clone molecolare di HTLV-1 p13-*knock-out*. Questi esperimenti verranno condotti in cellule T primarie ed in cellule dendritiche, che rappresentano il principale target infezione da HTLV-1 *in vivo*.

1. INTRODUCTION

1.1 Human T-cell leukemia virus type 1: taxonomy, epidemiology and pathogenesis.

Human T-cell leukemia virus type 1 (HTLV-1) was the first human retrovirus to be identified and is the only one with proven oncogenic properties (Poesz et al., 1980; Hinuma et al., 1981).

HTLV-1 belongs to the Retroviridae family, Oncovirinae sub-family, Deltaretrovirus genus, which also includes HTLV-2, -3, -4, simian T-Lymphotropic virus (STLV-1-5), and bovine leukemia virus (BLV). STLV and BLV infections are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, and -4 has not been clearly established (Araujo and Hall, 2004; Feuer and Green, 2005; Mahieux and Gessain, 2009). Deltaretroviruses are referred to as “complex” retroviruses. In fact, they present at the 3’ end of the genome, the so called “pX region” that encodes the regulatory proteins Tax and Rex and several accessory proteins. The oncogenic properties of Deltaretroviruses do not involve either the expression of a cell-derived viral oncogene (v-onc), or the activation of a cellular proto-oncogene (c-onc) through proviral integration, as in the case of acute and chronic transforming retroviruses, respectively. Instead, the oncogenicity of HTLV-1 is attributed mainly to the regulatory protein Tax (Paragraph 1.4.1).

HTLV-1 infects about 20 million people worldwide and is endemic in South-Western Japan, Central Africa, Iran, the Caribbean Basin, Central and South America and the Melanesian Islands. Sporadic infections occur in Europe and North America. Transmission of the virus may occur in a “vertical” manner from mother to newborn, mainly through breastfeeding and in few cases perinatally, or “horizontally” through exchange of biological fluids (sexual contact and parenteral transmission) (Proietti et al., 2005).

HTLV-1 is the causative agent of two distinct pathologies: adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4+ T-cells that is extremely refractory to current therapies (Uchiyama et al., 1977; Tsukasaki et al., 2009), and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM),

a demyelinating neurodegenerative disease (Gessain et al., 1985; Osame et al., 1986). The development of ATLL has been linked to the transmission through breast milk of infected mothers, whereas TSP/HAM is prevalent when the infection is transmitted to adults as in the case of transfusion of infected blood products (Uchiyama, 1997). HTLV-1 infection is also associated with a number of chronic inflammatory diseases such as uveitis (Mochizuki et al., 1992; Pinheiro, 1995), arthritis (Murphy et al., 2004b; Yakova et al., 2005) and infective dermatitis (LaGrenade et al., 1990). The majority of HTLV-1-infected individuals remain asymptomatic throughout life; approximately 3% develop ATLL or TSP/HAM after a latency period of decades or several years, respectively (Kawano et al., 1985). In spite of over 30 years of study, the molecular mechanisms leading to ATLL or TSP/HAM development have not been fully clarified.

HTLV-1 infection of peripheral blood mononuclear cells (PBMCs) leads to cell immortalization. After several months of culture, it is possible to detect mono- or oligoclonal provirus integration. This profile results from a selection process of one or few major clones that carry several genetic alterations and acquire the capability to grow in an interleukin-2 (IL-2)-independent manner. These cells usually show a CD3⁺ CD4⁺ IL-2R⁺ (IL-2 receptor, CD25), or, rarely, a CD3⁺ CD8⁺ IL-2R⁺ phenotype (Lairmore and Franchini, 2007).

The dynamics of infection and immortalization observed *in vitro* recapitulate at least part of the natural history of ATLL *in vivo*. ATLL is classified into four clinical forms: acute, chronic, smouldering and lymphoma (Tsukasaki et al., 2009). In some cases the acute phase of ATLL is preceded by peripheral lymphocytosis with poly- or oligoclonal integration of the viral genome. Although acute ATLL was characterized by the presence of a dominant proliferating leukemic clone with monoclonal integration of the provirus (Lairmore and Franchini, 2007), a recent study based on massive sequencing of integration sites in ATLL patients and asymptomatic carriers revealed an oligoclonal pattern of integration of the virus, with a large number of unique insertion sites (Gillet et al., 2011). This oligoclonal pattern differs between ATLL patients and asymptomatic carriers, with ATLL patients characterized by proviral integration near activating epigenetic region, that expand more frequently

than in asymptomatic carriers, suggesting a negative selection that shapes the distribution of abundance of HTLV-1 clones *in vivo*. This negative selection is probably due to the cytotoxic T-cell (CTL) response to HTLV-1, and is stronger in asymptomatic carriers than in patients with ATLL (Gillet et al., 2011). ATLLs cells possess multi-lobulated nuclei and are called “flower cells”; they are usually CD3+ CD4+ CD8- CD25+ and frequently accumulate in peripheral blood as well as in lymphoid organs and skin. ATLL cells express very little, if any, viral protein and frequently carry defective proviral copies integrated in the host genome. The propagation and persistence of the infected cells in the host relies mainly on “mitotic transmission” of the integrated viral genome to daughter cells, rather than on *de novo* infection of new host cells (Overbaugh and Bangham, 2001).

HTLV-1 infection is accompanied by a high frequency of Forkhead Box P3 positive (FoxP3+) T-cells (Chen et al., 2006; Kohno et al., 2005). FoxP3 is a marker of regulatory T cells (Treg), which play a critical role in suppressing the immune response. The frequency of FoxP3+ cells in the peripheral blood is especially high in patients with chronic ATLL. However, in ATLL, the FoxP3+ leukemic T-cell clones are distinct from the functional Treg population (Abe et al., 2008; Toulza et al., 2009). The increased frequency of FoxP3+ cells in ATLL results from the HTLV-1-mediated expression of the chemokine CCL22, which binds the CCR4 receptor on Treg cells, favouring their migration and survival. The FoxP3+ T cells suppress the growth of autologous ATLL clones, retarding the progression of ATLL; on the other hand, they suppress the host's CTL response, which normally limits HTLV-1 replication and reduces the risk of HTLV-1-associated diseases (Toulza et al., 2010; Bangham and Toulza, 2011).

One common feature of ATLL is hypercalcemia, which is due to the Tax-mediated transcriptional activation of a parathormone-like peptide. The prognosis of acute ATLL is extremely poor with an overall survival of a few months.

TSP/HAM is characterized by a slowly progressive spastic paraparesis, associated with bladder dysfunction and sensory disorders (Rodgers, 1965). Parenchymal and perivascular infiltration of mononuclear cells occurs in the white and gray matter of the spinal cord, resulting in demyelization and fibrosis (Iwasaki, 1990). The presence

of infiltrating T-cells in the spinal cord lesions and of Tax-specific CTL in the cerebrospinal fluid and in the peripheral blood suggests that TSP/HAM might have an autoimmune basis (Osame, 2002; Johnson-Nauroth et al., 2006; Verdonck et al., 2007). This hypothesis is consistent with the association between the human leukocyte antigen (HLA) haplotype and the risk of developing TSP/HAM (Jeffery et al., 1999; Barmak et al., 2003).

1.2 Infection and virus propagation

The HTLV-1 virion consists of a core that contains the viral-encoded enzymes (reverse transcriptase, integrase and protease) and the single-stranded diploid RNA genome surrounded by capsid and matrix proteins. A lipoproteic envelope, composed of a plasma membrane-derived lipid bilayer and the gp21 and gp46 envelope glycoproteins, surrounds the viral core (Figure 1) (Manel et al., 2005; Lairmore and Franchini, 2007). HTLV-1 presents a broad cell tropism *in vitro* (monocytes, microglial cells, epithelial cells, B- and T- lymphocytes), but it is mainly detected in CD4+ T-lymphocytes and dendritic cells of infected individuals (Manel et al., 2005; Macatonia et al., 1992). So far, three cellular receptors of HTLV-1 have been identified: the glucose transporter GLUT-1, heparan sulphate proteoglycan (HSPG), and neuropilin-1. Current models suggest that the virus may first contact HSPG on the cell surface, and then form complexes with neuropilin-1 through the viral envelope protein (Jones et al., 2005; Lambert et al., 2009). Afterwards, the interaction between the viral envelope protein gp46 and GLUT-1 would favour membrane fusion and entry into the cell (Manel et al., 2003). Consequently, glucose consumption in the infected target cell is inhibited and extracellular milieu acidification is reduced, possibly causing metabolic alterations in the infected cells (Manel et al., 2003; Manel et al., 2005). The gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membrane fusion with the formation of the virological synapse. The virological synapse is an organized contact area whose assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and genome at the cell junction. The virion components are then transferred to the uninfected cell as enveloped particles (Ikagura et al., 2003;

Majorovits et al., 2008). Tax is also involved in the formation of the virological synapse: it localizes in the contact region between infected and target cells (Nejmeddine et al., 2005) and enhances the microtubule-organizing centre (MTOC) formation by stimulating the CREB pathway (Nejmeddine et al., 2005; Nejmeddine et al., 2009). The MTOC polarization is induced by the engagement of intracellular adhesion molecule-1 (ICAM-1) by its cognate receptor lymphocyte function associated antigen-1 (LFA-1) (Barnard et al., 2005), and activation of the Ras-MEK-ERK pathway (Nejmeddine et al., 2009).

In addition to the formation of the virological synapse, two other mechanisms have been proposed to be involved in the cell-to-cell transmission of HTLV-1. The first involves the storage of viral particles from HTLV-1 infected cells in extracellular biofilm-like structures, composed of collagen, agrin, and linker-proteins. When infected cells attach to uninfected cells, those structures are rapidly transferred to the surface of the target cells, favouring infection (Pais-Correia et al., 2010). Another mechanism of virus spread involves the activity of the viral accessory protein p8. p8 enhances T-cell contact by interacting with LFA-1 and ICAM-1 and mediates formation of intracellular conduits among T-cells, through which virions may be transmitted (Van Prooyen et al., 2010). All these mechanisms are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects and cell-free blood products are not infectious (Fan et al., 1992; Derse et al., 2001).

A notable exception to the cell-to-cell mode of viral transmission is represented by dendritic cells, which can be infected by cell-free HTLV-1 virions (Jones et al., 2008). HTLV-1-positive dendritic cells have been detected in the peripheral blood of infected individuals (Macatonia et al., 1992). Moreover, HTLV-1-exposed dendritic cells are an efficient viral vector for autologous primary CD4⁺ T-cells. The most important antigen receptor mediating HTLV-1 binding, transmission, and cell-free infection in dendritic cells is the DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) (Jain et al., 2009). Heparan sulfate proteoglycans and neuropilin-1 also contribute to transmission of the virus to dendritic cells. DC-mediated transmission of HTLV-1 results in long-term productive

infection and interleukin-2-independent transformation of the CD4+ T-cells (Jones et al., 2008).

After virus entry into the target cell, the viral genome is reverse-transcribed by the viral reverse transcriptase (RT), producing an RNA-DNA hybrid. The ribonuclease H (RNase H) component of viral RT degrades the RNA strand, while the DNA strand is used as a template by RT, which also has DNA-polymerase-DNA-dependent activity, to synthesize a complementary DNA strand. The double-stranded DNA circularizes and transfers to the nucleus, where it integrates randomly in the host genome. Integration is mediated by the viral enzyme integrase and by the long terminal repeats (LTRs) located at both ends of the viral genome. Viral genes are then transcribed and translated by the cellular machinery. Virion assembly occurs through the interactions between the nucleocapsid and the genomic RNA, and the matrix and the host plasma membrane. Viral particles incorporate two copies of the single stranded RNA genome along with tRNA, RT, protease and integrase.

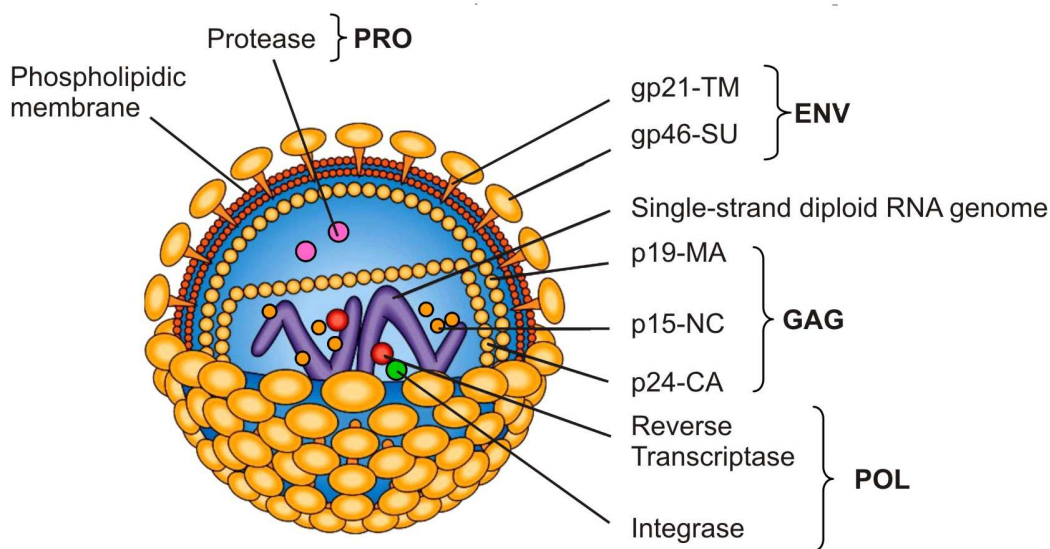


Figure 1. Schematic representation of the HTLV-1 virion (Adapted from Le Blanc et al., 2001).

1.3 HTLV-I genetic organization

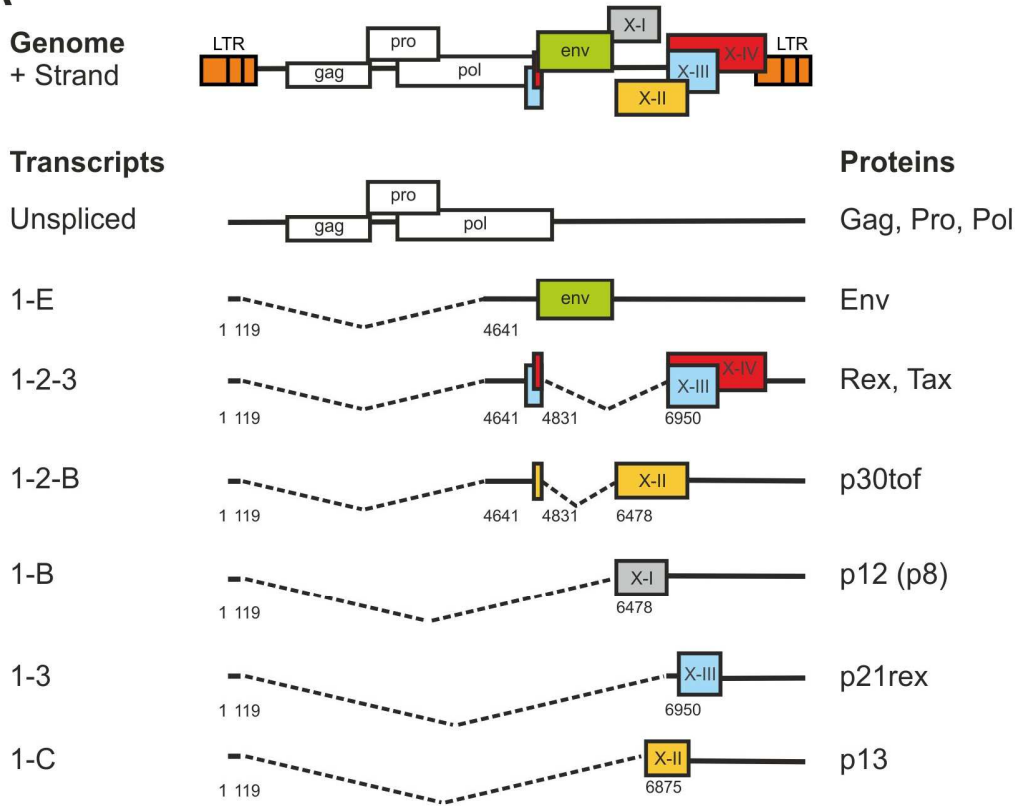
The genome of HTLV-1 reflects the basic structure of the Deltaretrovirus genus (Figure 2). At the 5' and 3' ends are located the long terminal repeat (LTR) sequences. The LTRs flank the partially overlapping open reading frames (ORFs) of the gag, pro, pol and env genes that code for enzymes and structural proteins of mature viral particles. The region between the end of the env gene and the 3' LTR is termed the pX region and contains four major partially overlapping ORFs, termed x-I through x-IV, coding for regulatory and accessory proteins (Figure 2A). The minus strand of HTLV-1 also contains an ORF located in the pX region (antisense orientation) (Larocca et al., 1989) which codes for the HTLV-1 bZIP factor protein (HBZ) (Figure 2B) (Gaudray et al., 2002). Expression of the highly condensed HTLV-1 genetic information is achieved through different mechanisms: (i) ribosomal frameshifting, which generates a Gag-Pro-Pol polyprotein from the full-length transcript; (ii) alternative splicing, which produces distinct mRNAs coding for the Env and pX region genes; and (iii) polycistronic translation, which produces the Tax and Rex proteins from the same mRNA. Minus strand transcription generates at least two different mRNAs encoding two isoforms of the HBZ protein.

The HTLV-1 transcripts can be grouped in four major classes (Figure 2A and B):

- a) plus strand unspliced mRNA (US), coding for Gag-Pro-Pol, and used as genomic RNA;
- b) plus strand singly-spliced mRNAs (SS), coding for the envelope glycoproteins (Env) and for the accessory proteins p21rex, p12 (p8) and p13;
- c) plus strand doubly-spliced mRNAs (DS), coding for the regulatory proteins p40 (Tax) and p27 (Rex), and for the regulatory/accessory protein p30/tof;
- d) minus strand mRNAs coding for HBZ proteins.

A recent study carried out on primary cells from infected patients and cells transfected with HTLV-1 molecular clone, indicated a two-phase pattern of gene expression, with the tax/rex mRNA preceding expression of other transcripts (Rende et al., 2011). This study also showed that over 90% of the HBZ mRNAs are localized in the nuclear compartment, suggesting their role as a nuclear non-coding transcripts (Paragraph 1.4.7).

A



B

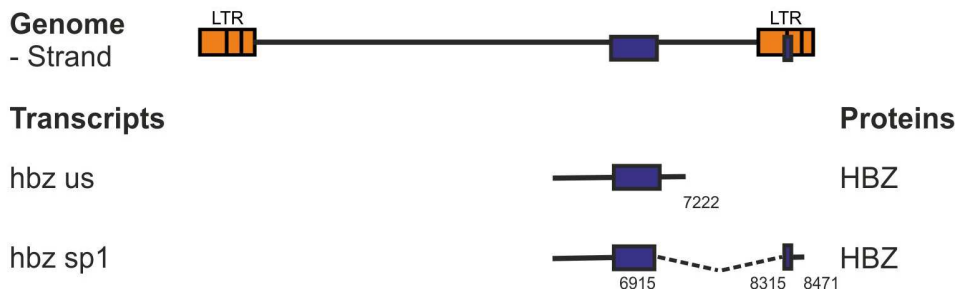


Figure 2. Organization and expression of the HTLV-1 genome. A: The transcriptional map of plus-strand mRNAs and proteins coded by each mRNA is shown. The numbers indicate the position of the splicing sites. Resulting exons are: 1 (1-119), 2 (4641-4831), 3 (6950-8493), B (6478-8493), C (6875-8493) and E (4641-8493). mRNAs are named according to their exon composition. **B:** The transcriptional map of the minus-strand mRNAs and proteins coded by each mRNA is shown. The numbers indicate transcription start sites and splicing sites. Resulting exons are: hbz us (7222-4834) and hbz sp1 (8471-8315 and 6915-4834) (Rende et al., 2011).

1.3.1 Expression of structural proteins

The full-length plus-strand US mRNA (8.6 kb) is packaged into virions as the genomic RNA and is also translated into the structural proteins (gag) and enzymes (protease and polymerase) of mature virus particles (Lairmore and Franchini, 2007). The gag gene codes for the 19 kDa matrix (MA), 24 kDa capsid (CA) and 15 kDa nucleocapsid (NC) structural proteins. p24-CA multimers form the inner core of the virion; p15-NC interacts with the genomic RNA inside the inner core of the virion; and p19-MA is involved in virus budding and assembly (Dorweiler et al., 2006; Heidecker et al., 2007), and has a role in cell-to-cell transmission of the virus (Le Blanc et al., 1999; Hémonnot et al., 2006). The pro gene encodes the viral protease. The 5' portion of the pol gene encodes reverse transcriptase (RT) and integrase. These genes are translated as polyproteic precursors (Gag, Gag-Pro and Gag-Pro-Pol) generated through ribosomal frameshifting at the gag-pro and/or gag-pro-pol junction. The precursors are post-translationally myristylated at the N-terminus, an essential step for their insertion in the inner layer of the plasma membrane of the infected cell. After virion budding, the precursors are cleaved by the viral protease to generate the single mature polypeptides.

The 4.2 kb SS mRNA codes for the env precursor, which is post-translationally modified by glycosylation and cleaved into the surface gp46 (SU) and transmembrane gp21 (TM) proteins.

1.3.2 Expression of proteins coded by the pX region

The pX region of the HTLV-1 genome contains at least four different partially overlapping open reading frames, termed x-I through x-IV, that code for non structural proteins of HTLV-1. As shown in Figure 2A, expression of the different pX region genes is accessed through alternative splicing and polycistronic translation. All these mRNAs contain exon 1, which is non coding.

Singly spliced mRNAs code for the accessory proteins p13 (mRNA 1-C, translated from a start codon located in the x-II ORF), p12/p8 (mRNA 1-B, translated from a start codon in the x-I ORF) and p21rex (mRNA 1-3, translated from a start codon located in the x-III ORF) (Ciminale et al., 1992; Koralnik et al., 1992).

Two doubly spliced mRNAs contain exons 1 and 2, and different 3' exons. mRNA 1-2-3 is a bicistronic transcript coding for the regulatory proteins Rex and Tax; Rex initiates at the first AUG in exon 2 and continues in the x-III ORF in exon 3, while Tax initiates at the second AUG and continues in the x-IV ORF in exon 3. The 1-2-B mRNA codes for the accessory/regulatory protein p30/tof, which is translated from the second start codon of exon 2 and continues in frame with the x-II ORF in exon B (Ciminale et al., 1992; Koralnik et al., 1992).

1.3.3 Expression of proteins coded by the minus strand of HTLV-1

The minus strand of HTLV-1 contains one ORF located in the pX region (antisense orientation) which generates two major transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). These transcripts code for two isoforms of HBZ protein that differ by seven amino acids at the N-terminus. Hbz sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the hbz us transcript initiates within the tax gene (Figure 2B). Both hbz sp1 and hbz us are transcribed from TATA-less promoters (Yoshida et al., 2008).

1.4 Functions of the non-structural proteins

1.4.1 Tax

Tax is a 353-amino acid 40-kDa phosphoprotein that transcriptionally controls the expression of viral genes and of a large number of cellular genes. Tax is predominantly a nuclear protein, although it can translocate to the cytoplasm. The following functional domains have been mapped in the Tax sequence (Boxus et al., 2008):

- a) a nuclear localization signal (NLS) located in the N-terminal domain;
- b) a zinc finger region that overlaps with the NLS and is crucial for Tax's interaction with the CRE-binding/activating transcription factor (CREB/ATF) and the serum responsive factor (SRF);
- c) a kinase-inducible domain (KID) and a dimerization domain localized in the central region of Tax. The KID domain mediates the interaction with the kinase-

inducible exchange (KIX) domain of the transcriptional co-activators CREB binding protein (CBP) and p300.

d) A domain involved in the interaction of Tax with the transcriptional co-activator p300/CBP-associated factor (P/CAF), located in the C-terminal region of the protein.

1.4.1.1 Effects of Tax on the CREB pathway

Tax was initially described as an activator of LTR-directed transcription (Felber et al., 1985). Three Tax responsive elements (TRE), within the U3 region of the LTR, are sufficient to confer Tax responsiveness (Brady et al., 1987). The TRE elements are imperfectly conserved sequences of 21 nucleotides. Each element contains an octamer motif TGACG(T/A)(C/G)(T/A) flanked by a GC stretch at the 5' and 3' ends (Jeang et al., 1988). The octamer motif shares homology with the consensus cAMP-responsive element (CRE) (5'-TGACGTCA-3'). Tax exhibits poor affinity for DNA and binds indirectly to the TRE element by interacting with members of the CREB/ATF family (Giam and Xu, 1989). These proteins share a common cluster of basic residues, allowing DNA binding, and a leucine zipper (b-Zip) domain involved in homo- and heterodimerization. Tax promotes formation of a Tax-CREB/ATF-TRE ternary complex by interacting with the b-Zip domain of CREB/ATF factors. Tax enhances the dimerization of CREB/ATF factors, increasing their affinity for the TRE (Wagner and Green, 1993; Anderson and Dynan, 1994; Perini et al., 1995; Yin and Gaynor, 1996), and further stabilizes the ternary complex through direct contact of the GC-rich flanking sequences (Kimzey and Dynan, 1998; Lundblad et al., 1998). Tax also recruits coactivators (CBP/p300 and P/CAF) to facilitate transcription initiation. The ability of Tax to dimerize is required for efficient ternary complex formation and for optimal transactivation (Jin and Jeang, 1997; Tie et al., 1996). Physiologically, the CREB-CBP/p300 interaction is controlled by CREB phosphorylation in response to different signal transduction pathways. Through its ability to bind both CREB and CBP/p300, Tax triggers CREB activation even in the absence of phosphorylation. In this way, viral gene transcription becomes independent from cellular signals.

Through its interactions with CREB/ATF proteins, Tax can both activate a variety of cellular genes, including interleukin 17 (IL-17) and c-fos (Dodon et al., 2004; Alexandre and Verrier, 1991) and repress the expression of other genes such as cyclin A, p53 and c-myc (Kibler and Jeang, 2001; Mulloy et al., 1998; Nicot et al., 2000).

1.4.1.2 Effects of Tax on the NF- κ B pathway

The oncogenic properties of Tax have been ascribed, mostly, to its ability to activate NF- κ B, a major survival pathway engaged by HTLV-1 infection (Saggiaro et al., 2009). The mammalian NF- κ B proteins consist of five structurally related members: p65/RelA, RelB, c-Rel, NF- κ B1 (p50/and its precursor p105) and NF- κ B2 (p52/and its precursor p100) which form homo-heterodimers that regulate the expression of target genes bearing a NF- κ B-responsive elements in their promoters (Hayden and Ghosh, 2012). In unstimulated cells, NF- κ B dimers are sequestered in the cytoplasm by inhibitory proteins called I κ Bs (comprising p105, p100, I κ B α , I κ B β , I κ B γ , I κ B ζ , and Bcl-3) that mask the nuclear localization signal of NF- κ B. Upon cell stimulation, I κ B proteins are rapidly phosphorylated and degraded by the proteasome, and NF- κ B translocates into the nucleus to regulate the expression of target genes coding for cytokines, chemokines, adhesion molecules, inhibitors of apoptosis, and other proteins (Hayden and Ghosh, 2012).

Two main signalling pathways lead to NF- κ B activation: the canonical (or classical) and the non-canonical (or alternative) pathways.

The canonical NF- κ B pathway is induced by a variety of innate and adaptive immunity mediators, such as pro-inflammatory cytokines (TNF- α , IL-1 β), and engagement of Toll-like receptors (TLRs) and antigen receptors (TCR, BCR) (Pahl, 1999; Bonizzi et al., 2004). The crucial step in the canonical NF- κ B pathway is the activation of the I κ B-kinase (IKK) complex, which consists of the two kinases, IKK α and IKK β (Zandi et al., 1997), and of one regulatory subunit IKK γ , also known as NF- κ B essential modulator (NEMO) (Yamaoka et al., 1998) (Figure 4A). Activated IKK phosphorylates I κ B α on serines 32 and 36, triggering its ubiquitination and proteasomal degradation. This unmasks the DNA binding activity of the p50/RelA

dimer, and allows its translocation into the nucleus where it activates the transcription of target genes (Beinke and Ley., 2004) (Figure 4A).

Tax intervenes at multiple levels to activate the canonical NF- κ B pathway. In the cytoplasm, Tax directly binds to NEMO and recruits the IKK complex to the perinuclear compartment, where it is phosphorylated and activated (Harhaj and Sun, 1999; Jin et al., 1999; Xiao et al., 2000). Tax also activates kinases upstream to the IKK complex, including MAPK/ERK kinase kinase 1 (MEKK1) and TGF- β activating kinase 1 (TAK1) (Yin et al., 1998; Wu and Sun, 2007), thus enhancing IKK α and IKK β phosphorylation, and I κ B α and I κ B β degradation (Harhaj and Sun, 1999; Jin et al., 1999). In addition, Tax binds IKK α and IKK β and activates their kinase activity independently of the upstream kinases (Chu et al., 1998). The binding of Tax to I κ Bs also enhances their degradation independently of IKK phosphorylation (Hirai et al., 1994; Suzuki et al., 1995). Tax thus promotes I κ Bs degradation at multiple levels, allowing nuclear translocation of NF- κ B independently of external stimuli. In the nucleus, Tax recruits RelA, CBP/p300 and PCAF (Bex et al., 1998; Bex and Gaynor, 1998) into discrete transcriptional hot spots termed Tax nuclear bodies, leading to NF- κ B transcriptional activation (Semmes and Jeang, 1996; Bex et al., 1997).

The non-canonical NF- κ B pathway is important for secondary lymphoid organ development and homeostasis. It is induced by B-cell activating factor (BAFF) (Claudio et al., 2002), lymphotoxin β (LT β) (Dejardin et al., 2002), and CD40 ligand (Coope et al., 2002; Xiao et al., 2001a). The crucial step in this pathway is the processing of p100 into p52 by IKK α , thus allowing p52 DNA binding in association with its partner RelB. The phosphorylation and activation of IKK α is mediated by the upstream NF- κ B inducing kinase (NIK) (Figure 4B) (Xiao et al., 2001b; Xiao et al., 2004). Interestingly, NIK can also induce long-term activation of the IKK complex and I κ B α degradation, thus activating also the canonical NF- κ B pathway (Zarnegar et al., 2008) (Figure 4A and B).

The induction of the non-canonical pathway is a hallmark of NF- κ B activation by HTLV-1 infection, because this arm of the pathway usually is not active in normal T cells (Xiao et al., 2001a). Tax induces the non-canonical NF- κ B pathway by

promoting the processing of p100 to p52 (Xiao et al., 2006). Tax-mediated processing of p100 requires NEMO as an adaptor protein in the assembly of the Tax/IKK complex, and is independent of the NIK kinase. In contrast to the canonical Tax/NEMO/IKK complex, which contains both the IKK α and β , the non-canonical complex contains only IKK α (Xiao et al., 2001b), which phosphorylates p100 leading to its processing to p52 (Qu et al., 2004).

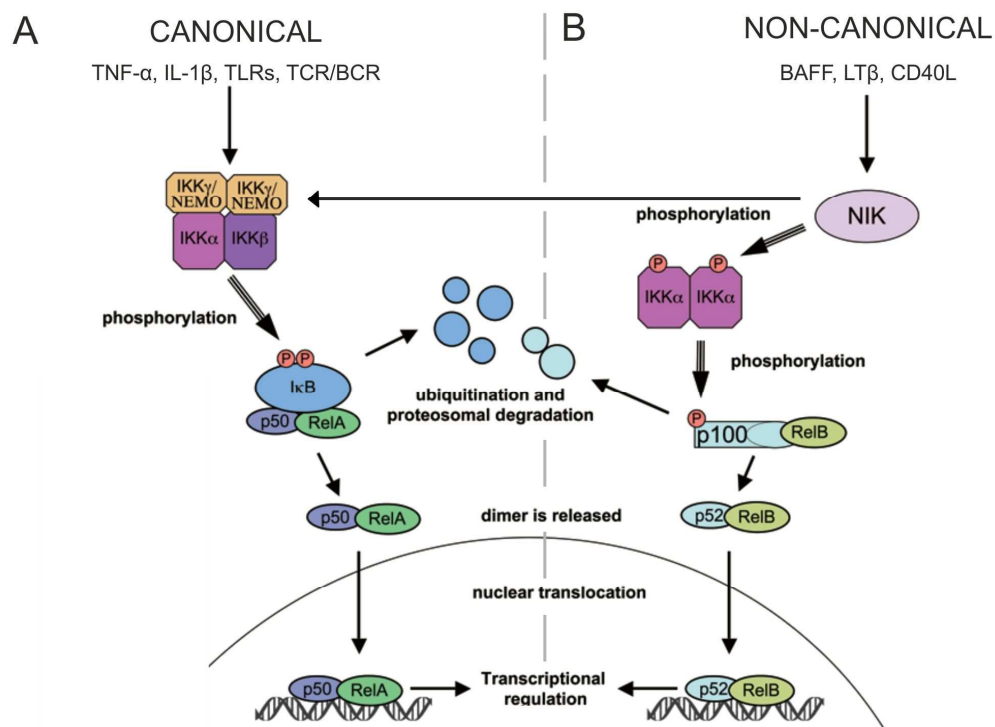


Figure 4. NF- κ B activation: the canonical and non-canonical pathways. **A:** The stimuli that induce the canonical NF- κ B pathway converge on the activation of the IKK complex, which consists of the two kinases, IKK α and IKK β , and one regulatory subunit IKK γ /NEMO. The IKK complex phosphorylates I κ B α , triggering its proteasomal degradation. This allows the translocation of NF- κ B1 (p50/RelA) in the nucleus, where it activates the transcription of target genes. **B:** The crucial step of the non-canonical NF- κ B pathway is the processing of p100 into p52 mediated by IKK α , thus allowing NF- κ B2 (p52/RelB) DNA binding. The phosphorylation and activation of IKK α is mediated by the upstream NIK kinase, which also induces I κ B α degradation, thus activating the canonical NF- κ B pathway (Adapted from Morgan and Liu., 2011).

Almost all steps of the NF- κ B pathway can be terminated through feedback inhibition mechanisms. These mechanisms are extremely important, since persistent activation of NF- κ B can result in cellular damage, death or induce cancer due to acute inflammation and uncontrolled cell turnover. Among the physiological NF- κ B termination mechanisms, the most rapid and essential is mediated by PDZ-LIM domain-containing protein 2 (PDLIM2). PDLIM2 is an ubiquitously expressed cytoplasmic-nuclear shuttling protein that terminates NF- κ B activation by shuttling RelA to the nuclear matrix, where PDLIM2 enhances RelA proteasome-mediated degradation through its E3 ubiquitin ligase activity (Fu et al., 2010; Tanaka et al., 2007). Tax directly shuts off this feedback inhibition mechanism by binding to PDLIM2, resulting in reduced RelA degradation, although this process results in the proteasomal degradation of Tax itself (Yan et al., 2009).

Tax-mediated activation of the NF- κ B pathway results in increased survival through the transcriptional activation of the anti-apoptotic factors Bcl-X_L, Bfl1 and HIAP-1 (Kawakami et al., 1999, Tsukahara et al., 1999; Nicot et al., 2000; De La Fuente et al., 2003) and the downregulation of the pro-apoptotic protein Bax (Brauweiler et al., 1997). In addition, Tax induces a physical interaction between RelA and p53 that inhibits p53 transcriptional activity (Jeong et al., 2004). Furthermore, Tax represses p53 at the protein level via NF- κ B using two different mechanisms: (i) activated IKK directly phosphorylates p53 to trigger p53 ubiquitination and proteasomal degradation by the β -TrCP ubiquitin ligase (Xia et al., 2009); (ii) activated NF- κ B induces expression of MDM2, a ubiquitin ligase for p53 ubiquitination and degradation (Busuttill et al., 2010).

1.4.1.3 Effects of Tax on the SRF/AP-1 pathway

Tax increases the expression of the transcription factor AP-1 (activator protein -1) a homo- or heterodimeric complex of Fos (c-Fos, FosB, Fra1 and Fra2) and Jun (c-Jun, JunB and JunD) (Fujii et al., 1991; Fujii et al., 2000). Fos and Jun transcription is positively regulated by the serum responsive factor (SRF) in response to various stimuli such as cytokines, growth factors, stress signals and oncogenes. SRF binds to

the Fos/Jun promoters through two SRF responsive elements (SRE): a CArG box (CC(A/T)6GG) and an upstream Ets box (GGA(A/T)). Once SRF occupies the CArG box, the ternary complex factor (TCF) establishes protein interactions with SRF and subsequently binds the upstream Ets site. This complex then recruits the co-activators P/CAF and CBP/p300 to activate transcription.

Tax activates the transcription of promoters under the control of SRE motifs through the interaction with transcription factors associated with the SRF pathway (Alexandre and Verrier, 1991; Fujii et al., 1991; Alexandre et al., 1991). This interaction results in increased binding of SRF to the SRE (Dittmer et al., 1997). Once the complexes are stabilized, Tax recruits the coactivators CBP/p300 and P/CAF and mediates transactivation (Shuh and Derse, 2000).

1.4.1.4 Non-transcriptional effects of Tax

Tax also controls the cell cycle by interacting with cyclins-D1, -D2 and -D3 as well as with cyclin-dependent kinases (CDKs) 4 and 6 (Neuveut et al., 1998; Haller et al., 2000; Haller et al., 2002; Fraedrich et al., 2005). Through these interactions, Tax stabilizes the cyclin D2/CDK4 complex and enhances its kinase activity, leading to hyperphosphorylation of the retinoblastoma protein (Rb). Tax also associates with the CDK inhibitors (CDKI) p15INK4b and p16INK4a and counteracts their CDK4-inhibitory activity (Suzuki et al., 1996; Suzuki and Yoshida, 1997; Low et al., 1997; Suzuki et al., 1999). In addition, Tax binds to Rb and enhances its proteosomal degradation (Kehn et al., 2005).

Tax also interacts with Tax1 binding protein (TAX1BP2) and Ran/Ran binding protein 1 (RanBP1), which control centrosome amplification during mitosis (Ching et al., 2006; Peloponese et al., 2005). Through these interactions, Tax induces supernumerary centrosomes and causes multipolar mitosis, thus contributing to aneuploidy, a hallmark of HTLV-1-infected cells (Marriott et al., 2002; Boxus and Willems 2009). Furthermore, Tax binds to and impairs the activity of Mad1, a constituent of the spindle assembly checkpoint (SAC) machinery (Jin et al., 1998). Tax interacts with the anaphase promoting complex (APC), which controls the metaphase-anaphase transition (Liu et al., 2005). APC directs the ubiquitination and

proteosomal degradation of cyclin B1 and Pds1p/securin. Securin and cyclin B1 inhibit separase, a protease that destroys the connection links of sister chromatids. In normal cells, chromosomes start to segregate only after the kinetochore is subjected to the mechanical tension generated by the mitotic spindle (Nasmyth, 2005). Tax activates APC in the S phase, before the cell enters mitosis, decreasing the levels of cyclin B1 and securin, and resulting in the premature activation of separase, thus leading to unequal chromosomal separation between cells (Liu et al., 2005).

Tax also promotes genome instability by targeting multiple components of the DNA damage repair pathway. Tax directly causes DNA damage generating double strand breaks both by modulating the timing of activation of the replication origins (Boxus et al., 2012) and by inducing oxidative stress (Kinjo et al., 2010). In addition, Tax attenuates the DNA damage response through sequestration and/or inhibition of critical proteins such as ATM, DNA-PK, CHK1-2 and p53 (Boxus and Willems, 2012).

Although Tax is a major target of CTLs, ATLL cells frequently lose the expression of Tax (see Paragraph 1.4.7) Thus, it is speculated that Tax plays an important role in the persistent proliferation of HTLV-1-infected cells mainly during the healthy carrier state. In this phase, the mutator phenotype conferred by Tax promotes accumulation of genetic and epigenetic changes that finally lead to Tax-independent proliferation and, following the silencing of Tax, escape from the host immune system (Yasunaga and Matsuoka, 2007).

1.4.2 Rex

Rex is a 189-amino acid, 27-kDa phosphoprotein coded by the x-III ORF of the pX region. Rex controls viral gene expression at the post-transcriptional level. The protein accumulates in the nuclei and nucleoli, and is able to shuttle between the nucleus and the cytoplasm (Palmeri and Malim, 1996; Narayan et al., 2003). This function of Rex is mediated through direct interaction with a 254-nucleotide stem-loop cis-acting RNA element termed the Rex-responsive element (RxRE), present in the U3/R region of the 3' LTR of all plus-strand HTLV-1 transcripts (Grone et al., 1994).

Different functional domains have been mapped in the Rex protein. The N-terminal arginin-rich region serves as nuclear localization signal (NLS) (Siomi et al., 1998; Nosaka et al., 1989) and as RNA binding domain (RBD) which mediates Rex binding to the RxRE (Grassmann et al., 1991; Bogerd et al., 1992). A leucine-rich sequence near the middle of the protein functions as activation domain (AD) (Weichselbraun et al., 1992b) and contains the nuclear export signal (NES) (Palmeri and Malim, 1996; Kim et al., 1996). The NES interacts with the protein chromosome region maintenance interacting protein 1 (CRM1/exporting 1) and allows export of the Rex-RNA complexes from the nucleus to the cytoplasm (Bogerd et al., 1995). CRM1 belongs to the importin- β family, whose members act as RNA transporters between nuclear and cytoplasmic compartments (Bogerd et al., 1998). Two regions adjacent to the NES are required for the assembly of Rex into multimeric structures upon binding to the RxRE (Bogerd et al., 1995; Bogerd and Greene, 1993). The formation of Rex multimers on the RxRE is critical for the nuclear export of viral mRNA, and mutations of the multimerization domain confer a dominant-negative phenotype to the protein (Bogerd et al., 1993). In addition to functioning as a Rex exporter, CRM1 serves as an inducing factor for Rex multimerization on viral mRNA by aiding in complex formation (Englmeier et al., 2001; Hakata et al., 1998). Collectively, the requirement for multiple bound copies of Rex on the RxRE is believed to protect the mRNA from being spliced, sequestered, or degraded in the nucleus.

After Rex-RxRE binding and Rex multimerization, CRM1 is recruited into the Rex-mRNA complex. The Rex-mRNA-CRM1 complex binds to RanGTP and translocates across the nuclear pore by interacting with phenylalanine-glycine rich nucleoporins. In the cytoplasm, RanGTP is then converted to RanGDP and is released from the Rex-mRNA complex. The conversion of GTP to GDP is catalyzed by the regulator of chromosome condensation 1 (RCC1) (Bischoff et al., 1991).

Rex's interaction with the RxRE is modulated by several cellular proteins. Heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) competes with and inhibits Rex-RxRE interaction (Dodon et al., 2002). On the contrary, both Ran Binding Protein 3 (RanBP3) and SRC-associated in mitosis 68 (Sam 68) favour Rex function.

RanBP3 stabilizes the RanGTP-CRM1-Rex-mRNA complex in the nucleus (Hakata et al., 2003; Englmeier et al., 2001), and Sam 68 enhances the RNA-binding activity of Rex in a CRM1-independent manner (Reddy et al., 1999; Reddy et al., 2000). Experiments performed with protein kinase inhibitors indicated that Rex function is modulated by phosphorylation on serine/threonine residues (Adachi et al., 1990; Kesic et al., 2006), a property that highlights the importance of cellular factors in the multi-level regulation of HTLV-1 gene expression (Younis and Green, 2001). In addition to its role in RNA trafficking, Rex inhibits splicing *in vitro* during the initial phase of spliceosome formation (Bakker et al., 1996) and increases mRNA stability in the nucleus (Grone et al., 1996).

Furthermore, Rex affects the expression of cellular genes. Rex is able to augment Tax-mediated upregulation of IL-2 and stabilize the IL-2 receptor- α chain (IL-2R α) mRNA, thereby prolonging its half-life independently of nuclear-cytoplasmic transport (McGuire et al., 1993; Kanamori et al., 1990). Rex has also been shown to increase FynB (p59fyn) expression, possibly by affecting its splicing (Weil et al., 1999). FynB is a src family protein-tyrosine kinase that regulates T-cell receptor stimulation (Cooke et al., 1991). Co-transfection experiments in Jurkat T-cells demonstrated that Rex augments Tax's ability to increase expression of vascular cell adhesion molecule-1 (VCAM-1) and lymphocyte function-associated antigen-3 (LFA-3) (Valentin et al., 2001). Both VCAM-1 and LFA-3 are important proteins in T-cell adhesion that favour proliferation of uninfected cells and virus spread.

Although Rex is not required for cellular immortalization *in vitro*, it is necessary for infectivity and viral persistence *in vivo* (Ye et al., 2003), since expression of the US and SS viral RNAs encoding structural proteins is necessary for the assembly of virions. The fact that these mRNAs depend on Rex for expression suggests that the Rex-RxRE interaction may function as a molecular switch controlling the transition between productive and latent phases of HTLV-1 infection. This hypothesis was recently tested in a study that analysed the temporal sequence of HTLV-1 gene expression in primary cells from infected patients using splice site-specific quantitative RT-PCR. This study revealed a two-phase kinetics of HTLV-1 expression, with the tax/rex mRNA preceding expression of other viral transcripts.

Studies carried out with Rex knock-out molecular clones of HTLV-1 demonstrated the strict Rex-dependency of the two-phase kinetics (Rende et al., 2011).

1.4.3 p21rex

p21rex is a truncated isoform of Rex lacking the N-terminal arginine-rich domain. It was hypothesized that it might act as a repressor of full-length Rex, inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins (Heger et al., 1999). However, this function of p21rex as a latency-inducing factor in HTLV-1 life cycle has been questioned by other authors (Bai et al., 2012).

1.4.4 p30/tof

p30/tof is a 241-amino acid protein coded by the x-II ORF of the pX region. p30/tof is a nucleolar-nuclear non-shuttling protein (Ciminale et al., 1992; D'Agostino et al., 1997) whose sequence contains three NLS and an arginine-rich nucleolar retention signal (NoRS) (D'Agostino et al., 1997; Ghorbel et al., 2006). An HTLV-1 molecular clone containing a mutation in the x-II ORF is still able to produce infective virions and immortalize human T-lymphocytes (Robek et al., 1998), but shows a drastic reduction in *in vivo* infectivity in animal models (Bartoe et al., 2000; Valeri et al., 2010).

p30/tof functions at the post-transcriptional level by inhibiting the nuclear export of the tax/rex mRNA; this results in a global inhibition of viral gene expression, suggesting that p30/tof might act as a latency factor (Nicot et al., 2004).

Additional studies shed light on the mechanism used by the virus to discriminate between viral mRNAs that are p30/tof responsive (tax/rex) and those that are Rex responsive (gag/pro/pol and env). p30/tof interacts with the RNA-binding domain of Rex and with the tax/rex mRNA, building a p30/tof-Rex-tax/rex mRNA ternary complex that prevents Rex-RxRE binding to the tax/rex mRNA; p30/tof efficiently interacts with Rex only when it is bound to RNA (Sinha-Datta et al., 2007). Two domains are crucial to the formation of the ternary complex: the Rex binding domain (RexBD) on the p30/tof protein and the p30 mRNA responsive element (p30RE) on the tax/rex mRNA. The ability of p30/tof to interact with tax/rex but not with

gag/pro/pol or env mRNAs explains why p30/tof is able to prevent tax/rex nuclear export but has little or no effect on other viral mRNAs (Sinha-Datta et al., 2007).

Although it is critical for HTLV-I to reduce its expression to evade immune detection and clearance, complete latency would not benefit the virus, which needs Tax expression to promote clonal expansion of infected cells. In agreement with this model, it was demonstrated that Rex permits export of residual p30/tof-bound tax/rex mRNA allowing a low level of virus expression (Sinha-Datta et al., 2007). Rex may oppose p30/tof function by recruiting a limiting cellular factor and inducing a conformational change in p30/tof or in the local RNA structure, thus releasing p30/tof from its p30 responsive element (p30RE) and allowing export of some tax/rex mRNA to the cytoplasm.

In addition to its post-transcriptional functions, p30/tof modulates transcription from CREB-responsive promoters with cellular CRE and viral TRE sequences, by interacting with the co-activator CBP/p300 (Zhang et al., 2000; Zhang et al., 2001). p30/tof interacts with the kinase-inducible exchange domain (KIX) of p300/CBP, also known to bind to CREB and Tax. p30/tof modulation of the transcription from the CRE sequences depends on its expression levels and on the presence of Tax. At low levels of expression and in the absence of Tax, p30/tof activates the transcription from the HTLV-I LTR. On the other hand, at high levels, p30/tof represses Tax-mediated LTR transcription. Furthermore, p30/tof can disrupt the assembly of the CREB–Tax–p300/CBP complex on TRE sequence. Taken together, these data suggest that p30/tof might decrease transcription of the viral genome, thereby facilitating viral latency.

p30/tof also influences the expression of a number of cellular genes at the transcriptional and post-transcriptional level, including adhesion molecules and genes involved in T-cell activation and apoptosis (Michael et al., 2004; Taylor et al., 2009).

Finally, p30/tof recruits the co-activator Tat-interacting protein 60 (TIP60) and promotes the formation of the Myc/TIP60 transcription complex on the Myc-response E-box element, thus transactivating transcription (Awasthi et al., 2005). Due to the importance of Myc as a proto-oncogene, p30/tof may contribute to the transformation of the HTLV-1-infected cell. In fact, p30/tof modulates expression of cellular genes

involved in the cell cycle and apoptosis (Michael et al., 2004; Taylor et al., 2009; Datta et al., 2007). p30/tof-mediated alteration of cell cycle events was proposed to promote early viral spread and T-cell survival (Datta et al., 2007).

1.4.5 p13

p13 is a 87-amino acid protein coded by the x-II ORF of the pX region. p13 corresponds to the C-terminal portion of p30/tof (Koralnik et al., 1992) and accumulates prevalently in mitochondria (Ciminale et al., 1999).

Studies of a p13-knockout virus showed that although the protein is dispensable for viral replication in cultured cells (Derse et al., 1997; Robek et al., 1998), it is required for establishing a persistent infection in a rabbit experimental model (Hiraragi et al., 2006).

Functional mapping of p13 revealed a mitochondrial targeting signal (MTS) spanning amino acids 21-30 (Ciminale et al., 1999). Upon exposure to membrane-mimetic solutions, the MTS (LRVWRLCTRR) folds in an amphipathic α -helix, with a positively charged face of four arginine residues (Figure 4A, left) (D'Agostino et al., 2002). Unlike canonic MTS, the MTS of p13 is not cleaved upon import and substitution of the four arginines with glutamines, prolines, or alanines and leucines does not affect mitochondrial targeting (Ciminale et al., 1999) (Figure 4A, right).

Immunoelectron microscopy and fractionation experiments demonstrated that p13 is an integral membrane protein and accumulates mainly in the inner mitochondrial membrane. p13 induces specific alterations in mitochondrial morphology, resulting in isolated clusters of round-shaped, swollen mitochondria (Ciminale et al., 1999; D'Agostino et al., 2002; D'Agostino et al., 2005). Site-directed mutagenesis experiments demonstrated that the arginines of the amphipathic α -helical domain are essential to induce mitochondrial fragmentation/swelling (D'Agostino et al., 2002). The subcellular localization of p13 could be influenced by its expression level and the physiological state of the cell; in fact, although the protein has been localized mainly in the mitochondria, occasionally it may be detected also in the nucleus (Koralnik et al., 1993; D'Agostino et al., 1997). Interestingly, a recent study has demonstrated

that, upon co-expression with Tax, p13 is partially routed to nuclear speckles (Andresen et al., 2011).

In vitro assays carried out using isolated mitochondria and purified full-length synthetic p13 demonstrated that it triggers an influx of K^+ in mitochondria, leading to membrane depolarization, increased activity of the electron transport chain (ETC) and mitochondrial reactive oxygen species (ROS) production (Silic-Benussi et al., 2009). The increased ROS production, along with membrane depolarization, lowers the opening threshold of the permeability transition pore (PTP), a mitochondrial channel controlling apoptosis (Figure 4B) (Rasola and Bernardi, 2007). p13 mutants in the amphipathic α -helix have little or no effects on ROS production. The effects of p13 on inner membrane potential ($\Delta\psi$) and ROS production were also observed in living cells (Biasiotto et al., 2010; Silic-Benussi et al., 2010b).

Studies of the influence of p13 on ROS indicate that the protein may have a distinct impact on cell survival and proliferation, depending on the cell's inherent ROS levels, with death-promoting effects in transformed cells and activation predominating in normal resting T-cells (Silic-Benussi et al., 2010a). p13 sensitizes transformed cells to proapoptotic stimuli such as Fas ligand (FasL) and ceramide (Hiraragi et al., 2005; Silic-Benussi et al., 2004) or glucose deprivation (Silic-Benussi et al., 2010b). Interestingly, the effect of p13 on FasL-mediated apoptosis is enhanced by Ras overexpression and blocked by inhibitors of Ras (Hiraragi et al., 2005; Silic-Benussi et al., 2004). Furthermore, p13 interacts with farnesyl pyrophosphate synthase, a key enzyme in the synthesis of substrates required for the prenylation of Ras (Lefèbvre et al., 2002a; Lefèbvre et al., 2002b), suggesting that p13 may affect the Ras signal transduction pathway. These results suggest that, in the context of transformed cells, p13 might act as a viral tumor suppressor reducing proliferation and favoring cell death especially in conditions of reduced substrate availability.

In contrast, expression of p13 in primary quiescent T-cells results in ROS-dependent activation (Silic-Benussi et al., 2010b). The dual effect of p13 in normal versus transformed cells is consistent with a current model that compares the effects of ROS on cell turnover to a rheostat (Rustin, 2002). According to this model, an increasing gradient of ROS is found from resting- to activated- to transformed- to apoptotic cells.

This model is also relevant in the context of T-cell activation, as engagement of the TCR induces rapid generation of ROS, in part produced by mitochondria. ROS act as powerful second messengers controlling redox-sensitive pathways such as the NF- κ B, MEK, ERK and LCK, and modulate the opening threshold of the permeability transition pore (PTP) (Williams and Kwon, 2004).

Another mechanism through which p13 might control T-cell activation and death is by influencing calcium (Ca^{2+}) homeostasis at the mitochondrial level. Ca^{2+} uptake in the mitochondrial matrix controls the activity of key metabolic enzymes, stimulating ATP production and aerobic metabolism. Excessive calcium accumulation in mitochondria triggers PTP opening and apoptosis (Giacomello et al., 2007). The analysis of Ca^{2+} concentration in different intracellular compartments (endoplasmic reticulum, mitochondria and cytosol) following histamine stimulation revealed that p13 specifically reduces mitochondrial Ca^{2+} uptake (Biasiotto et al., 2010).

Taken together, these findings suggest that in the context of the HTLV-1 propagation strategy, p13 could provide a mechanism to increase the pool of normal infected T-lymphocytes while promoting the elimination of cells occasionally acquiring a transformed phenotype, thus favouring life-long persistence of the virus in the host in the absence of disease.

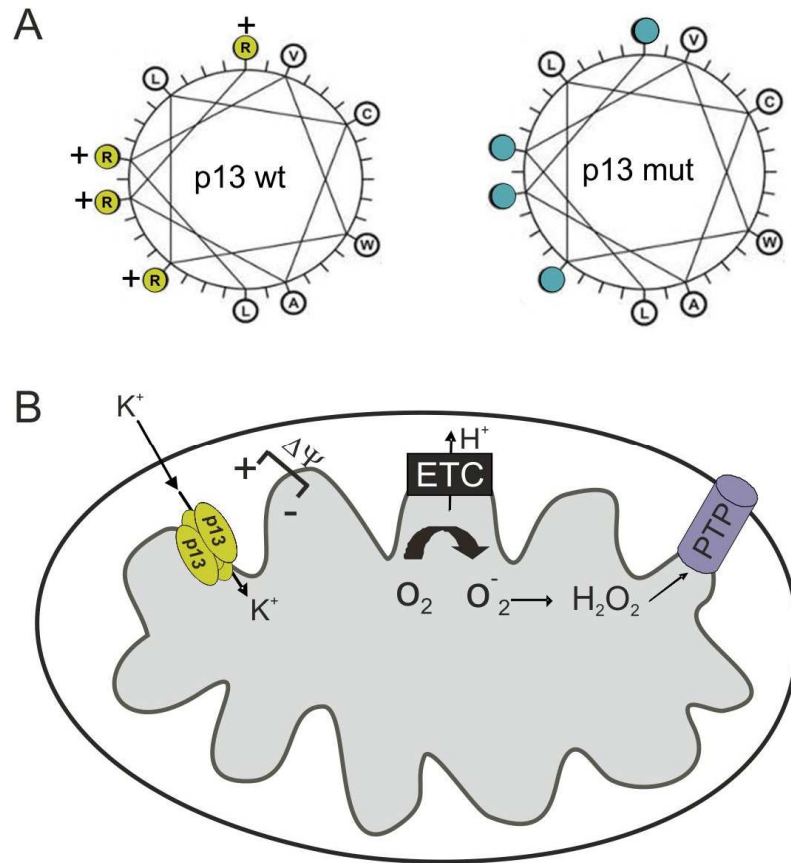


Figure 4. Schematic model of p13 structure and mitochondrial functions. **A:** The amphipathic α -helix of p13. Rs indicate the four positively charged arginine residues in positions 22, 25, 29 and 30 of p13 (left). The substitution of these arginines with glutamines, prolines, or alanines and leucines is indicated as blue dots in the p13 mutant (right). These substitutions do not affect mitochondrial targeting, but are essential for function. **B:** In the inner mitochondrial membrane p13 triggers an inward K^+ current that leads to depolarization and increased respiratory chain activity (ETC), reactive oxygen species (ROS) production and lowering of the opening threshold of the permeability transition pore (PTP). wt, wild type; mut, mutant; K^+ , potassium, $\Delta\Psi$ mitochondrial membrane potential; ETC, electron transport chain, H^+ , protons, O_2 , molecular oxygen, O_2^- , superoxide anion, H_2O_2 hydrogen peroxide, PTP, permeability transition pore. (Adapted from Silic-Benussi et al., 2010a).

1.4.6 p12

p12 is a 99-amino acid hydrophobic protein coded by the x-I ORF. p12 localizes in the endoplasmic reticulum (ER) and in the Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001; Johnson et al., 2001). The protein is dispensable for HTLV-1 replication *in vitro*, but plays a key role in establishing a productive viral infection *in vivo* (Collins et al., 1996; Derse et al., 1997; Robek et al., 1998 Albrecht et al., 2000; Valeri et al., 2010).

p12 contains four proline-rich Src homology 3 (SH3) domains, two trans-membrane domains, two leucine zipper motifs and a calcineurin-binding motif. The SH3 domains are responsible for binding cellular signaling proteins (Koralnik et al., 1993). Together the trans-membrane domains and the leucine zipper motifs mediate targeting of the protein to the ER and other endomembrane compartments and dimerization (Ding et al., 2001; Johnson et al., 2001). The calcineurin-binding motif allows p12 to bind calcineurin, a Ca^{2+} -responsive protein phosphatase that controls the nuclear factor of activated T-cells (NFAT) activity, thus influencing gene expression of infected T-lymphocytes (Kim et al., 2003; Albrecht et al., 2002). In addition, p12 interacts with calreticulin and calnexin, two ER-resident proteins that regulate Ca^{2+} storage and signalling, and enhances Ca^{2+} release from the ER stores, resulting in increased cytosolic Ca^{2+} levels (Ding et al., 2001; Ding et al., 2002). This contributes to p12-mediated NFAT activation and ultimately decreases the threshold for T-cell activation (Nicot et al., 2005).

In the context of the whole virus, it is possible that p12 might act in concert with p13 to control intracellular Ca^{2+} homeostasis (Biasiotto et al., 2010). Combined expression of p12 and p13 might in fact enhance the amplitude and duration of cytosolic Ca^{2+} transients in response to TCR stimulation, resulting in enhanced NFAT activation.

p12 also binds the β and γ_c chains of the interleukin-2 receptor (IL-2R), thus reducing dependency on IL-2 for T-cell activation (Mulloy et al., 1996; Nicot et al., 2001). The IL-2R β chain in fact is involved in the activation of the Janus-associated kinases 1 and 3 (Jak1 and Jak3), which phosphorylate and activate the signal transducers and activators of transcription-5 (STAT-5). By binding to the cytoplasmic domain of IL-

2R β , p12 determines an increase in STAT-5 activity in the absence of IL-2, thus providing a proliferative advantage to T-cells (Nicot et al., 2001).

p12 was also shown to sequester free major histocompatibility complex class I heavy chains (MHC-I-Hc), preventing their binding to β 2-microglobulin. p12-bound MHC-I molecules are translocated in the cytosol and degraded by the proteasome. The overall result of this interaction is a decrease in functional MHC-I on the surface of HTLV-1-infected cells, which would favour their escape from CTL recognition and clearance by the immune system (Johnson et al., 2001). Furthermore, p12 causes a reduction in the expression of intracellular adhesion molecule 1 and 2 (ICAM-1 and ICAM-2), which mediate adhesion of natural killer (NK) cells to the infected cells, resulting in the protection of HTLV-1-infected CD4⁺ T-cells from NK-mediated cytotoxicity (Banerjee et al., 2007).

p12 is proteolytically cleaved to generate p8, a protein that enhances T-cell contact by interacting with lymphocyte function-associated antigen-1 (LFA-1) and ICAM-1 and mediates formation of intracellular conduits among T-cells, thereby increasing viral transmission (Van Prooyen et al., 2010) (see also Paragraph 1.2).

1.4.7 Hbz

The minus strand of the HTLV-1 genome contains one ORF located in the pX region. Transcription from antisense promoters generates at least two different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) (Figure 2B). (Cavanagh et al., 2006; Murata et al., 2006; Satou et al., 2006). HBZ is not necessary for viral replication or immortalization *in vitro*, but increases infectivity and viral persistence *in vivo* (Arnold et al., 2006).

Hbz sp1 is translated into a protein of 206 amino acids, while hbz us produces a protein of 209 amino acids. The two HBZ isoforms differ by seven amino acids in the N-terminus (Murata et al., 2006). The HBZ SP1 protein is more abundant and has a longer half-life than the HBZ US isoform (Yoshida et al., 2008). The HBZ protein contains an N-terminal transcriptional activation domain (AD), a central domain (CD) and a C-terminal basic ZIP domain (bZIP) (Gaudray et al., 2002). HBZ accumulates

in nuclear speckles through its NLS which contains two regions rich in basic amino acids and a DNA binding domain (Hivin et al., 2005).

The transcription of *hbz sp1* is dependent on Sp1 (Yoshida et al., 2008), a basal transcription factor critical for many TATA-less promoters (Boam et al., 1995; Liu and Cowell, 2000). The TRE sequence in the 3' LTR enhances transcription of the *hbz* anti-sense transcripts (Yoshida et al., 2008; Landry et al., 2009). However, this enhancing activity is relatively weak compared to that mediated by the TREs present in the 5' LTR promoter. This is consistent with the finding that transcription of the *hbz* gene is relatively constant in ATLL cases regardless of the expression levels of Tax (Saito et al., 2009).

HBZ interacts with a number of transcription factors, including CREB-2, p300/CBP, Jun family members, and NF- κ B (Matsuoka and Green, 2009).

HBZ binds CREB-2 and CBP/p300, respectively, through its bZIP domain (Lemasson et al., 2007; Hagiya et al., 2011) and via LXXLL-like motifs in its N-terminal region (Clerc et al., 2008). The interaction of HBZ with CREB-2 and CBP/p300 inhibits Tax-induced viral transcription from the 5' LTR by dissociating CREB-2 from TREs and counteracting the binding between CBP/p300 and Tax (Gaudray et al., 2002). Similarly, HBZ alters the effect of CREB proteins upon cellular gene transcription.

Through its bZIP domain, HBZ forms heterodimers with several AP-1 transcription factors, such as c-Jun, JunB and JunD, and modulates their activity (Basbous et al., 2003; Thebault et al., 2004). Binding of HBZ to JunB and c-Jun decreases their DNA binding activity by preventing their interaction with Fos (Matsumoto et al., 2005). Furthermore, HBZ reduces c-Jun stability by targeting it for proteasomal degradation (Matsumoto et al., 2005), and sequestres JunB within nuclear bodies (Hivin et al., 2007). In contrast, the interaction of HBZ with Jun-D stimulates its transcriptional activity (Thebault et al., 2004), and results in the activation of JunD-dependent cellular genes including human telomerase reverse transcriptase (hTERT) (Kuhlmann et al., 2007).

HBZ suppresses the activation of the canonical NF- κ B pathway through inactivation of the NF- κ B transcription factor p65. Inhibition of p65 by HBZ is mediated by two

different mechanisms: it inhibits the DNA binding of p65 and induces p65 ubiquitination and degradation by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65 (Zhao et al., 2009). Interestingly, HBZ does not inhibit the non-canonical NF- κ B pathway.

HBZ expression is associated with proliferation of ATLL cells *in vitro* and *in vivo* (Satou et al., 2006; Arnold et al., 2008). Mutational analyses of the *hbz* gene showed that *hbz* mRNA, rather than HBZ protein, has a growth-promoting effect on T-cells, possibly by upregulating the transcription of the E2F1 gene and its downstream targets (Satou et al., 2006). This function of HBZ as a non-coding transcript is consistent with the fact that most of the HBZ mRNAs are detected in the nuclear compartment (Rende et al., 2011).

An intriguing aspect of ATLL pathogenesis is the fact that Tax expression is not detected in about 60% of leukemia cases. Three mechanisms for inactivating Tax expression in ATLL cells have been described: 1) genetic changes (nonsense mutation, deletion, and insertion) of the *tax* gene (Takeda et al., 2004; Furukawa et al., 2001), 2) deletion of the 5' LTR (Tamiya et al., 1996; Miyazaki et al., 2007) and 3) DNA methylation of the 5' LTR (Koiwa et al., 2002; Taniguchi et al., 2005). Since Tax is the major target of CTLs *in vivo* (Kannagi et al., 1991), one hypothesis is that these mechanisms disrupting Tax expression facilitate the escape of ATLL cells from host CTL. Interestingly, analyses of HTLV-1 proviruses in ATLL cells revealed an intact *hbz* gene and lack of deletion or methylation of the 3' LTR, suggesting that *hbz* expression may be essential for the maintenance of the ATLL phenotype (Fan et al., 2010; Yoshida et al., 2008). A recent study described a significant association between the detection of HBZ-specific CD8⁺ T-cell responses with low viral load and asymptomatic carriage of HTLV-1. This result is consistent with the finding that the HLA class 1-restricted T-cell response plays an important part in limiting HTLV-1 viral load and the risk of HAM (Macnamara et al., 2010).

1.5. Role of the NF- κ B pathway in HTLV-1 pathogenesis

1.5.1 NF- κ B activation in ATLL

NF- κ B is involved in all stages of the natural history of ATLL, from initiation to invasion and metastasis (Xiao and Fu, 2011). Tax is the major driver of NF- κ B persistent activation during HTLV-1 infection (Paragraph 1.4.1.2), although Tax-independent mechanisms are also important, especially in acute ATLL (Paragraph 1.5.2).

During the early stages of ATLL, NF- κ B induces expression of genes involved in T-cell proliferation and survival such as IL-2R α (Ballard et al., 1988), IL-6, CD30, CD40, OX40/OX40L (Pankow et al., 2000; Ohtani et al., 1998), TNF- α , 4-1BB (Pichler et al., 2008), Bcl-xL, (Tsukahara et al., 1999); and cIAP (Wäldele et al., 2006). Furthermore, NF- κ B is important in Tax-induced IL-2-dependent and -independent cell growth as well as in HTLV-1-induced T-cell immortalization (Iwanaga et al., 1999; Robek and Ratner, 1999).

Persistent NF- κ B activation in ATLL also results in the bypass of cell cycle checkpoints and p53-mediated apoptosis through Tax-mediated effects (Paragraph 1.4.1.2) and through the mutual repression of NF- κ B and p53, resulting from the competition for CBP/p300 proteins (Webster and Perkins, 1999).

NF- κ B may also contribute to ATLL progression by causing DNA damage and oncogenic mutations through the induction of inflammation-mediated production of ROS and reactive nitrogen species. The pro-inflammatory effects of NF- κ B are also likely to play a role in the development of HAM/TSP (Banerjee et al., 2010). Interestingly, NF- κ B also activates many other pro-oncogenic molecules, such as c-Myc and phosphatidylinositol-3-kinase (PI3K) to induce expression of human telomerase reverse transcriptase (hTERT) (Sinha-Datta et al., 2004), and promotes the expression of genes involved in tumor progression and metastasis such as matrix metalloproteinase-9 (MMP-9) (Mori et al., 2002) and Fascin (Kress et al., 2011).

1.5.2 Tax-independent mechanisms of NF- κ B activation

Although Tax expression is lost in about 60% of ATLL cases, both the canonical and non-canonical NF- κ B pathways are still strongly activated in Tax-negative ATLL cells, suggesting Tax-independent mechanisms of NF- κ B activation (Hironaka et al., 2004). A recent study revealed the downregulation of miR-31 as an example of these mechanisms (Yamagishi et al., 2012). miR-31 specifically binds to the 3' UTR of the mRNA of the NIK kinase, and represses its expression, leading to the inhibition of the non-canonical NF- κ B pathway. In ATLL patients, miR-31 is profoundly repressed through the action of the Polycomb family of repressors, resulting in constitutive activation of the NF- κ B pathway.

Other mechanisms of Tax-independent NF- κ B activation may be attributed to stress conditions and epigenetic or genetic alterations caused by HTLV-1 infection. For example, DNA damage can lead to strong NF- κ B activation (Miyamoto, 2011). Furthermore, other viral proteins may play an indirect role by influencing cellular pathways that modulate NF- κ B activity (e.g. ROS homeostasis, Paragraph 1.6).

1.6 Crosstalk between NF- κ B signalling and ROS homeostasis

The NF- κ B pathway and ROS homeostasis are connected by a complex reciprocal crosstalk. On one hand, the expression of both pro-oxidant and antioxidant proteins is transcriptionally controlled by NF- κ B (Table 1); thus NF- κ B can influence intracellular ROS levels (Morgan and Liu, 2011).

NF- κ B target genes involved in redox homeostasis	
Pro-oxidants	Antioxidants
NADPH oxidase (NOX2)	Manganese Superoxide Dismutase (MnSOD)
Xanthine Oxidase/Dehydrogenase (XOR)	Copper-Zinc Superoxide Dismutase (Cu,Zn-SOD)
Inducible Nitric Oxide Synthase (iNOS)	Ferritin Heavy Chain (FHC)
Neuronal Nitric Oxide Synthase (nNOS)	Catalase
Cyclooxygenase-2 (COX-2)	Thioredoxin-1 (Trx1)
Arachidonate 12-lipoxygenase (LOX-12)	Thioredoxin-2 (Trx2)
Arachidonate 5-lipoxygenase (LOX-5)	Glutathione S-transferase pi (GST-pi)
Cytochrome p450 enzyme	Metallothionein-3 (MT3)
	NAD(P)H dehydrogenase [quinone] 1 (NQO1)
	Heme Oxygenase-1 (HO-1)
	Glutathione peroxidase-1 (Gpx1)
	Dihydrodiol dehydrogenase (DDH1)

Table 1. NF- κ B target genes involved in redox homeostasis. (Adapted from Morgan and Liu, 2011).

On the other hand, NF- κ B was the first transcription factor shown to be regulated by ROS (Schreck et al., 1991). ROS can modulate the NF- κ B pathway both positively and negatively, depending on the cellular context and on the nature of the oxidative stress.

In the nucleus, ROS-mediated oxidation of p50 on cysteine 62 results in reduced DNA binding capacity (Toledano et al., 1993; Matthews et al., 1993). A similar effect may result from S-nitrosylation of cysteine 62 by nitric oxide (NO) produced by the inducible nitric oxide synthase (iNOS). Since iNOS is an NF- κ B target gene (Table 1), this mechanism acts as a negative feedback loop of the NF- κ B pathway in the nucleus (Matthews et al., 1996). NF- κ B oxidation can be counteracted by the ROS scavenger

thioredoxin (TRX) and by the nuclear apurinic apyrimidinic endonuclease redox effector factor-1 (APE1-/Ref-1) (Matthews et al., 1992; Ando et al., 2008).

Interestingly, in Jurkat T-cells hydrogen peroxide (H_2O_2) mediates the phosphorylation of I κ B α on tyrosine 42 (rather than the canonic serine 32 and 36) by the Syk tyrosine kinase, resulting in calpain-mediated degradation of I κ B α (Schreck et al., 1991; Takada et al., 2003; Schoonbroodt et al., 2000). This atypical mechanism of NF- κ B activation has been observed in other cellular contexts after treatment with pervanadate (a potent tyrosine phosphatase inhibitor) and after hypoxia/reoxygenation (Imbert et al., 1996; Livolsi et al., 2001). In this case, c-Src phosphorylates I κ B α on tyrosine 42 and the phosphorylated I κ B α binds the SH2 domains of the p85 α regulatory subunit of PI3K, thus releasing NF- κ B and allowing its nuclear translocation (Béraud et al., 1999; Fan et al., 2003; Lluís et al., 2007).

In addition, the canonical IKK-mediated phosphorylation of I κ B α on serine 32 and 36 is enhanced by H_2O_2 in HeLa cells and in the CEM T-cell line. In HeLa cells, IKK activation is mediated by protein kinase D (PKD), which tyrosine-phosphorylates IKK β (Storz et al., 2003). In CEM T-cells, IKK activation relies on the expression of the lipid phosphatase SH2-containing inositol phosphatase-1 (SHIP-1) (Krystal, 2000). The mechanism through which SHIP-1 activates the IKK complex is still unknown. Other studies have shown that H_2O_2 inactivates IKK through oxidation of cysteine 179 of IKK β (Reynaert et al., 2006).

Other oxidants, such as hypochlorous acid (HOCl) (Barua, et al., 2001; Ogino et al., 2005), singlet oxygen (O_2^{\cdot}) (Volanti et al., 2002; Reelfs et al., 2004) and peroxynitrites (ONOO $^-$, PN) (Cooke et al., 2002; Jozsef and Filep, 2003; Hattori et al., 2004; Levrant et al., 2005) modulate NF- κ B with different mechanisms and outcomes depending on the cell type.

In addition to the direct effect of ROS on the NF- κ B pathway, pro-inflammatory cytokines such as interleukine 1 β (IL-1 β) and tumor necrosis factor α (TNF- α) induce NF- κ B activation in a ROS-dependent manner. The redox dependence of NF- κ B activation by IL-1 β is cell-type specific. In lymphoid cells, the main generator of ROS after exposure to IL-1 β is 5-lipoxygenase (5-LOX) (Bonizzi et al., 2000). In monocytic cells, ROS are produced mainly by the NADPH oxidase (Nox) complex

(Bonizzi et al., 1999); in epithelial cells, the major source of ROS is Nox2, a phagocytic NADPH oxidase also expressed in non-phagocytic cells (Li et al., 2006).

ROS play an important role in the TNF-induced cross-talk between JNK and NF- κ B activation. These two pathways have opposite biological roles: JNK activation promotes apoptosis through the mitochondrial-dependent pathway (Karin et al., 2005), whereas NF- κ B activation promotes cell survival. NF- κ B down-regulates JNK activation by suppressing TNF-induced ROS accumulation (Sakon et al., 2003; Ventura et al., 2004).

These powerful crosstalk mechanisms suggests that viral proteins controlling ROS homeostasis may add a further layer of complexity in the deregulation of the NF- κ B pathway in infected cells. Testing this hypothesis was a major aim of the present study.

2. AIM OF THE STUDY

HTLV-1 is the causative agent of adult T-cell leukemia/lymphoma (ATLL), an aggressive malignancy of mature CD4⁺ T-cells. Tax-mediated NF- κ B activation is a key determinant of HTLV-1 pathogenicity, especially in the initial phase of the neoplastic transformation process. Tax activates both the canonical and non-canonical NF- κ B pathways, promoting NF- κ B translocation to the nucleus and transcription of genes that favour T-cell proliferation and survival. Our previous studies showed that the p13 protein of HTLV-1 enhances mitochondrial ROS production, resulting in activation of primary T-cells. ROS constitute a homeostatic rheostat that controls the activity of several key pathways, including NF- κ B.

The work described in the present thesis was aimed at testing the hypothesis that Tax and p13 might act in concert to activate the NF- κ B signal transduction pathway in primary T-cells. To this end, we optimized a transfection protocol for primary T-cells using an innovative approach based on the electroporation of *in vitro*-transcribed RNA. The activation of the NF- κ B pathway was analysed by measuring the expression of the NF- κ B target genes CD25 by flow cytometry and 4-1BB by quantitative RT-PCR.

3. MATERIALS AND METHODS

3.1 Cell culture

The Jurkat T-cell line was maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS) (Life Technologies), 2 mM L-glutamine (GIBCO), 100 units/mL penicillin, and 20 units/mL streptomycin (Sigma-Aldrich). Peripheral blood mononuclear cells (PBMCs) from healthy donors were isolated by Ficoll-Hypaque gradient centrifugation using a standard protocol. After transfection, Jurkat cells were cultured in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine, whereas PBMCs were cultured in RPMI 1640 or IMDM (LONZA) supplemented with 10% FCS and 2 mM L-glutamine.

3.2 Plasmids

RNA to be transfected was *in vitro*-transcribed using a system based on vector pST1-GFP (Figure 5) (Holtkamp et al., 2006). pST1-GFP contains the T7 phage promoter sequence at the 5' end and a 120 base pair-long poly(A) tail at the 3' of the coding sequence of interest. In addition, the pST1-vector contains two tandemly repeated human β -globin untranslated regions (UTRs) between the coding sequence and the poly(A) tail that increase the stability of the *in vitro*-transcribed RNA. This vector can be used to *in vitro*-transcribe genes of interest, ensuring high transcript stability and translational efficiency (Holtkamp et al., 2006). The vector can be linearized by restriction digestion with the Sap I enzyme, whose unique site is located downstream of the poly(A).

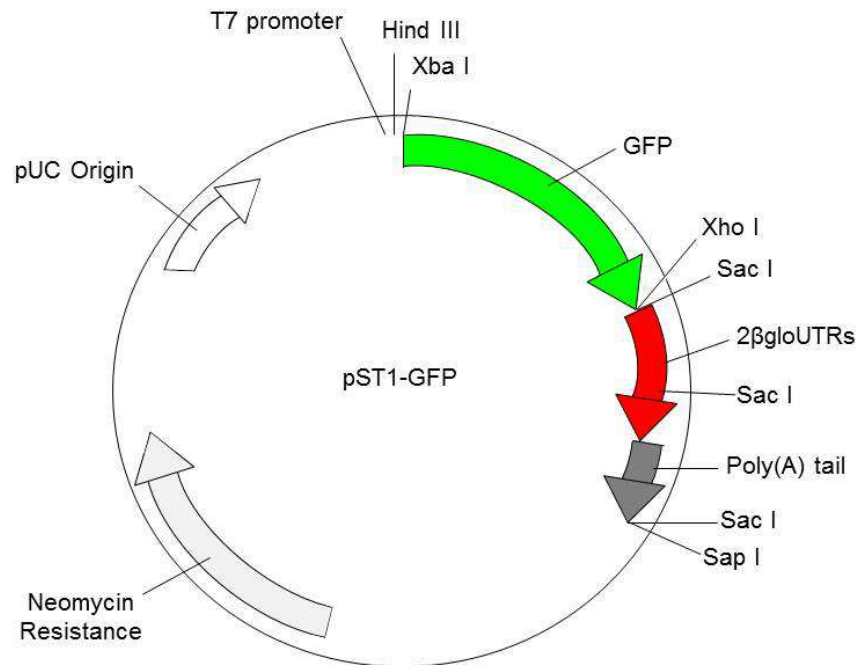


Figure 5. The pST1-GFP plasmid. The vector contains the T7 phage promoter sequence at the 5' and a 120 base pair-long poly(A) tail (grey arrow) at the 3' of the coding sequence of the green fluorescent protein (GFP, green arrow). Two tandemly repeated human β -globin UTRs (2 β gloUTRs, red arrow), between the GFP coding sequence and the poly(A) tail, increase the stability of the *in vitro*-transcribed RNA. The Sap I unique site, located downstream of the poly(A) tail, can be used to linearize the vector (Adapted from Holtkamp et al., 2006).

The coding sequence of Tax was amplified by end-point PCR from the pcTax plasmid (Rimsky et al., 1988) using the following 5' tagged set of primers: Xba I-Tax forward, 5'-ATATCTAGACTGCCATGGCCCACTT-3' and Tax-Xho I reverse, 5'-GTCTCTCGAGTCAGACTTCTG-3'. The resultant Xba I-Tax-Xho I fragment was cloned in the intermediate vector pCR-Blunt (Life Technologies) and digested with Xba I and Xho I. The product of the digestion was then cloned in the final vector pST1-delta-GFP. The pST1-delta-GFP vector was generated from the pST1-GFP following deletion of the green fluorescent protein (GFP) coding sequence through an Xba I-Xho I restriction digestion.

The coding sequence of p13GFP was obtained from the pcDNAp13GFP plasmid (Silic-Benussi et al., 2010b) through an Hind III-Xho I restriction digestion. The p13GFP fragment was cloned in the intermediate pCR-Blunt vector and cut with Hind III and Xho I to generate the Hind III-p13GFP-Xho I fragment. This fragment was then ligated to the pST1-delta-GFP2 vector previously obtained from the pST1-GFP digested with Hind III-Xho I. Attempts to express untagged p13 using this system were unsuccessful.

The pST1-Tax and pST1-p13GFP plasmids, together with the control pST1-GFP plasmid, were linearized using the FastDigest Sap I (Lgu I) restriction enzyme (Fermentas, Thermo Scientific), purified from an agarose gel using the GENECLAN Turbo kit (MP Biomedicals) and quantitated using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

3.3 *In vitro* transcription and capping

The Sap I-linearized pST1-Tax, pST1-p13GFP, and pST1-GFP plasmids were subjected to *in vitro*-transcription and capped using the mMACHINE T7 kit (Ambion, Life Technologies). The reaction was performed for 2 hours at 37°C following the manufacturer's recommendations (Table 2). Thereafter, 1 µl of TURBO DNase (Ambion, Life Technologies) was added, and samples were incubated for 15 minutes at 37°C to remove DNA templates. The *in vitro*-transcribed RNAs were then purified using the RNeasy MinElute Cleanup kit (Qiagen) according to the manufacturer's instruction, and used in transfections.

Component	Volume
Nuclease-free Water	to 20 μ l
2X NTP/CAP	10 μ l
10X Reaction Buffer	2 μ l
Linear template DNA	1 μ g
Enzyme Mix	2 μ l

Table 2: *In vitro* transcription and capping reactions. The 2X NTP/CAP is a buffered solution that contains 15 mM ATP, 15 mM CTP, 15 mM UTP, 3 mM GTP and 12 mM cap analog [m7G(5')ppp(5')G]; the 10X reaction buffer contains salts, buffer, dithiothreitol, and other components; the enzyme mix is a 50% glycerol buffered solution containing the T7 RNA polymerase, an RNase inhibitor and other components.

3.4 Transfections

The transfection experiments described in the present thesis were realized as part of a collaborative project with Dr. A. Kress at the Institute of Clinical and Molecular Virology, University of Erlangen-Nuremberg, Erlangen, Germany, in the context of the DAAD-Vigoni Program, German-Italian University Center. The techniques described in the present paragraph were developed and carried out jointly by the two laboratories.

The RNA transfection protocol was set up in Jurkat T-cells and freshly isolated PBMCs using the Neon transfection system (Life Technologies). For Jurkat cells, the day of the transfection cells were counted and resuspended in aliquots of 5×10^6 cells in 100 μ l of T buffer (Neon, Life Technologies) for each electroporation. Transfections were carried out in 100 μ l tip-electrodes of the Neon transfection system with one 1410-V, 30-msec pulse, following the manufacturer's recommendations. For PBMCs, 3×10^6 cells were resuspended in 10 μ l of T buffer for each electroporation, and transfections were carried out in 10 μ l tip-electrodes of the Neon transfection system with one 1910-V, 30-msec pulse. The same amount of GFP RNA or pmaxGFP plasmid (Amaxa, LONZA) were transfected to a total of 4 μ g for Jurkat cells and 3 μ g for PBMCs. After transfections, cells were seeded in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamine, to a final density of 1.25

$\times 10^6$ cells/ml for Jurkat cells and 1×10^6 cells/ml for PBMCs and harvested at different time points. Cell viability and transfection efficiency were evaluated by flow cytometry.

In order to investigate the functional interactions of Tax and p13 on the NF- κ B pathway, the *in vitro*-transcribed Tax and p13GFP RNAs were transfected in freshly isolated PBMC and Jurkat T-cells in parallel. The day of the transfection cells were counted, centrifuged and resuspended in aliquots of 6×10^6 cells for each electroporation in 800 μ l of Optimem reduced medium without phenol red (GIBCO, Life Technologies). Cells were incubated for 30 minutes at 37°C before transfection. Meanwhile, RNA was prepared for the following transfection mixtures: (1) GFP; (2) Tax + GFP; (3) p13GFP + GFP; (4) Tax + p13GFP. A 1-to-1 ratio for Tax and p13GFP RNA was maintained. A total of 6 μ g RNA for Jurkat and 15 μ g for PBMCs were transfected. Transfections were carried out in 4-mm cuvettes using the Gene Pulser Xcell electroporation system (Biorad) with one 500-V, 5-msec square-wave pulse, following the manufacturer's recommendations. After transfection, cells were seeded as described in Paragraph 3.1, to a final density of 1×10^6 cells/ml for Jurkat cells and 2×10^6 cells/ml for PBMCs and harvested after 24, 48 and 72 hours.

3.5 Analysis of the NF- κ B pathway by flow cytometry

The activation of the NF- κ B pathway was analyzed by flow cytometry, through the detection of the CD25 marker, which is a known target of NF- κ B (Ballard et al., 1988, Ruben et al., 1988). Cell activation was also monitored using the CD38 marker. 500,000 cells were harvested 24, 48 and 72 hours after transfection, washed in PBS and incubated for 30 min on ice with mouse anti-human CD38-PE-Cy7 monoclonal antibody (1:10, in 10% FCS-PBS) (Biolegend) and mouse anti-human CD25-PE monoclonal antibody (1:10, in 10% FCS-PBS) (MACS, Miltenyi Biotec). CD38 (45-kDa type II transmembrane glycoprotein) is expressed on activated T-cells, plasma cells, monocytes and macrophages. CD25, the interleukin-2 receptor alpha chain (IL-2R α), is expressed on activated T- and B-cells and at a lower level on activated monocytes. In order to identify the T-cell population, PBMCs were also stained with mouse anti-human CD3-Alexa Fluor 700 (1:50, in 10% FCS-PBS) (Biolegend).

Samples were analysed by flow cytometry using a BD LSRII apparatus (BD Biosciences) equipped with 488-nm, 633-nm, and 405 nm lasers. For each sample, 10,000 GFP positive/transfected events were examined. Data were analyzed using the FACSExpress flow cytometry analysis software.

The specific effects of the transfection on the percentage of CD25-positive cells was expressed as a fold change (CD25+FC) and calculated as follows:

$$CD25+FC = \frac{\frac{(\% CD25+GFP^+)}{(\% CD25+GFP^+) + (\% CD25^- GFP^+)}}{\frac{(\% CD25+GFP^-)}{(\% CD25+GFP^-) + (\% CD25^- GFP^-)}}$$

The same formula was applied to calculate the CD38+FC.

3.6 Analysis of the NF-κB pathway by quantitative RT-PCR

The activation of the NF-κB pathway was further analyzed by quantitative RT-PCR of 4-1BB, which codes for a co-stimulatory molecule of the tumor necrosis factor receptor family 9 (TNFRSF9). 4-1BB is up-regulated in HTLV-1-infected cells and patients and its expression is stimulated by Tax through the NF-κB pathway (Pichler et al., 2008).

RNA from transfected samples was extracted using the RNeasy Micro kit (Qiagen), which includes a DNAase treatment step, and quantitated by spectrophotometry. RNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen) and random hexamers.

The 4-1BB target gene was analysed using a PrimeTime qPCR Assays kit (IDT, Integrated DNA Technology). The kit contained two primers and a double-quenched probe for the gene of interest. The double-quenched probe was 5' end-labeled with FAM, 3' end-labeled with Iow Black FQ (IBFQ) and contained an internal ZEN quencher linked to a position nine base pairs downstream of the 5' end. The shortened distance from FAM, combined with the 3' quencher led to an increased signal-to-

noise ratio of the system. As an internal control, GAPDH mRNA was analyzed in parallel using a GAPDH PrimeTime qPCR Assays kit (IDT, Integrated DNA Technology). The GAPDH probe was 5' end-labeled with HEX, 3' end-labeled with IBFQ, and contained an internal ZEN quencher.

PCR reactions were performed with a Light Cycler II 480 thermal cycler (Roche) using 4 µl of each diluted RT sample, 10 µl of Light Cycler 480 Master Mix (Roche), and 1 µl of primers and probes mix; each reaction was performed in duplicate. The cycling conditions comprised an initial denaturation step at 95°C for 10 minutes and 40 cycles at 95°C for 10 seconds, 60°C for 30 seconds, 72°C for 1 second. The absolute quantification method was applied by using standard curves constructed from 5-fold serial dilutions of plasmids containing the GAPDH and 4-1BB amplicons.

The absolute copy number of the 4-1BB transcript was divided by the GAPDH copy number to generate the normalized copy number (NCN) value. The NCN for 4-1BB was then divided by the fraction of GFP-positive cells measured by flow cytometry for each sample.

Tax and p13 expression was verified by end-point RT-PCR using the following set of primers: Tax forward, 5'-CCTGTCTTTTCCAGACCCC-3'; Tax reverse, 5'-GGGTGGCAAAAATCACGTG-3'; p13GFP forward, 5'-CTATGTTTCGGCCCGCCT-3'; p13GFP reverse, 5'-CTGGGTGCTCAGGTAGTGGT-3'. In order to discriminate Tax and p13 in the co-transfected samples, the Tax primers were designed in a region downstream of the p13 stop codon, which is not present in the p13 expression plasmid.

4. RESULTS

4.1 Introduction of Tax and p13 into primary T-cells

The aim of this study was to test the hypothesis that HTLV-1 Tax and p13 might act in concert to activate the NF- κ B signal transduction pathway in normal T-cells. Tax is the major driver of NF- κ B's persistent activation, which promotes survival of infected T-cells and tumorigenesis (Sun and Yamaoka, 2005). Our previous studies showed that the p13 protein of HTLV-1 enhances mitochondrial ROS production, resulting in activation of normal T-cells (Silic-Benussi et al., 2010b). ROS constitute a homeostatic rheostat that controls the activity of several key pathways, including the NF- κ B pathway. Thus, we hypothesized that ROS production mediated by p13 could contribute to Tax's induction of the NF- κ B pathway in primary T-cells.

To this end, we optimized a transfection protocol for primary T-cells using an innovative approach based on the electroporation of *in vitro*-transcribed RNA. The coding sequences of Tax and p13 were cloned into the pST1-vector (Figure 5, Materials and Methods, Par 3.2), which allows efficient production of highly stable *in vitro*-transcribed RNA (Holtkamp et al., 2006). Transfection of *in vitro*-transcribed RNA has emerged in recent years as a promising technique for gene transfer in cells refractory to standard methods of DNA transfection, including primary T-cells and dendritic cells (Van Tendeloo et al., 2001; Holtkamp et al., 2006; Zhao et al., 2006; Rowley et al., 2009). We first tested the protocol in Jurkat T-cells, which can be easily transfected through electroporation, and then applied it to freshly isolated peripheral mononuclear cells (PBMCs).

4.1.2 Electroporation of *in vitro*-transcribed RNA in Jurkat T-cells

Jurkat T-cells were transfected with the plasmid pmaxGFP or with GFP RNA, obtained from the *in vitro* transcription of the pST1-GFP vector (Materials and Methods, Par 3.2). Cell viability and the percentage of transfected cells were estimated through flow cytometry, by measuring the changes in side scatter (SSC)/forward scatter (FSC) (Figure 6, left) and GFP fluorescence (Figure 6, right), respectively, 24 hours after the transfection.

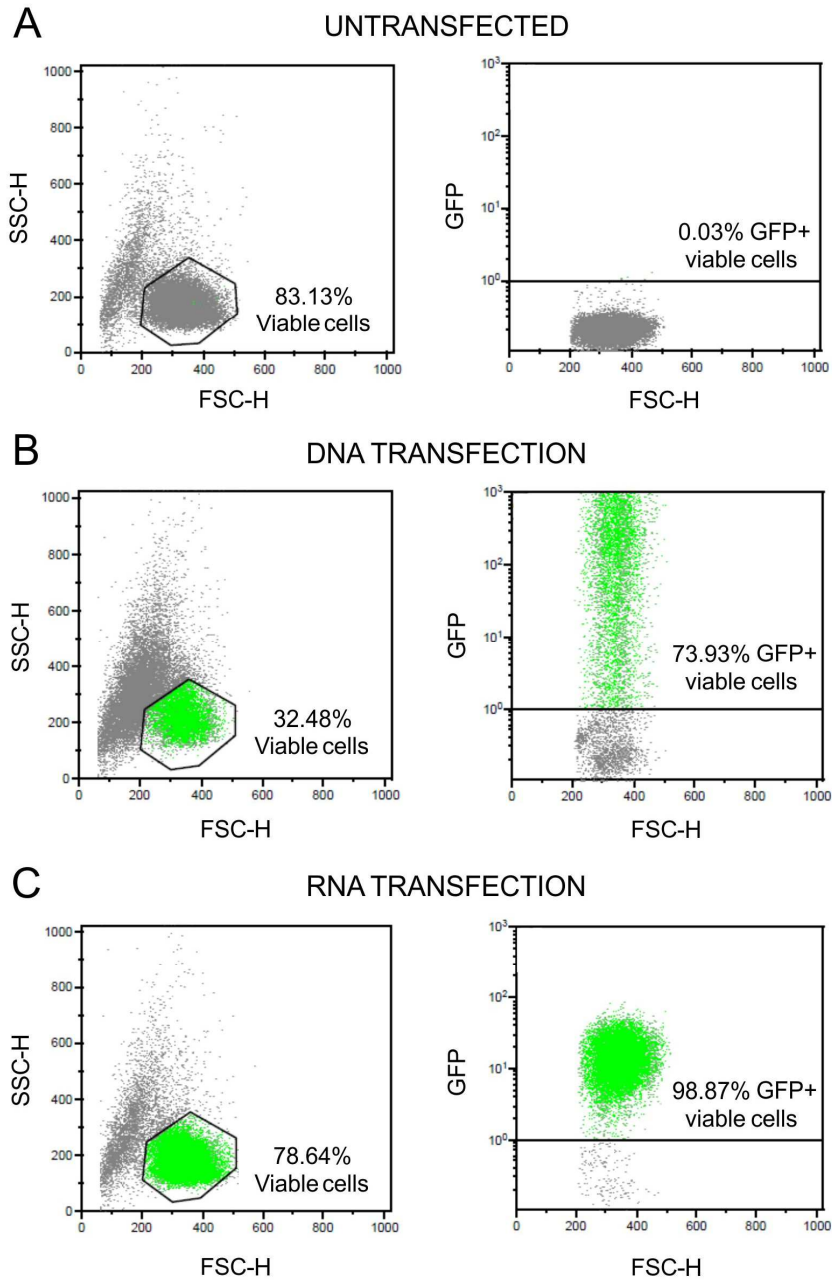


Figure 6. pmaxGFP DNA and GFP RNA transfection in Jurkat T-cells. Dot plot of the side/forward scatter (left) and GFP fluorescence (right) of untransfected (**A**), pmaxGFP - (**B**) and GFP RNA-transfected cells (**C**) 24 hours after electroporation. In the side/forward scatter plots (left) viable cells are gated and their percentage is shown. GFP-positive viable cells are indicated as green dots and their percentage is shown (right).

Figure 7 shows the analysis over time of the viability (estimated through the changes in the side/forward scatter) (Figure 7A) and of the transfection efficiency (Figure 7B), measured as percentage of GFP positive cells in the viable fraction, 24, 48 and 72 hours after transfection.

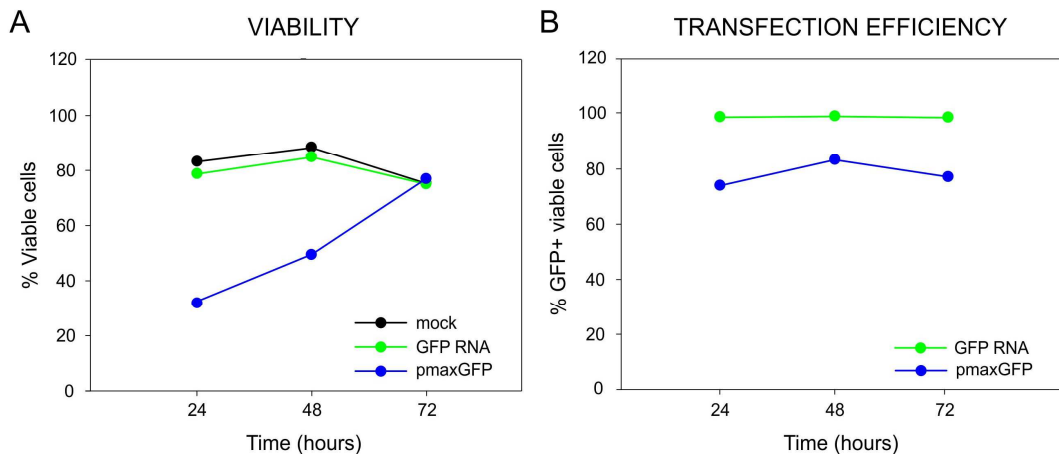


Figure 7. Viability and transfection efficiency of pmaxGFP- and GFP RNA- transfected Jurkat T-cells. A: Temporal variation in the percentage of viable cells (24, 48 and 72 hours) in untransfected (black line), pmaxGFP- (blue line) or GFP RNA-transfected samples (green line) Viability was measured from the changes in the side/forward scatter by flow cytometry. **B.** Percentage of GFP-positive cells in the viable fraction 24, 48 and 72 hours after transfection of pmaxGFP (blue line) or GFP RNA (green line).

Results showed that 24 hours after transfection, the percentage of viable cells in the RNA-transfected population was comparable to the untransfected sample (Figure 6A and C). This was also confirmed at the later time points (Figure 7A), indicating that RNA transfection does not significantly affect cell viability. On the contrary, pmaxGFP transfection dramatically reduced cell viability 24 hours after transfection (Figure 6B), although viability increased at the later time points, possibly due to the proliferation of surviving cells and loss of signal from dead cells as they were reduced to debris (Figure 7A).

RNA electroporation reached a higher transfection efficiency (>90% GFP-positive cells), compared to DNA transfection (~70% GFP-positive cells) (Figure 7B). Furthermore, RNA transfection produced a more homogenous level of expression,

with the GFP fluorescence signal spanning one log of the intensity scale (Figure 6C), compared to the pmaxGFP signal that spanned over three logs (Figure 6B). These results confirmed that the RNA transfection technique combines a high transfection efficiency with low transfection-related toxicity.

4.1.2 Electroporation of *in vitro*-transcribed RNA in normal T-cells

The RNA transfection protocol was then tested on freshly isolated PBMCs, which are usually refractory to standard DNA transfection techniques. In particular, electroporation of DNA often results in a high toxicity combined with a low transfection efficiency, thus rendering subsequent analyses extremely difficult. Given the high transfection efficiency and good viability observed in Jurkat T-cells electroporated with RNA, we decided to apply this method of transfection to normal T-cells.

Freshly isolated PBMCs were electroporated with pmaxGFP or *in vitro*-transcribed GFP RNA and viability and transfection efficiency were assessed, as described for Jurkat cells (Paragraph 4.1.1). Figure 8 shows the side scatter (SSC)/forward scatter (FSC) values (left) and the GFP fluorescence (right) of untransfected (Figure 8A), pmaxGFP- (Figure 8B) or GFP RNA-transfected PBMCs (Figure 8C), 24 hours after electroporation.

As previously observed for Jurkat cells, RNA transfection yielded superior results compared to plasmid DNA transfection, both in terms of cell viability (48.94% RNA versus 8.21% DNA) and transfection efficiency (76% RNA versus 29.53% DNA) (Figure 8B and C). In this case, the percentage of viable cells was dramatically reduced in the DNA-transfected population, which also affected the transfection efficiency (Figure 8B). Again, RNA transfection led to a more homogenous level of expression, with the GFP fluorescence signal spanning one log of the intensity scale (Figure 8C) compared to over three logs in the DNA-transfected sample (Figure 8B). These results confirmed that RNA electroporation was a suitable technique for experiments aimed at investigating the functional interaction of Tax and p13 in the context of primary T-cells, a major target of viral infection *in vivo*.

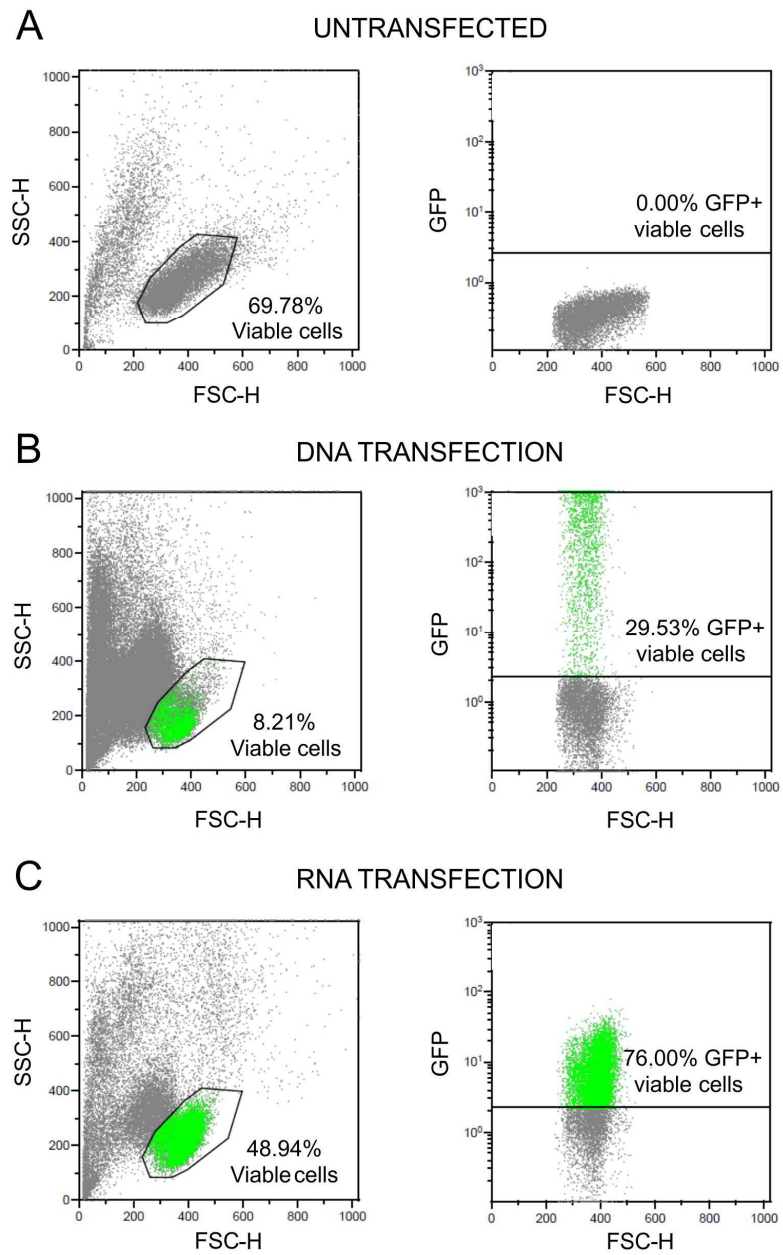


Figure 8. pmaxGFP DNA and GFP RNA transfection in PBMCs. Dot plot of the side/forward scatter (left) and GFP fluorescence (right) of untransfected (**A**), pmaxGFP - (**B**) and GFP RNA-transfected cells (**C**) 24 hours after electroporation. In the side/forward scatter plots (left) viable cells are gated and their percentage is shown. GFP-positive viable cells are indicated as green dots and their percentage is shown (right).

4.2 Functional interactions of Tax and p13 in T-cells

In order to investigate the functional interactions between Tax and p13 on the NF- κ B signal transduction pathway, freshly isolated PBMCs from healthy donors and Jurkat T-cells were transfected with the Tax and/or p13GFP RNA. An RNA expressing GFP was included in the control and in the transfections containing Tax or p13GFP alone. Cells were harvested at three time points (24, 48, 72 hours after transfection). The activation of the NF- κ B pathway was evaluated by flow cytometry through the detection of CD25, and by quantitative RT-PCR to detect expression of the 4-1BB mRNA. Cell activation was monitored by the CD38 marker.

4.2.1 Effects of Tax and p13 on CD25 expression

CD25 (alfa-chain of the IL-2 receptor) is expressed on activated T- and B-cells and at a lower level on activated monocytes. The IL-2R α promoter was the first shown to be activated by Tax in an NF- κ B-dependent manner (Ballard et al., 1988, Ruben et al., 1988). The CD3 marker was used in order to identify the T-cell population during the analysis. We estimated the effects on CD25 expression in the population of transfected cells by calculating the percentage of CD25 positive (CD25+) cells among the GFP-positive (GFP+) population and the percentage of CD25+ cells among the GFP-negative (GFP-) population of T-cells (CD3+) as described in Materials and Methods, Paragraph 3.5. These percentages were used to calculate the Fold Change in CD25+ cells (CD25+FC) (Figure 9).

Results obtained showed that neither Tax nor p13 was sufficient to activate the NF- κ B pathway in primary T-cells (Figure 9A and B). However, the co-transfection of the two RNAs resulted in a synergistic activation of the pathway that was significant at the later time point observed (72 hours) (Figure 9A and B, $p = 0,025$ Student T-test). This result was consistent with our hypothesis that p13 renders primary T-cells permissive for Tax-triggered activation of the NF- κ B pathway.

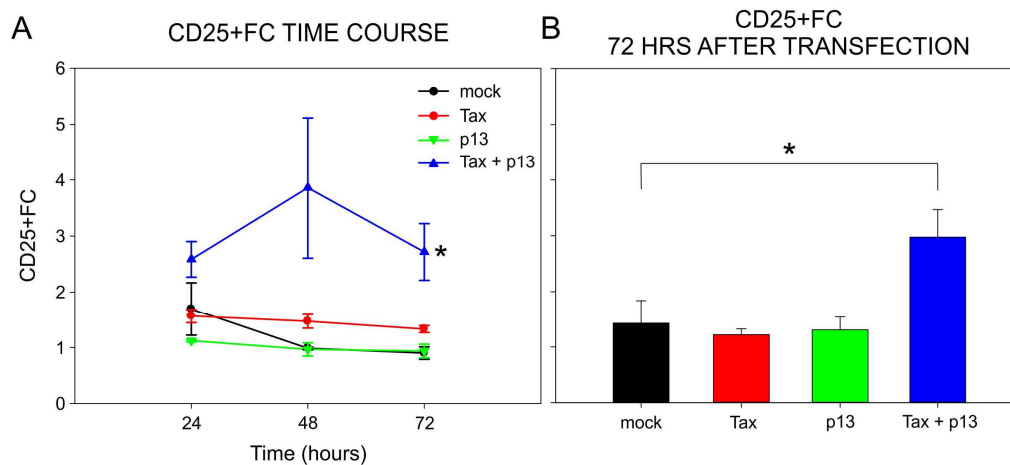


Figure 9. Effects of Tax and/or p13 expression in normal T-cells. **A:** Temporal variation in the CD25+FC of normal T-cells (CD3+) transfected with GFP alone (black line), or with GFP plus Tax (red line), p13GFP (green line), or Tax and p13GFP RNAs (blue line). **B:** CD25+FC of normal T-cells (CD3+) transfected with GFP alone (black bar), or with GFP plus Tax (red bar), p13GFP (green bar), or Tax and p13GFP RNAs (blue bar) 72 hours after transfection. Mean values of 3 independent experiments and standard error bars are shown. The asterisks indicate a statistically significant difference calculated with the Student T-test.

In addition to being a transcriptional target of NF- κ B, CD25 is also an early marker of T-cell activation. To test the possible effects of Tax and p13 on T-cell activation, we used flow cytometry to measure the expression of CD38. CD38 is 45-kDa type II transmembrane glycoprotein expressed in the intermediate-late stages of T-cell activation, as well as in plasma cells, monocytes and macrophages. Jurkat cells, which exhibit a constitutively activated CD38+ phenotype, were used as a control. Also in this case, the CD3 marker was used in order to identify the T-cell population within PBMCs. The CD38+FC was calculated as described in Paragraph 3.5, Materials and Methods. Results of this analysis confirmed the synergy of Tax and p13, although the effect on normal T-cell activation was not as prominent as that measured with CD25 (Figure 10A and B), suggesting that, within the time frame of our experiments, Tax and p13 drove T-cells into an early-intermediate stage of activation. As expected, in Jurkat T-cells, which exhibit a constitutively CD38+ activated phenotype, the co-expression of Tax and/or p13 had no effect on CD38 expression (Figure 10C).

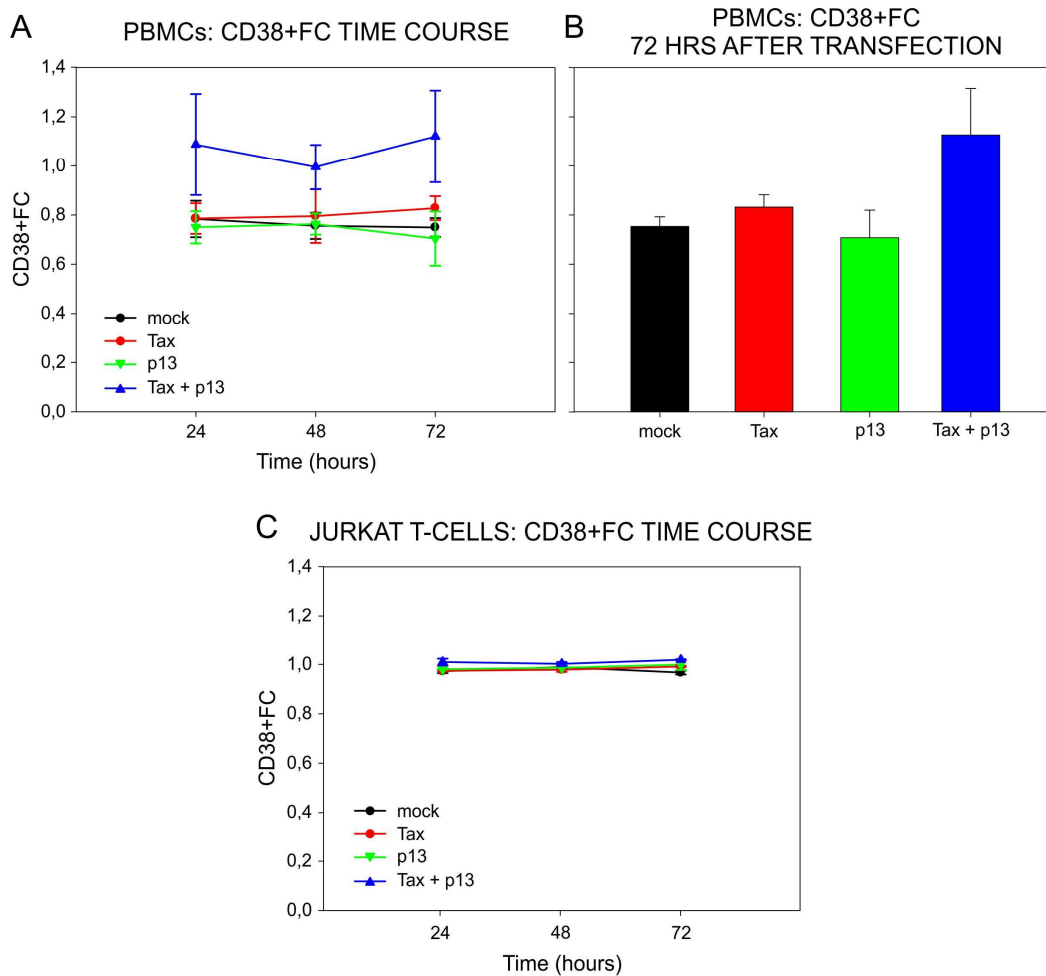


Figure 10. Effects of Tax and/or p13 on CD38 expression. **A:** Temporal variation in the CD38+FC of normal T-cells (CD3+) transfected with GFP alone (black line), or with GFP plus Tax (red line), p13GFP (green line), or Tax and p13GFP RNAs (blue line). **B:** CD38+FC of normal T-cells (CD3+) transfected with GFP alone (black bar), or with GFP plus Tax (red bar), p13GFP (green bar), or Tax and p13GFP RNAs (blue bar) 72 hours after transfection. **C:** Temporal variation in the CD38+FC of Jurkat T-cells transfected with GFP alone (black line), or with GFP plus Tax (red line), p13GFP (green line), or Tax and p13GFP RNAs (blue line). Mean values of 3 independent experiments and standard error bars are shown.

4.2.2 Effects of Tax and p13 on 4-1BB expression

To further investigate the effects of Tax and/or p13 on the NF- κ B pathway, we analysed the expression of 4-1BB, a known NF- κ B target gene, that is conspicuously activated by Tax in HTLV-1 infected cells and patients (Pichler et al., 2008). The expression of 4-1BB was analysed by quantitative RT-PCR as described in Paragraph 3.6, Materials and Methods. The absolute copy number of the 4-1BB transcript was divided by the GAPDH copy number to generate the normalized copy number (NCN) value. As all transfected cells were expected to show a GFP signal, we estimated the effects on 4-1BB expression in the transfected cell population, by dividing the 4-1BB NCN for the percentage of GFP positive cells measured by flow cytometry. Figure 11 shows the results of this analysis in PBMCs (Figure 11A) and Jurkat T-cells (Figure 11B) 24 hours after transfection.

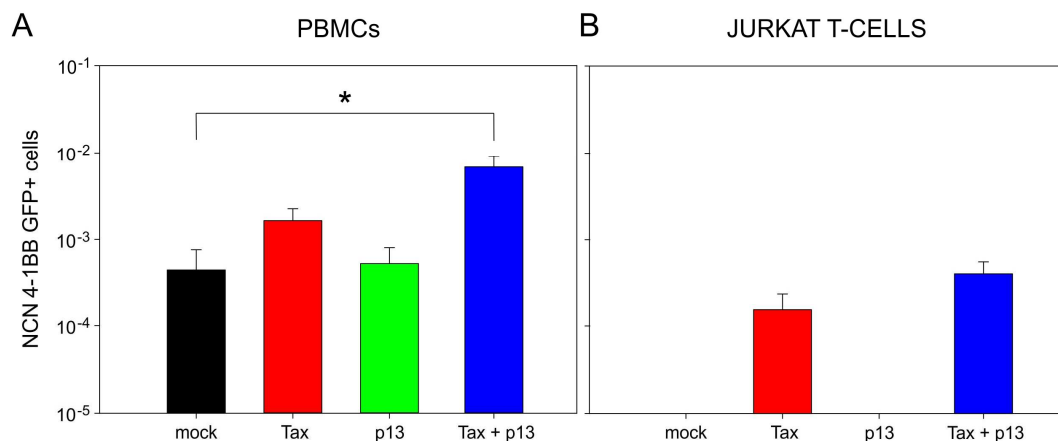


Figure 11. Effects of Tax and/or p13 on the expression of 4-1BB. **A:** NCN of 4-1BB divided for the percentage of GFP-positive PBMCs transfected with GFP alone (black bar), or with GFP plus Tax (red bar), p13GFP (green bar), or Tax and p13GFP RNAs (blue bar) 24 hours after transfection. **B:** NCN of 4-1BB divided for the percentage of GFP-positive Jurkat T-cells transfected with GFP alone (black bar), or with GFP plus Tax (red bar), p13GFP (green bar), or Tax and p13GFP RNAs (blue bar) 24 hours after transfection. Mean values of 3 independent experiments and standard error bars are shown. The asterisk indicates a statistically significant difference calculated with the Student T-test.

Pilot experiments indicated that the effects on 4-1BB expression were rapid and transient, with expression decreasing after 24 hours (data not shown). However, 24 hours after transfection, the level of 4-1BB expression induced by Tax and p13 co-transfection in normal T-cells was significantly higher than in the control (Figure 11A, $p=0,010$, Student T-test), while Tax or p13 alone did not significantly affect 4-1BB expression.

Interestingly, in Jurkat T-cells, Tax alone was able to induce a powerful upregulation of 4-1BB, which was expressed at levels below the threshold of detection in the mock-transfected sample; p13 alone had no effect on 4-1BB expression, and co-transfection of p13 did not significantly change the effect of Tax (Figure 11B).

Taken together, these findings suggest that, in contrast to the well-established role of Tax as an activator of the NF- κ B pathway in tumor cell lines (e.g. Jurkat), in the context of normal T-cells, the induction of NF- κ B target genes requires the concerted action of Tax and p13.

5. DISCUSSION

The aim of the present study was to gain insights into the interplay of HTLV-1 proteins in the persistent activation of the NF- κ B pathway, which represents a hallmark of HTLV-1-infected cells (Watanabe et al., 2005). According to current models, Tax is the major driver of the persistent activation of both the canonical and non-canonical NF- κ B pathways, resulting in increased survival of infected T-cells and tumor promotion (Sun and Yamaoka, 2005). Evidence for Tax-independent mechanisms for NF- κ B activation came from the finding that both NF- κ B pathways are strongly activated in Tax-negative ATLL cells, which are detected in approximately 60% of the patients (Hironaka et al., 2004). A recent study demonstrated the frequent loss of miR-31 expression in ATLL. As miR-31 is a physiological negative regulator of the NIK kinase, its loss could contribute to the Tax-independent activation of NF- κ B (Yamagishi et al., 2011). In addition, the viral protein HBZ was shown to modulate the canonical arm of the pathway by inhibiting the DNA binding capacity of p65 and promoting its degradation (Zhao et al., 2009). This inhibitory role of HBZ is of particular interest in the context of Tax-negative cells, which usually express HBZ protein (Fan et al., 2010; Yoshida et al., 2008). In this context in fact, the HBZ-mediated inhibition of the canonical NF- κ B may render the non-canonical pathway (activated by Tax-independent mechanisms) predominant. In the present study, we asked whether p13 might play a role in NF- κ B activation through its ability to modulate ROS homeostasis, which is connected to the NF- κ B pathway through a complex reciprocal crosstalk (Morgan and Liu, 2011). p13 activates primary T-cells in a ROS-dependent manner (Silic-Benussi et al., 2010b). Thus, we hypothesized that p13 could act in concert with Tax on the NF- κ B pathway in primary T-cells.

To this end, we optimized a transfection protocol for primary T-cells using an innovative approach based on the electroporation of *in vitro*-transcribed RNA. Transfection of *in vitro*-transcribed RNA has recently emerged as a promising technique for gene transfer in cells refractory to standard methods of DNA transfection, including primary T-cells and dendritic cells (Van Tendeloo et al., 2001;

Holtkamp et al., 2006; Zhao et al., 2009; Rowley et al., 2009). The RNA transfection protocol was first tested in Jurkat T-cells, and then applied to freshly isolated peripheral mononuclear cells (PBMCs). The comparison of the results obtained with an *in vitro*-transcribed RNA coding for GFP to those obtained with a plasmid coding for GFP revealed that the former technique yielded far better results in terms of percentage of transfected cells, homogeneity of transgene expression levels and cell viability. Building on these encouraging results, we employed RNA electroporation to express Tax and p13 in primary T-cells and investigated their functional interaction on the NF- κ B pathway.

As a readout of NF- κ B activation, we measured the expression of CD25 by flow cytometry and 4-1BB by quantitative RT-PCR. CD25 (alfa-chain of the IL-2 receptor) is a marker of ATLL cells and its promoter was the first shown to be activated by Tax in an NF- κ B-dependent manner (Ballard et al., 1988; Ruben et al., 1988). The 4-1BB gene codes for a co-stimulatory molecule of the tumor necrosis factor receptor family 9 (TNFRSF9). Co-stimulatory receptors of the TNFR superfamily are crucial mediators of proliferation and survival signals mediating T-cell activation and maintenance of long-lived T-cell subtypes. 4-1BB is up-regulated in HTLV-1-infected cells and patients and its expression is stimulated by Tax through the NF- κ B pathway (Pichler et al., 2008). The analysis of CD25 positivity and of 4-1BB expression in Tax- and/or p13-transfected primary T-cells revealed that Tax alone is not sufficient to activate the expression of these NF- κ B targets, suggesting that a further stimulus is needed to prime T-cells for Tax-activity. Our results suggest that this stimulus may be provided by p13. In fact, Tax and p13 expression resulted in a significant synergistic activation of the NF- κ B pathway in primary T-cells. The timing of NF- κ B activation was earlier for the 4-1BB mRNA (24 hours after transfection) than for the CD25 surface marker (72 hours after transfection). This could be explained by the difference in the read-out systems used (one detecting mRNA, the other protein levels) or to different kinetics of activation of the two NF- κ B-target genes. Interestingly, the synergistic effect of Tax and p13 was observed only in primary T-cells, whereas in Jurkat T-cells, Tax expression alone was sufficient to increase 4-1BB expression and p13 co-expression did not produce any

additive effect, suggesting that Jurkat T-cells are already permissive for Tax activity. These findings are consistent with previous reports on Tax-mediated activation of 4-1BB expression in Jurkat T-cells (Pichler et al., 2008).

As the CD25 is also a T-cell activation marker, the co-expression of Tax and p13 proteins also resulted in T-cell activation. To better investigate this aspect, we also evaluated the effects of Tax and p13 on the expression of CD38, a type II transmembrane glycoprotein expressed on activated T-cells, plasma cells, monocytes and macrophages. Results revealed an increase in CD38 positivity upon Tax and p13 co-expression, compared to cells expressing the individual proteins; however, the cooperative effect of Tax and p13 was less prominent compared to their upregulation of CD25 expression. A different timing of expression of the two activation markers could explain the difference in the magnitude of the effects observed. In Jurkat T-cells, which exhibit a constitutively CD38-positive phenotype, the expression of Tax and/or p13 had no effect on CD38 expression.

In conclusion, the experiments carried out on primary T-cells demonstrated that Tax is not sufficient to induce the expression of NF- κ B target genes and that p13 is required to render T-cells permissive for Tax-activity, thus adding a further layer of complexity to the regulation of this pathway by HTLV-1. It is possible that physiological mitogenic stimulation might render T-cells permissive for Tax activity on the NF- κ B pathway. In this scenario, p13 might be particularly relevant in resting T-cells, where it might contribute to NF- κ B activation even in the absence of T-cell receptor engagement.

Several studies demonstrated that the NF- κ B pathway is involved in p53-mediated-apoptosis (Ryan et al., 2000) and p53-p21-induced senescence (Rovillain et al., 2011). Interestingly, Tax-mediated persistent NF- κ B activation leads to cellular senescence in a p53-independent, p21-dependent manner (Zhi et al., 2011). These findings suggest that the contribution of p13 to the Tax-dependent activation of NF- κ B in primary T-cells could result in different outcomes on cell fate depending on the magnitude and duration of the activation of the pathway: on one hand, persistent NF- κ B activation could promote cell survival and the initial steps of leukemogenesis; on the other hand, an excessive level of NF- κ B activation could promote cell death or

senescence of infected cells. In this last case, p13 might act as a viral tumor suppressor that reduces proliferation and favors cell death or growth arrest of Tax-expressing cells. Interestingly, Tax-induced senescence can be counteracted by the HBZ viral protein, which down-regulates NF- κ B (Zhi et al., 2011), suggesting that it would be of particular interest to investigate the combined effects of Tax, p13 and HBZ expression on the NF- κ B pathway and on the fate of primary T-cells.

Current studies are aimed at verifying the ROS-dependence of the synergistic effect of Tax and p13 by using ROS scavengers and ROS-inducing agents. In addition, we will test the validity of the functional interaction of Tax and p13 in the context of the complete HTLV-1 genome. To this end, we will compare the effects of wild type HTLV-1 and a p13-knock-out HTLV-1 molecular clone on NF- κ B activation. These experiments will be carried out in primary T-cells as well as in primary dendritic cells, which have recently emerged as an important target of the virus *in vivo* (Jones et al., 2008).

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