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Role of HTLV-1 p30 during infection of human monocytes and dendritic cells

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Abstract

ABSTRACT

Human T-Lymphotropic virus type 1 (HTLV-1) is the causative agent of two distinct pathologies, adult T-cell leukemia (ATL), an aggressive malignancy of mature CD4$^+$ T-cells, and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a demyelinating neurodegenerative disease. An estimated 10–20 million people worldwide are infected with HTLV-1. While the majority of HTLV-1-infected individuals remain asymptomatic, a low percentage of patients develop either ATL (3–5%) or TSP/HAM (0.3–2%) after a long period of clinical latency. HTLV-1 primarily infects CD4+ T-cells and has been detected in ex vivo CD8$^+$ T-cells, B-cells, monocytes and dendritic cells (DC) from infected individuals. These cells may contribute, more than previously thought, to viral persistence and pathogenesis. DCs isolated from HTLV-1-positive patients are defective in IFN-α response upon in vitro stimulation and their ability to activate T-cells is impaired. In addition, in HTLV-1 infected patients alteration in monocytes differentiation and activation has been reported. Contrarily to how observed in T or B cell, where HTLV-1 p30 is not required in vitro for an efficient viral replication, p30 seems to play a key role during HTLV-1 infection of DCs. In the macaque model, the ablation of p30 expression within the HTLV-1 provirus (p30-KO) or p12/p8 (p12-KO) severely affects infectivity. Furthermore, the infectivity of p30-KO in human primary monocyte-derived dendritic cells (Mo-mDCs) is severely impaired and is not sustained over time. Prior data demonstrated that p30, by interacting with the cellular transcription factor PU.1 in human monocytes, affects TLR4 signaling and the expression of several genes involved in apoptosis, cell cycle, and transcription, pointing to p30 as a regulator of innate response to HTLV-1. The interaction of p30 and PU.1 might lead to inhibition of the transcriptional activity of PU.1.

We used a human monocytic cell line, THP-1 and primary dendritic cells, to study the mechanism of p30 and p12/p8 requirement in these cell types. We hypothesized that p30 and/or p12/p8 affected not only TLR3/4 but also TLR7/8 signaling in virus infected monocytes and in dendritic cells, and that by affecting the type 1 IFN response p30 and/or p12/p8 affects the activation and differentiation of these cells and ultimately the host response to the virus allowing viral replication and spread throughout the body.

We confirm that p30 decreases the expression of TLR4 on THP-1 surface and the production of IL12 and TNFα by these cells. p30 is also able to inhibit the expression of interferon responsive genes (ISG) following stimulation with LPS of the TLR4 and with polyI:C of TLR3, but not of TLR7/8 with Imiquimod. Results in THP-1 mirrored those in ex-vivo human primary monocyte-derived dendritic cells (Mo-mDC). The effect of p30 on TLRs signaling was also demonstrated by ablating its expression within a molecular clone of HTLV-1. HTLV-1 infection of monocytes inhibited TLR3 and TLR4–induced ISGs expression by 50 to 90%, depending on the genes, whereas the isogenic clone p30 knockout (KO) virus was less effective in inhibition of TLR3 and TRL4 signaling and displayed lower infectivity. Viral expression and inhibition of ISGs transcription, was however, rescued by restoration of p30 expression. A chromatin immunoprecipitation (ChIP) assay demonstrated that p30 inhibits initiation and elongation of PU.1 dependent transcription of interferon-α1 (IFNα1), IFNβ, and TLR4 genes upon TLRs stimulation. In contrast, experiments conducted with p12/p8 did not demonstrate an effect on IFN response gene expression. These results provide a mechanistic

explanation on the requirement of p30 for HTLV-1 infectivity in vivo, and suggest that dampening interferon responses in monocytes and DCs is specific for p30 and represent an essential early step for permissive HTLV-1 infection and persistence.

SOMMARIO

HTLV-1 (Human T-lymphotropic virus type 1), o Virus T-linfotropico dell'uomo di tipo 1 e' un retrovirus oncogene scoperto nel 1979. HTLV-1 è causa di due distinte patologie: la leucemia dell'adulto a cellule T (ATL), un tumore maligno molto aggressive che colpisce I linfociti T CD4$^+$ maturi, e la paraparesi spastica tropicale/mielopatia associata ad HTLV-1 (TSP/HAM), una malattia neurodegenerative demielinizzante. Circa 10-20 milioni di persone sono infette con HTLV-1; tuttavia, la maggior parte di esse rimane asintomatica e solo una bassa percentuale di pazienti sviluppa la malattia dopo un lungo periodo di latenza clinica (nel 3-5% ATL e nel 0.3-2% TSP/HAM).

I linfociti T CD4$^+$ sono le cellule target inizialmente infettate da HTLV-1. Il virus, tuttavia, e' stato ritrovato ex vivo anche nei linfociti T CD8$^+$, nei linfociti B, nei monociti e nelle cellule dendritiche di pazienti infetti. Queste cellule potrebbero contribuire, piu' di quanto finora si pensava, all'infettivita', alla persistenza e alla patogenesi del virus. Diversi studi hanno dimostrato che cellule dendritiche (DC) di pazienti HTLV-1 positivi hanno una ridotta capacita' di attivare I linfociti T e una diminuita produzione di IFN-α in vitro. Inoltre e' stato osservato che in pazienti HTLV-1 infetti si ha spesso un'alterazione nel differenziamento e nell'attivazione dei monociti.

Contrariamente a quanto osservato nei linfociti T e B, nei quali la proteina virale di HTLV-1 p30 non e' risultata necessaria per ottenere un'efficiente replicazione virale in vitro, p30 sembra giocare un ruolo chiave durante l'infezione da parte di HTLV-1 di DC. Nel modello animale (Rhesus macaque), la delezione delle proteine virali p30 o p12/p8 causa una diminuzione dell'infettivita' dei virioni. Risultati simili sono stati osservati anche in cellule dendritiche umane infettate in vitro con cloni molecolari di HTLV-1 privati dell'espressione di p30.

Dati precedenti hanno dimostrato inoltre che p30, interagendo con il fattore trascrizionale PU.1 nei monociti, influenza il pathway di signaling del TLR4 e l'espressione di diversi geni coinvolti nell'apoptosi, nel ciclo cellulare e nella trascrizione. L'interazione tra le due protein p30 e PU.1 potrebbe portare all'inibizione dell'attivita' trascrizionale di quelst'ultima.

Per studiare la funzione di p30 e p12/p8 in questo studio abbiamo usato una linea cellular monocitica umana (THP-1) e cellule dendritiche umane primarie. Abbiamo ipotizzato che p30 e/o p12/p8 possano influenzare sia il pathway di signaling di diversi TLR (TLR3, 4, 7 e 8) sia la risposta antivirale mediate dagli interferoni di tipo 1 nei monociti e nelle cellule dendritiche HTLV-1 infette e possano quindi permettere la replicazione virale e la diffusione del virus nel corpo.

Abbiamo inizialmente confermato, come dimostrato da dati pubblicati precedentemente, che p30 downregola l'espressione del TLR4 sulla superficie delle THP-1 e la produzione di IL12 e TNFα da parte delle stesse cellule. p30 e' anche in grado di inibire l'espressione di geni responsive agli interferoni (ISG) in seguito a stimolazione del TLR4 con LPS, del TLR3 con polyI:C; ma non del TLR7/8 con Imiquimod. I risultati ottenuti con le cellule dendritiche umane derivate dai monociti (mdDC) sono simili a quelli descritti per le THP-1. L'effetto di p30 sul pathway di signaling dei TLR e' stato anche confermato deletando l'espressione della proteina virale in un clone molecolare di HTLV-1. Mentre l'infezione dei monociti da parte del virus wild type inibiva l'espressione di ISG nel 50-90% dei casi in base al gene analizzato; il clone molecolare virale che non esprime la

proteina p30 ha mostrato una minor capacita' di inibizione del signaling dei TLR3 e 4 e quindi dell'espressione di ISG e una conseguente minore capacita' di infezione. Riaggiungendo alla coltura la proteina p30, l'espressione del virus e degli ISG e' tornata ai livelli precedentemente osservati (rescue). Mediante un saggio di immunoprecipitazione cromatinica (ChIP) e' stato dimostrato che p30, dopo stimolazione dei TLR, inibisce sia l'inizio, sia l'allungamento della trascrizione di geni sotto il controllo di PU.1 quali IFNα1, IFNβ, e TLR4.
Questi risultati suggeriscono che la proteina virale p30 sia da ritenere fondamentale per l'infettivita' del virus in vivo e suggeriscono che p30 sia responsabile di una downregolazione della risposta antivirale mediate dagli interferoni nei monociti e nelle cellule dendritiche e che questo fatto rappresenti uno degli step fondamentali perche' si instauri un'infezione da HTLV-1 persistente.

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List of abbreviations

Ab: Antibody
ACs: asintomatic carriers
AP1: Activating Protein 1
ATF: Activator of Transcriptional Factors
ATL: Adult T-cell Leukaemia
A3G: Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G
BLV: Bovine Leukemia Virus
CA: Capsid
CAT: Computed Axial Tomography
CD: Cluster of Differentiation
CD4+: Lymphocytes T CD4 positive
CD8+: Lymphocytes T CD8 positive
c-onc: Cellular proto- Oncogene
CRS: Cis-acting Repressing Sequences
CTD: Cistein
CTL: Cytotoxic T Lymphocyte
DC: Dendritic Cell
FBS : Fetal Bovine Serum
FoxP3: Forkhead box P3
Env: Envelope
GFP: Green Fluorescent Protein
GLUT-1: Glucose Transporter-1
GM-CSF: Granulocyte Macrophage – Colony Stimulating Factor
gp: GlycoProtein
HA: influenza hemagglutinin
HAM/TSP: HTLV-associated myelopathy/tropical spastic paraparesis
HBZ: HTLV-1 bZIP factor
hbz sp1: spliced hbz
hbz us: unspliced hbz
HRP: HorseRadish Peroxidase
HTLV-1: Human T-cell leukemia/lymphoma virus type-1
IFNAR: IFN-a receptor
IL: InterLeukine
INF: INterFeron
ISG: Interferon Stimulated Genes
IRF: IFN Regulatory Factor
LDH: Lactate Dehydrogenase
LPS: LipoPolySaccharide
LTR: Long Terminal Repeat
LV: LentiVirus
MA: Matrix
MAPK: Mitogen-Activated Protein Kinase
Mo-mDC: Monocytes derived Dendritic Cells
MTOC: MicroTubule Organizing Center
MxA: Myxovirus resistance GTPase protein A
NC: NucleoCapsid
NF-kB: Nuclear Factor kappa B
NK: natural killer cells

NLS: Nuclear Localization Sequence
OAS: 2', 5' Oligoadenylate Synthetase
ORF: Open Reading Frame
PAMP: Pathogen Associated Molecular Pattern
PBMCs: peripheral blood mononuclear cells
PET: Positron Emission Tomography
PMA: Phorbol Myristyl Acetate
Pol: Polymerase
Pro: Protease
PRR: Pattern Recognition Receptor
RNAse H: ribonuclease H
ROS: reactive oxygen intermediates
RT: reverse transcriptase
RT: Room Temperature
RxRE: Rex Response Element
Ser: Serin
SRF: Serum Responsive Factor
STLV: Simian T-Lymphotropic Virus
TBK1: Tank Binding Kinase 1
TCR: T Cell Receptor
TGF-β: Transforming Growth Factor-beta
TLR: Toll Like Receptor
TNFα: Tumor Necrosis Factor alpha
Treg: T regulatory lymphocytes
v-onc: Viral Oncogene
WT: WildType

Introduction

1. INTRODUCTION

1.1 Human T-lymphotropic virus type 1: taxonomy, epidemiology and pathogenesis

Human T-cell leukemia/lymphoma virus type-1 (HTLV-1) is the first retrovirus discovered in 1979 in T-lymphocytes of a patient with cutaneous T-cell lymphoma [1; 2]. This retrovirus is able to establish a persistent infection in humans and is the only one with oncogenic properties [3].

HTLV-1 belongs to the Retroviridae family, Oncovirinae sub-family, Deltaretrovirus genus, which also includes HTLV-2, -3, -4, simian T-Lymphotropic virus (STLV), and bovine leukemia virus (BLV). STLV and BLV infections are associated with neoplastic diseases, while the pathogenicity of HTLV-2, -3, -4 has not been clearly established [4; 5; 6].

Deltaretroviruses have two main peculiarity: they present at the 3' end of the genome, the "pX region" that encodes the regulatory proteins Tax and Rex and different accessory proteins. Another characteristic of the Deltaretroviruses is represented by their oncogenic mechanism of cell transformation, which does not involve the expression of a cellular-derived viral oncogene (v-onc), as in the case of acute-transforming retroviruses, or integration in the proximity of a cellular proto-oncogene (c-onc), as in the case of chronic-transforming retroviruses. The mechanism of cell transformation by Deltaretroviruses is instead determined by the ability of Tax protein to de-regulate the expression of a wide range of cellular genes.

HTLV-1 primarily infects $CD4^+$ T-cells and has been detected in *ex vivo* $CD8^+$ T-cells, dendritic cells (DC), and B-cells from infected individuals. While cell-free virions have been shown to efficiently infect DCs *in vitro*, HTLV-1 is believed to be transmitted to T-cells and DCs mostly by cell-to-cell contact through a virological synapse, biofilm-like extracellular viral assemblies, or cellular conduits [7; 8; 9; 10].

Based on epidemiology studies it has been estimated that approximately 15 to 20 million HTLV-1 carriers exist throughout the world [11]. This virus is

endemic in South-Western Japan, Central Africa, the Caribbean Basin, Central and South America and the Melanesian Islands. Sporadic infection occurs in Europe and North America. In these endemic areas there is a wide range of seroprevalence rates ranging from 0.1 to 30%. HTLV-1 is spread through contact with bodily fluids containing infected cells. Contaminated whole blood or whole blood products represent the most common form of HTLV-1 transmission in the United States of America, typically from sharing of needles among intravenous drug users [12; 13; 14] or transfusion of infected blood products. However the more natural route of HTLV-1 transmission is through infected mothers who breast-feed their children resulting in the transfer of infected maternal lymphocytes to their infant [15]. Japan has educated HTLV-1 infected mothers about the possible risks of transmitting HTLV-1 through breast-feeding, effectively reducing transmission in endemic regions [16;17]. Perinatal contamination of the fetus from infected maternal blood occurs, but does not represent a significant mode of HTLV-1 transmission [18]. The transmission of HTLV-1 through sex is a less efficient route of transmission, however male to female transmission via semen is four-times as likely to lead to transmission as female to male [19].

While the majority of HTLV-1-infected individuals remain asymptomatic, a low percentage of patients develop after prolonged latency periods (as long as 20 to 60 years) two major diseases: adult T-cell leukemia (ATL), a disease characterized by malignant proliferation of $CD4^+$ T-lymphocytes, and tropical spastic paraparesis/HTLV-1-associated myelopathy (TSP/HAM), a neurodegenerative disorder [20; 21; 22; 23; 24; 25; 26]. In spite of over 30 years of study, the molecular mechanisms determining ATL or TSP/HAM have not been yet fully clarified. HTLV-1 is also associated with other clinical disorders including HTLV-1-associated arthropathy, HTLV-1-associated uveitis [27; 28], infective dermatitis [29], and polymyositis [30;31].

1.2 Human T-lymphotropic virus type 1 associated diseases

As mentioned before, HTLV-1 is causatively associated with a number of pathologies; the two most common are adult T-cell leukaemia (ATL) and HTLV-associated myelopathy/tropical spastic paraparesis (HAM/TSP).

Only a minority of HTLV-1-infected individuals develops disease. Depending on ethnicity and gender, approximately 2–3% of infected individuals develop ATL and 0.25–4% develop HAM/TSP [32;33;34]. Other associated diseases include HTLV-associated uveitis and infective dermatitis. The majority of infected individuals remain lifelong asymptomatic carriers (ACs). The mechanisms by which HTLV-1 causes such diverse clinical diseases are not understood and it is also not known why disease typically occurs decades after initial infection and affects less than 10% of carriers. Understanding of HTLV-1-associated disease is further limited by the lack of suitable animal models and inaccessibility of tissue from the central nervous system of individuals. Since no viral genotype has been associated with any particular disease [35] and there is a large antiviral immune response, the currently accepted hypothesis is that the host immune response is the main determinant of the risk of disease [36;37].

1.2.1 Adult T-cell leukemia (ATL)

Adult T-cell leukemia/lymphoma, in its acute form, is an aggressive T-cell malignancy that typically occurs 20 to 30 years after infection with HTLV-1 [16]. Neoplastic disease associated with HTLV-1 is exhibited in a variety of clinical forms, but is characterized by a monoclonal population of T-cells that express $CD3^+/CD4^+/CD8^-/CD25^+$/HLA-DR^+ cell surface markers [17–19], which frequently accumulate in peripheral blood as well as in lymphoid organs and skin.

Clinically, ATL occurs in at least four different forms: (1) smoldering; (2) chronic; (3) lymphoma and (4) acute [17]. Patients afflicted with the acute form of ATL make up approximately 55 to 75% of all ATL cases and

present with fever, malaise, skin lesions, lymphadenopathy, leukocytosis and hepatosplenomegaly.

Type of ATL	Characteristics
ACUTE	In individuals with acute ATLL, symptoms develop rapidly and may include fatigue, skin rash, and enlarged lymph nodes in the neck, armpit, or groin. The hallmarks of acute ATLL are a high white blood cell count often accompanied by an elevated level of calcium in the blood (hypercalcemia), which can cause irregular heart rhythms and severe constipation. Patients present also organomegaly and high lactate dehydrogenase (LDH).
CHRONIC	This slow-growing type of ATL can result in elevated lymphocytes in the blood, enlarged lymph nodes, skin rash, or fatigue. It can also be found in other areas of the body such as the spleen and liver. Patients present lymphocytosis $>4x10^9$/L with ATL cells, skin, lung, liver or node involvement, normal calcium level, LDH normal or less that twice in upper normal limit.
SMOLDERING	This slow-growing type of ATL is associated with very mild symptoms, such as a few skin lesions. Skin and/or lung infiltrating and no other organ involvement. Patients present normal lymphocyte count (1-5% ATL cells), normal calcium and LDH.
LYMPHOMATOUS	This subtype of ATLL is found primarily in the lymphnodes. Patients present organomegaly. Less than 1% circulating leukemia cells, high LDH and possible hypercalcaemia.

TABLE 1: the four subtypes of ATLL: acute, lymphomatous, chronic, and smoldering. Acute and lymphomatous are fast-growing forms of ATLL, whereas chronic and smoldering are less aggressive.

Depending on the subtype, diagnosing ATLL may require removing a small sample of tumor tissue or abnormal skin tissue (biopsy). A blood test may also be necessary to measure the white blood cell count and calcium levels. Other tests, such as a bone marrow biopsy, a computed axial tomography (CAT) scan of the chest, abdomen, liver, and spleen, and/or a positron emission tomography (PET) scan may be used to determine if, or how far, the cancer has spread.

In some cases the acute phase of ATL is preceded by peripheral lymphocytosis characterized by poly- or oligoclonal integration of the viral genome. The prolonged and complex interactions between the host and the virus that lead to development of ATL have not been elucidated. HTLV-1 infected neoplastic monoclonal T-cells originate from polyclonal populations of infected T-cells. Selective pressures such as the anti-HTLV-1 adaptive immune response of an infected individual promote an oligoclonal population of infected T-cells with survival advantages to emerge. From this oligoclonal population, a neoplastic T-cell clone emerges typically with a variety of somatic genetic mutations. Clonal expansion is thought to be driven by the expression of proviral genes (Tax/ HBZ) counter-balanced by the HTLV-1-specific immune response. Clones that become long lived may undergo genetic damage and subsequent malignant transformation [38]. These cells possess multi-lobulated nuclei and are called "flower cells" (Figure 1).

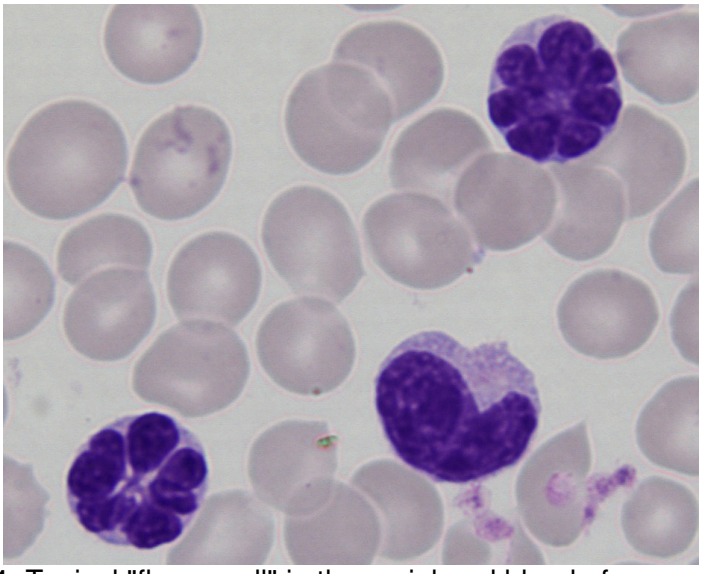


Figure 1: Typical "flower cell" in the peripheral blood of an acute ATL patient. (Matsuoka *et al.* 2005)

Furthermore, HTLV-1 infection is accompanied by a high frequency of T-cells expressing the surface marker Forkhead Box P3 (FoxP3) [39;40],

which play a critical role in suppressing the immune response. The increase in FoxP3$^+$ cell frequency in HTLV-1 infection results from the upregulation of the CCL22 chemokine production by HTLV-1-infected cells that express Tax. CCL22 engages the CCR4 receptor expressed on the functional T regulatory (Treg) cell population, resulting in an enhancement of the migration and survival of FoxP3$^+$ cells *in vitro*. These FoxP3$^+$ cells may both retard the progression of ATL and HTLV-1-associated inflammatory diseases and contribute to the immune suppression seen in HTLV-1 infection, especially in ATL [41].

Until recently the protein Tax was considered to be critical both for leukemogenesis and viral persistence because of its interactions with host genes responsible for cell proliferation, DNA repair, cell cycle control, and apoptosis [42] and because of its associations with cell proliferation *in vitro* [43] and *in vivo* [44]. However, Tax expression is only detected in about 30% of freshly isolated samples from ATL cases. Tax expression can be disrupted by deletion or CpG hypermethylation of the 5'LTR leading to silencing of forward strand transcription [45] and genetic mutations in Tax itself leading to silencing. These findings rise up doubt on the hypothesis that continued expression of Tax is essential in the late stages of leukaemogenesis [46;47]. In contrast to Tax, HTLV-1 bZIP factor (HBZ) mRNA has been detected in 100% of ATL cells [48;49]. Anyway, current opinion favors the role of Tax in the initiation of ATL and HBZ to maintain the transformed phenotype.

1.2.2 HTLV-1 associated myelopathy/tropical spastic paraparesis (HAM/TSP)

In 1985, Gessain *et al.* reported that a group of HTLV-1-seropositive patients in French Martinique suffered from a neurodegenerative disorder called tropical spastic paraparesis (TSP). Osame *et al.* subsequently described a similar clinical disorder in Japanese patients and termed it HTLV-1 associated myelopathy (HAM). TSP/HAM is characterized by a

slowly progressive spastic paraparesis, associated with bladder dysfunction and sensory disorders. The onset of HAM/TSP typically occurs in younger subjects infected with HTLV-1 and is more closely linked to the transfusion of HTLV-1 infected blood products, whereas ATL has been linked to transmission through breast milk of infected mothers. Parenchymal and perivascular infiltration of HTLV-1 specific $CD4^+$ and $CD8^+$ T lymphocytes occurs in the white and gray matter of the spinal cord, resulting in severe inflammation from production of *pro-inflammatory* cytokines such as IL-1, IL-6, IFN-γ, and TNF-α. Accumulation of *pro-inflammatory* cytokines leads to demyelization fibrosis and lymphocytic meningomyelitis [50]. The presence of infiltrating T-cells in the spinal cord lesions and of Tax-specific cytotoxic T- lymphocytes (CTL) in the cerebrospinal fluid and in the peripheral blood mononuclear cells (PBMCs) suggests that TSP/HAM might have an autoimmune base [51;52]. This hypothesis is consistent with the association between HLA haplotype and the risk of developing TSP/HAM [53].

The detailed mechanism of HAM/TSP development like ATL has yet to be elucidated. However HTLV-1 proteins utilizing molecular mimicry or acting as auto antigens have been postulated as factors that contribute to the development of HAM/TSP. Risk factors for the development of HAM/TSP such as high proviral loads have been linked with the development of HAM/TSP.

TSP/HAM CLINICAL CHARACTERISTICS

Spasticity lower extremities, hypereflexia, muscle weakness, and sphincter disorders, including dysfunction of the urinary bladder and intestines; clinically may overlap with multiple sclerosis
Progressive chronic myelopathy, with preferential damage of the thoracic spinal cord
Early lesion development characterized by infiltrates composed predominantly of $CD4^+$ T-cells, and macrophages with detectable levels of HTLV-1 tax RNA in lesions

Characterized by multiple white matter lesions in both the spinal cord and the brain involving perivascular demyelination and axonal degeneration; rarely, cerebellar syndrome with ataxia and intention tremor
Late lesions (>4 years) predominantly $CD8^+$ T-cells with less tax RNA
Cerebrospinal fluid contain high levels of *pro-inflammatory* cytokines, including IFN- γ, TNF-α, IL-1, and IL-6, as well as increased numbers of activated lymphocytes

TABLE 2: HTLV-1-Associated Myelopathy/ Tropical Spastic Paraparesis (HAM/TSP).

1.2.3 Other disorders associated with HTLV-1 infection

A number of other immune-mediated chronic inflammatory conditions are associated with HTLV-1 infection. However, it is less clear what specific role HTLV-1 infection plays in the initiation or development of these diseases. Arthropathies, uveitis, infective dermatitis, polymyositis, chronic respiratory disease, Sjogren's syndrome, lymphadenitis, and certain acute myeloid leukemias have been associated with HTLV-1 infection. It has been hypothesized that the deregulation of the immune system in chronic HTLV-1 infection promotes diseases. HTLV-1 related uveitis are characterized by infiltration of HTLV-1-infected lymphocytes and deregulated production of cytokines. Increased proviral load found in patients with HAM/TSP and uveitis results from clonal expansion of the HTLV-1-infected T-cells, which has been implicated in the pathogenesis of HTLV-1-associated diseases [54].

1.3 Infection and virus propagation

HTLV-1 is an enveloped virus that contains two identical copies of a plus single-stranded RNA genome that carries genetic information for structural proteins and enzymes (Gag, Env, reverse transcriptase (RT), protease, integrase) and is surrounded by capsid and matrix proteins and by an outer lipoproteic envelope containing protruding viral glycoproteins gp21 and gp46 (Figure 2) [55;56]. HTLV-1 can infect a wide range of human cell

types *in vitro* (monocytes, microglial cells, epithelial cells, B- and T-lymphocytes), but *in vivo* the virus is almost confined to the $CD4^+$ T lymphocyte subset [57]. $CD8^+$ T lymphocytes can also carry the virus, but at a consistently lower frequency than $CD4^+$ T cells. Viral spread is mediated through the interaction between the viral envelope protein gp46 and the glucose transporter GLUT-1 [57]. Consequently, glucose consumption of the infected target cell is inhibited and extracellular ambient acidification is reduced, possibly causing metabolic alterations in the infected cells [55; 57]. The gp46-GLUT-1 interaction allows the envelope protein gp21 to mediate cellular membranes fusion with the formation of the "virological synapse" which may be defined as a virus-induced, specialized area of cell-to-cell contact that promotes the directed transmission of the virus between cells. Its assembly results from the polarization of the cytoskeleton of the infected cell and the accumulation of HTLV-1 core complexes and the HTLV-1 genome at the cell junction; the virion components are then transferred to the uninfected cell as enveloped particles [58;59]. Tax is involved in the formation of the virological synapse: it localizes in the contact region between infected and target cells [60] and enhances MTOC (microtubule organizing center) formation by stimulating the CREB pathway [61]. Proteins that mediate antigen recognition and cell adhesion (e.g. hDlg, neuropilin-1, heparan sulphate proteoglycans) also contribute to HTLV-1 binding and entry into the target cell and are part of the "virological synapse" [62;63;64].

Another mode of HTLV-1 transmission is mediated through an extracellular biofilm-like structure that stores viral particles, facilitating virus spread after cell-to-cell contact [65]. Both the virological synapse and biofilm-mediated transmission are consistent with the fact that cell-free HTLV-1 particles are usually undetectable in the serum of HTLV-1 infected subjects [66; 67]. After virus entry into the target cell, the viral genome is reverse-transcribed by 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. RT 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 in the cytoplasm, through the interaction between the viral nucleocapsid and the plasma membrane, and incorporation of two copies of the single stranded RNA genome along with tRNA, RT, protease and integrase.

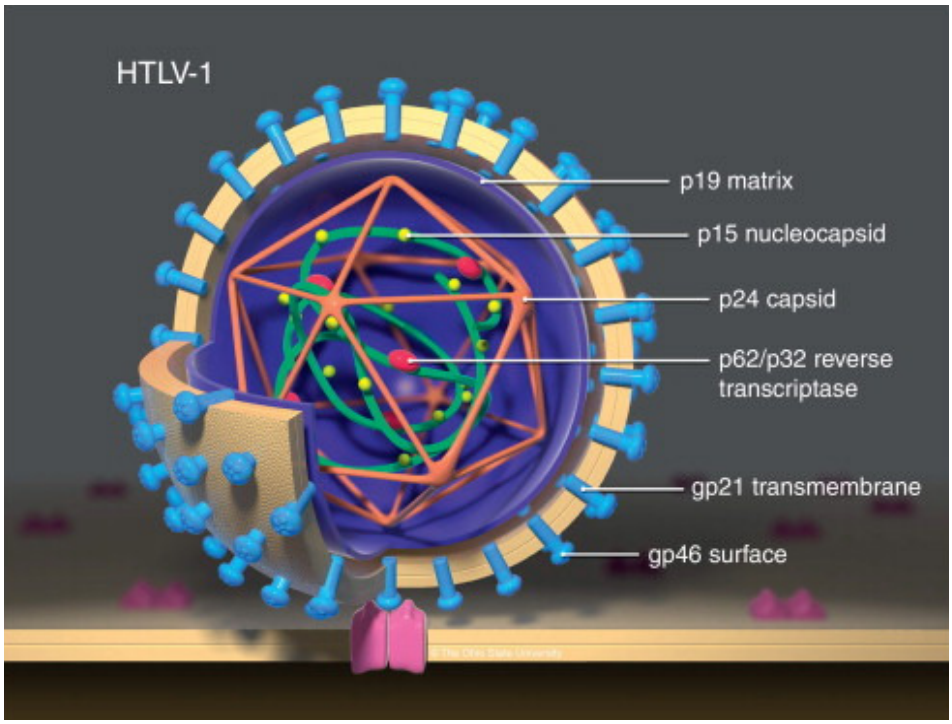


Figure 2. Schematic representation of the HTLV-1 virion (Lairmore *et al.* 2012).

1.3.1 HTLV-1 target cells

T-cells have been considered the key target cells for HTLV-1 infection, as the virus is almost always present in the ATLL cells and is found in the T-cells of TSP/HAM patients. However, whether cells other than T-cells also contribute to the pathogenesis of HTLV-1 infection remains unclear. Indeed, HTLV-1 also infects monocytes/macrophages and dendritic cells [7;12], that are professional antigens presenting cells and shape the host response to viruses as well as to other pathogens. DCs isolated from HTLV-1-positive patients are defective in IFN-α response upon *in vitro* stimulation and their ability to activate T-cells is impaired. In addition, several studies suggest that impairment of DC function after HTLV-1 infection plays a part in pathogenesis [7]. In addition, in HTLV-1 infection, alteration in monocytes differentiation and activation has been reported. Although the majority of HTLV-1 DNA is found in both $CD4^+$ and $CD8^+$ T-cells, up to 20 % of the total virus burden is found in monocytes (our unpublished data).

Cell-free human T-lymphotropic virus type 1 (HTLV-1) virions are poorly infectious *in vitro* for their primary target cells, $CD4^+$ T cells, efficient viral spread instead requires direct cell-to-cell contact. HTLV-1 can efficiently infect in a cell free manner myeloid and plasmacytoid dendritic cells (DCs). Moreover, DCs exposed to HTLV-1, both before and after being productively infected, can rapidly, efficiently and reproducibly transfer virus to autologous primary $CD4^+$ T cells. This DC-mediated transfer of HTLV-1 involves heparan sulfate proteoglycans and neuropilin-1 and results in long-term productive infection and IL2–independent transformation of the $CD4^+$ T cells. So we can say that DCs have a central role in HTLV-1 transmission, dissemination and persistence *in vivo* [7].

1.4 HTLV-1 genetic organization

The genome of HTLV-1 is approximately 9032 nucleotides long and it contains the typical retroviral structural and enzymatic genes Gag, Pro, Pol, and Env [68]. In addition, a region located between Env and the 3' long terminal repeat (LTR), contains four partially overlapping open reading frames (ORF) and is termed the pX region. This unique region encodes several regulatory and non-structural proteins through the use of alternative splicing and internal initiation codons [69; 70; 71].

Orf-I produces the p12 protein which can be proteolytically cleaved at the amino terminus to generate the p8 protein, while differential splicing of mRNA from Orf-II results in production of the p13 and p30 proteins [71; 72]. Orf-III and Orf-IV encode for the Rex and Tax proteins, respectively, and an antisense mRNA transcribed from the 3' LTR that generates the HTLV-1 basic leucine zipper (HBZ) protein [73; 74; 75]. Expression of the highly condensed HTLV-1 genetic information is achieved through ribosomal frame shifting (which generates a Gag-Pro-Pol polyprotein from the full-length transcript), by alternative splicing and polycistronic translation (which produces distinct mRNAs coding for the Env and pX region genes) and through minus-strand transcription, which generates at least 2 different transcripts encoding 2 isoforms of the HBZ protein (Figure 3).

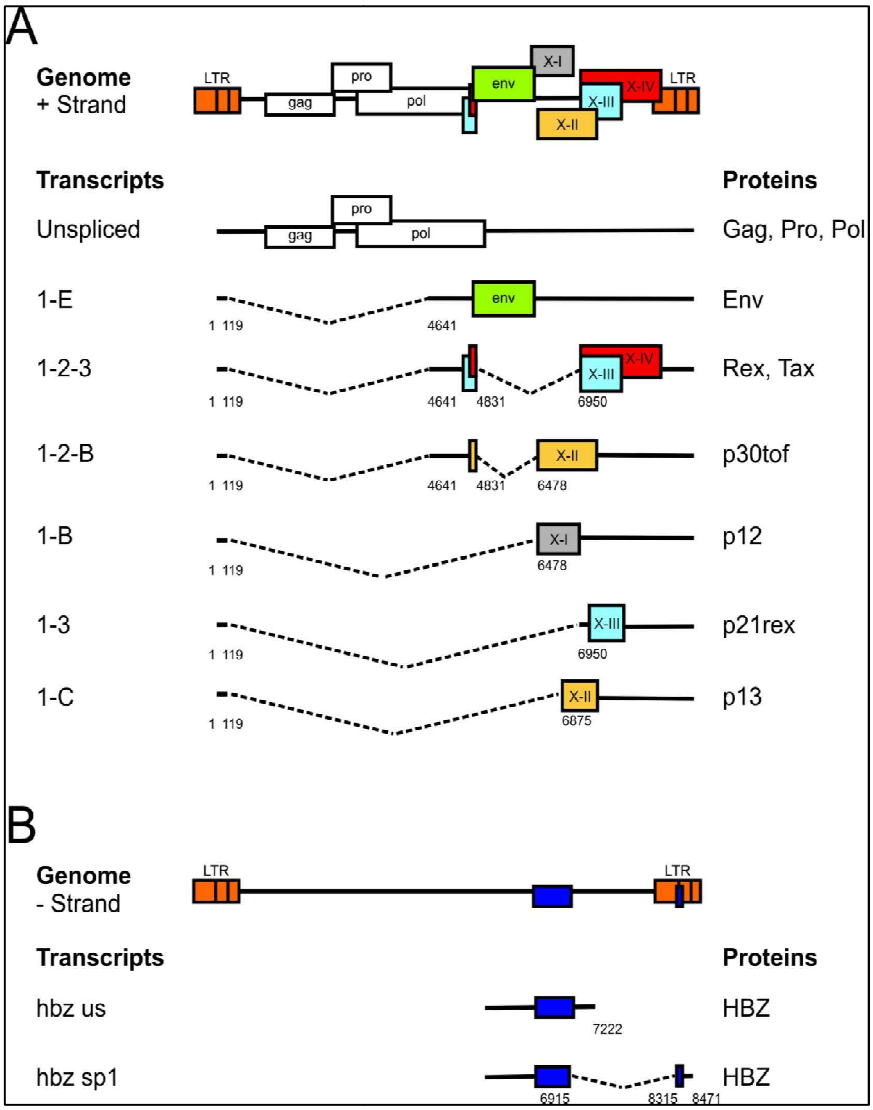


Figure 3. A scheme of the human T-cell leukemia/lymphoma virus type-1 (HTLV-1) genome. (A) Plus-strand ORFs, transcriptional map and proteins coded by each mRNA are shown. The numbering indicates splicing sites used for the generation of the mature mRNAs. Orf-I encodes for the p12 and p8 proteins. The p30 protein is translated from doubly spliced mRNA transcribed from Orf-II and the 5' end of Env. The p13 protein is translated from singly spliced mRNA transcribed from Orf-II and corresponds to the carboxyl terminus of p30. (B) For the minus-strand, the ORF, transcriptional

map and proteins coded by each mRNA are shown. The numbering indicates the start sites used for the generation of the mature mRNAs. Resulting exons are: hbz us and hbz sp1.

1.4.1 Expression of structural proteins

The full-length HTLV-1 mRNA (8.6 kb) is packaged into virions and is also translated into the structural proteins (Gag) and enzymes (Protease and Polymerase) of mature virus particle. HTLV-1 Gag or p55 is produced as a single precursor polyprotein. The Gag gene codes for the 19 kDa matrix (MA), 24 kDa capsid (CA) and 15 kDa nucleocapsid (NC) structural proteins. The pro gene encodes the viral protease. The 5' portion of the Pol gene encodes the reverse transcriptase protein, which converts the viral single-stranded RNA genome into double stranded DNA through its DNA polymerase and RNaseH activities. Sequences downstream code for Integrase, which is responsible for the integration of the reverse-transcribed viral genome in the host cell genome.

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 (Figure 4).

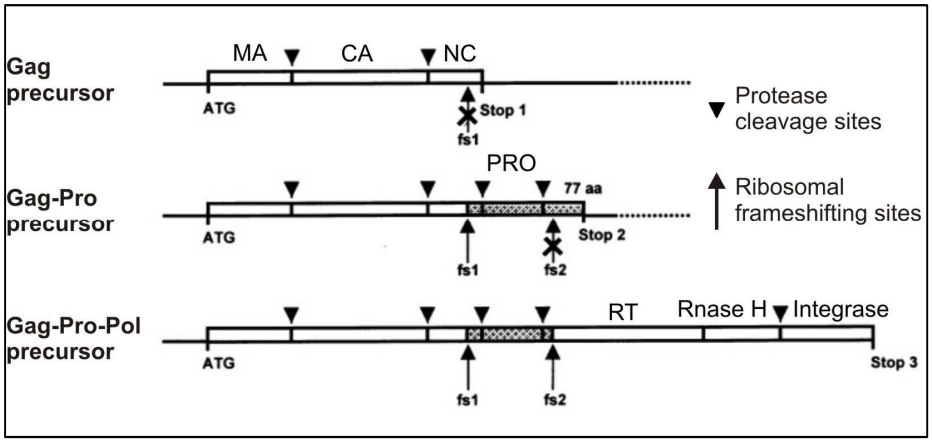


Figure 4. Schematic representation of the Gag-Pro-Pol polyprotein precursors of HTLV-1. Fs1 and fs2: ribosomal frameshifting sites; MA:

matrix, CA: capsid; NC: nucleocapsid, PRO: protease, RT: reverse transcriptase (Le Blanc *et al*., 2001).

The polyprotein is myristylated, post-translationally, and targeted for the inner lipid plasma membrane of the cell. At the inner membrane of the plasma membrane Gag is cleaved by viral proteases into its functional units: CA, NC and MA. Capsid interacts with itself to form the inner core of the virion. Nucleocapsid interacts with the genomic RNA inside the inner core of the virion. The proper spatial and temporal events of viral assembly and budding play a critical role in the ability of HTLV-1 to be transmitted from one cell to another. In contrast to HIV-1 Gag, the interaction of HTLV-1 MA appears to be independent of plasma membrane phospholipid, phosphatidylinositol-(4,5)-bisphosphate used by HIV-1 in particle assembly. HTLV-1 MA contains a PPPY domain that assists in virus budding by targeting cellular proteins Nedd4.1 and Tsg101. In addition to assisting in virus budding and assembly, MA appears to have a role in cell-to-cell transmission of the virus. Thus, like other retroviruses, phosphorylation of HTLV-1 L-domain proteins appears to be important in regulation of viral budding and thus cell-to-cell transmission. In addition, HTLV-1 uses a C-terminal peptide region of NC to block the action of the host restriction factor ABOBEC3G. Protease is produced from ribosomal frame shifting initially as an immature form that is inactive until self-cleavage activates the protease after viral budding.

Reverse transcriptase and IN are generated from proteolytic cleavage of the Gag/Pol precursor polyprotein. RT is responsible for transcribing the RNA template and IN acts as a catalyst in the integration of the dsDNA viral template into the cellular genomic DNA.

The 4.2 kb SS mRNA, transcribed from the Env gene, is translated into a 68 kDa precursor which is post-translationally modified by glycosylation and cleavage into two proteins named gp46-SU, localized at the surface of the

infected cells and virions and responsible for the binding to the GLUT-1 receptor, and gp21-TM, a transmembrane protein that mediates membrane fusion and formation of the virological synapse. HTLV-1 SU is a 312 amino acid protein. The C-terminal half of SU is highly antigenic and is recognized by serum antibodies from approximately 95% of HTLV-1 infected individuals. Early studies using site directed mutagenesis demonstrated functional domains within SU involved in intracellular maturation, syncytium formation, and the association between SU and TM Subsequent development of a cell transmission assay allowed for separation of fusion events from infectivity events.

The HTLV-1 TM contains YSLI amino acid sequences, known to interact with cellular adaptor protein complexes, and a PDZ-binding motif (ESSL) at the C terminus of Env. Alterations of the YSLI motif increased Env expression on the cell surface and increased cell fusion activity, whereas mutations of the ESSL motif reduce Env expression in cells. HTLV-1 SU and TM form as heterodimers at the surface of virions and are responsible for initiating binding, fusion with target cell and entry.

The mechanism of action that facilitates cell-to-cell transmission of the HTLV-1 is not resolved, but recently several groups have reported data on three main cellular receptors: glucose transporter (GLUT-1), heparin sulfate proteoglycans and neuropilin-1. Previous studies have shown GLUT-1 to be involved in envelope mediated cell-to-cell fusion. Heparin sulfate proteoglycan binds virus particles on cell surfaces and facilitates entry. In addition to being the main receptor, removal of heparin sulfate proteoglycan from primary lymphocytes significantly reduced binding of SU. Neuropilin-1 is part of the immunological synapse and is a binding partner of Env. Ectopic expression of neuropilin-1 significantly increased HTLV-1 Env-dependent syncytium formation. Further studies will be required to identify specific envelope motifs that both alter receptor binding and influence viral transmission and spread *in vivo.*

1.4.2 Expression of proteins coded in the pX region

The pX region of the HTLV-1 genome contains at least four different partially overlapping open reading frames that code for non structural proteins of HTLV-1. As shown in Figure 2, 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 contain exon 1 and different 3' exons, and code for the accessory proteins p13 (translated from a start codon located in the ORF II) and p12 (translated from a start codon in the ORF I) and for the accessory/regulatory protein p21rex (translated from a start codon located in the ORF III) [71; 76].

Two doubly spliced mRNAs contain exons 1 and 2, and different 3' exons. Exon 2 contains 2 start codons (AUGs). The regulatory proteins Rex and Tax are transcripted from a bicistronic mRNA. Rex initiates at the first AUG in exon 2 and continues in the ORF III in exon 3, while Tax initiates at the second AUG and continues in the ORF IV in exon 3. The accessory/regulatory protein p30, which is translated from the second start codon of exon 2 and continuing in frame with the x-II ORF in exon B [82;83]. Tax and Rex are required for viral replication.

Tax is a potent transcriptional transactivator of viral gene expression. Tax also regulates the expression of several cellular genes, including those involved in cell proliferation, cell cycle progression, apoptosis, and DNA damage responses.

Rex is a post-transcriptional regulator that facilitates nuclear export of unspliced and singly spliced viral mRNA. In addition, Rex inhibits splicing and transport of doubly spliced mRNA [77]. In contrast to Tax and Rex, Orf-I and Orf-II are dispensable for viral replication *in vitro* but are important for viral persistence *in vivo* [78]. Early work demonstrated that in the rabbit model, Orf-I was required for viral infectivity while Orf-II was required to maintain high viral load [79; 80]. Further work in the rabbit model showed

reversion of HTLV-1 clones lacking p30 to the wildtype p30-expressing virus, suggesting the importance of p30 to HTLV-1 viral persistence [81]. However, in these early studies the HTLV-1 clones that were used contained a frameshift that affected HBZ, making it unclear as to whether these effects were due to the loss of hbz or Orf-I and Orf-II-encoded proteins. In a more recent study, the ablation of p12/p8, p30, or HBZ impaired the establishment of persistent infection in the macaque model [82]. Nevertheless, ablation of these proteins did not affect viral replication in the rabbit model [82]. The orf-I and orf-II-encoded proteins are able to modulate a diverse range of viral and cellular mechanisms including transcriptional regulation, mitochondrial function, cell cycle progression, host cell activation and proliferation, apoptosis, virus infectivity and transmission, and host immune responses. Though these proteins are not essential for virus replication *in vitro*, p8, 12, p13, and p30 have an important role in the establishment and maintenance of HTLV-1 infection *in vivo*.

1.4.3 Expression of proteins coded by the negative strand of HTLV-1

The negative strand of the HTLV-1 genome contains one ORF located in the pX region (antisense orientation) which generates at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) [83; 84]. These transcripts code for 2 isoforms of HBZ protein that differ by 7 amino acids at the N-terminus due to the presence of the first exon only in the former transcript. 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. Both hbz sp1 and hbz us have TATA-less promoters [85].

1.5 Functions of the pX region proteins

1.5.1 Tax

The HTLV-1 proto-oncogene Tax, a potent transcriptional activator of cellular and viral genes, is thought to play a pivotal role in the transforming properties of the virus by deregulating intracellular signaling pathways. Tax is a 40-kDa protein mainly localized in the nucleus translated from a doubly-spliced mRNA from the ORF IV [86 ; 87]. Tax is able to transcriptionally activate the expression of cellular genes involved in growth and proliferation [88] and to repress the expression of tumor suppressor genes [89]. Tax is responsible for initiating viral transactivation from the LTR of the provirus by binding the GC-rich regions of the TRE-1, within the U3 region of the LTR [90; 91; 92; 93]. From the TRE-1, Tax can stabilize the CREB/ATF (activator of transcriptional factors) dimers, which are part of the transcriptional machinery needed for viral gene expression [94]. Tax also can recruit and bind CBP/p300 to the TRE-1. Phosphorylation of CREB by PKA leads to recruitment of CBP/p300 in normal cells; however in HTLV-1 infected T-cells Tax can bypass PKA-mediated phosphorylation of CREB. The ability of Tax to recruit and stabilize CREB-CBP/p300 and other factors like P/CAF (CBP/p300 associated factor) allows for efficient transcription of the provirus [95].

Tax can also bind TRE-2 in the LTR, which is located central and proximal to TRE-1. Tax recruits transcriptional co-activators like P/CAF and p300 (via KIX domain), Ets family transcription factors (Ets-1, -2, Elf-1, Tif-1) and c-Myc transcription factors to the TRE-2 region [96]. Tax can also bind the basic region of cellular basic leucine zipper transcription factors (bZIP), which aid in DNA binding. The presence of TRE-1 and -2 allows for Tax to mediate a number of processes and facilitate viral transcription bypassing cellular signals.

Tax dysregulates multiple cellular transcriptional signaling pathways including nuclear factor kappa B (NF-kB), cyclic AMP response element-binding protein (CREB), serum responsive factor (SRF) and activator protein 1 (AP-1) [97]. These signaling pathways are responsible for the

expression of multiple pro-inflammatory and anti-inflammatory cytokines. Tax influences also the expression of several transcription factors like c-Myc, c-Fos, c-Sis, Erg-1, c-Rel, and Lck and apoptosis and DNA repair genes like Bcl-XL, Bax and PCNA (proliferating cell nuclear antigen) [98].

The transforming ability of Tax is most likely attributable to its influence over the expression of these important cellular genes. These pleiotropic properties of Tax confer to this protein a pivotal role in the viral pathogenicity and allow this viral protein to promote immortalization/transformation of infected cell, causing the onset of the HTLV-1-associated diseases [89; 97; 99]. Indeed, Tax activity is also associated with production of reactive oxygen intermediates (ROS), chromosomal instability and DNA damage. Changes in the intracellular redox status induced by Tax promote DNA damage. Tax-mediated DNA damage is believed to be essential in initiating the transformation process by subjecting infected T cells to genetic changes that eventually promote the neoplastic state. Apoptosis and immune surveillance would then exert the necessary selection pressure for eliminating the majority of virally infected cells, while escape variants acquiring a mutated phenotype would constitute a subpopulation of genetically altered cells prone to neoplasia. Thus, the cooperation of Tax with other viral proteins determines the fate and progression of HTLV-1-infected cells through DNA damage, apoptosis, survival and transformation (Figure 5).

In contrast to other tumor viruses, which generally require continuous expression of viral oncoproteins to sustain transformation, *tax* gene transcripts were detected in only 34% of fresh ATLs [100], suggesting that Tax may be needed to initiate the transformation process, but may not be necessary for maintenance of the transformed phenotype. Interestingly, the HBZ antisense gene, transcribed from the 3′-LTR appears to be the only gene that is constitutively expressed in HTLV-1-infected and ATL cells [101]. Tax expression is typically low or undetectable, whereas HBZ gene

expression is maintained, suggesting that Tax and HBZ may cooperate in the process of leukemogenesis. Tax may initiate the transformation process inducing genetic instability, and HBZ expression may support survival of the infected cells.

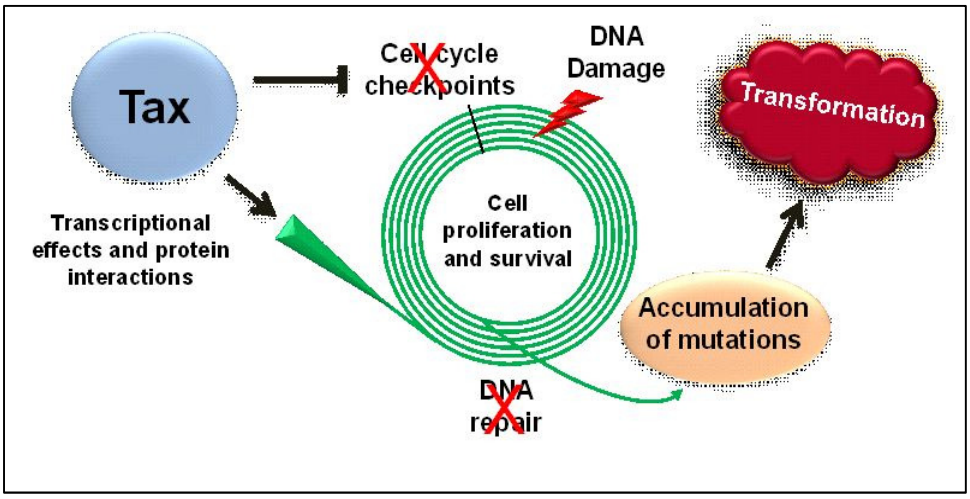


Figure 5. Pleiotropic functions of HTLV-1 Tax contribute to cellular transformation (Kendle Pryor and Susan J. Marriott, 2008).

While the role of Tax in cell gene expression, proliferation, and transformation have been extensively studied, the role of Tax in viral transmission is less clear. Presumably, HTLV-1 would not be able to accomplish viral replication and spread without Tax function, but specific Tax determinants in viral transmission and spread are difficult to study. Infectious clones of the virus that have Tax mutations would fail to enhance needed viral gene expression during early stages of cell-to-cell transmission. Interestingly, prostaglandins enhance viral expression via the HTLV-1 LTR through the protein kinase A signaling and Tax transactivates a promoter for cyclooxygenase 2, a prostaglandin synthetase, and induces PGE2 expression in peripheral and cord blood mononuclear cells [102].

This reciprocal interaction has been postulated to promote viral transmission *in vivo.*

Several studies showed Tax involvement in promoting cell adhesion and thereby cell-to-cell transmission. Significant correlation exists in cell lines comparing expression of HTLV-1 Tax and CCL22 suggesting an active role of Tax in selective $CD4^+$ T-cell viral transmission [103]. This conclusion is supported by transient Tax expression in an HTLV-1-negative T-cell line that induced CCL22 promoting CCR4 redistributed to cell contact points during *in vitro* transmission, and chemotaxis assays. Thus, HTLV-1-infected T-cells may selectively attract $CCR4^+CD4^+$ T-cells [103]. Similarly, Tax induced enhancement of ICAM-1 on the surface of T-cells has been shown to facilitate the formation of viral synapses and therefore may contribute to T-cell tropism and viral transmission [104].

The detailed mechanisms of how specific Tax induced alterations of the host influence viral transmission and spread wait specific testing of tax mutations in context to full length and infectious viral clones.

1.5.2 Rex

Rex is a 27 kDa nuclear/nucleolar phosphoprotein encoded by ORF III and it is expressed from a dicistronic doubly-spliced mRNA which contains exon 1, 2 and 3, starting from the first start codon of exon 2 [105; 106]. Rex contains multiple functional domains including a RNA binding domain (RBS), nuclear localization sequence (NLS), a multimerization domain; and an activation domain, which includes the nuclear export signal (NES) [107]. Several studies indicate that Rex localization to its correct subcellular compartment is critical for its proper function [108]. Unlike Tax, Rex is able to shuttle between the nucleus and the cytoplasm [109], allowing the nucleo-cytoplasmic export of unspliced incompletely spliced and singly spliced mRNAs (Gag, Env, Pol) into the cytoplasm viral RNA, controlling in this way viral gene expression at the post-transcriptional level.

The formation of Rex multimers on the RxRE is critical for the nuclear export of viral mRNA since mutations of Rex that failed to form multimers act as dominant negative mutants. 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. Residues 411 and 414 of CRM1 are critical for Rex multimerization, a region distinct from the one involved in the export of Rex. Mutations in the multimerization domains render Rex non-functional. The activation domain (AD) of Rex was originally identified as the minimal region that could functionally replace the HIV Rev activation domain in cis [110]. Subsequent studies showed that the sequence responsible for nuclear export of Rex, NES, is in fact its minimal effector domain. The NES in Rex is a leucine rich motif involved in protein-protein interactions that are critical for its function [111]. In addition to containing a unique Rex response element (RxRE) in the U3/R 3' LTR, these mRNAs have cis-acting repressive sequences (CRS) that retain and stabilize the unspliced mRNA in the nucleus to ensure the availability of sufficient amount of Rex substrate [112; 113].

Figure 6 summarizes the Rex-mediated nucleocytoplasmic export of incompletely spliced viral mRNAs. After Rex-RxRE binding and Rex multimerization, CRM1 is recruited into the complex. CRM1 uses guanosine diphosphate/guanosinetriphosphate (GDP/GTP) guanine nucleotide exchange of the GTPase Ran to function. CRM1 binds to RanGTP, along with the Rex-mRNA complex, and translocates this complex 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 regulator of chromosome condensation 1 (RCC1) catalyzes the conversion of GTP to GDP. Other proteins affecting Rex function include Ran Binding Protein 3 (RanBP3), a scaffold protein that stabilizes the RanGTP-CRM1-Rex-mRNA complex in the nucleus, and SRC- associated in mitosis 68 (Sam 68), which is able to increment tRex-mRNA binding in a CRM1-independent fashion (Figure 6).

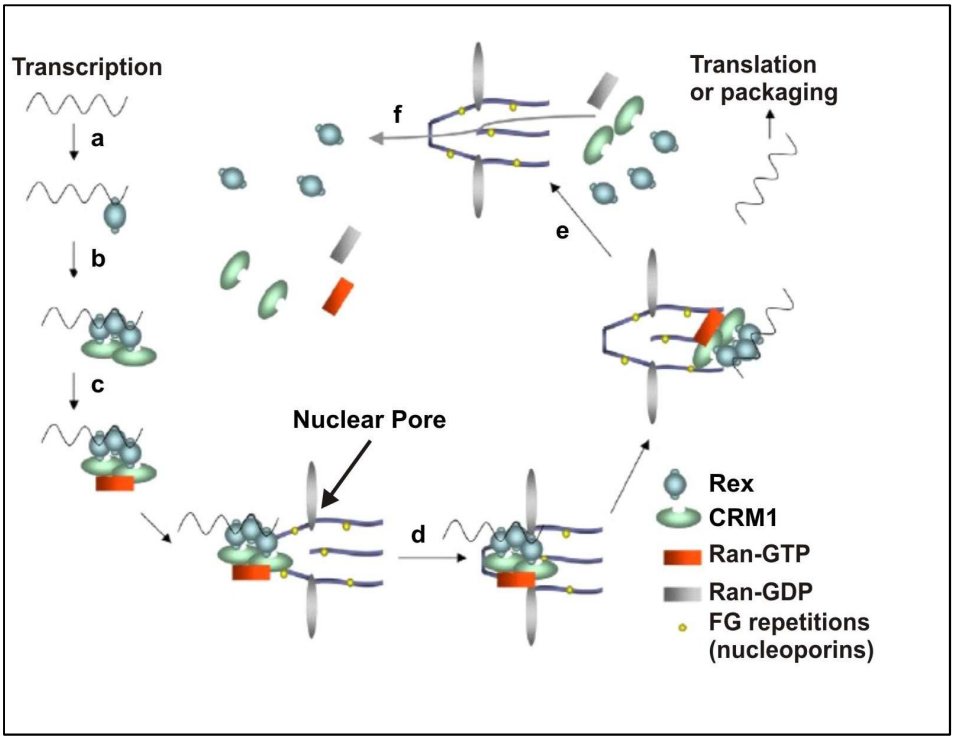


Figure 6. Rex-mediated nucleocytoplasmic export of viral mRNAs. Rex binding to the RxRE sequence in the viral mRNA after transcription (a). Rex multimerization (b) and viral mRNA/Rex/CRM1/RanGTP complex formation (c). Complex translocation from the nucleus to the cytoplasm is mediated by the interactions with phenylalanine-glycine rich nucleoporins (d). Conversion of RanGTP to RanGDP in the cytoplasm with subsequent complex dissociation and viral mRNA release (e). The other components of the complex return into nucleus to start another cycle (Younis and Green, 2005).

Rex, like HIV-1 Rev, appears to utilize the CRM1/exportin pathway [114; 115]. There are two suggested models for the mechanism by which Rex induces cytoplasmic expression of unspliced mRNA. The first model proposes that Rex actively transports the unspliced mRNA to the cytoplasm where it is translated. Here, Rex would directly bind to the RxRE on the mRNA, override the nuclear retention signals, and carry this mRNA cargo

through the nuclear pore to the cytoplasm. The specific binding of Rex to its RxRE is a critical step in its transactivation of mRNA export by allowing the mRNA to overcome nuclear retention due to the inhibitory CRS [116]. The second model suggests that Rex actively inhibits splicing of mRNA by stripping it of splicing factors [117]. Once the mRNA is free of splicing factors the cellular machinery would recognize it as a processed mRNA and export it efficiently to the cytoplasm. The essential role of Rex for viral infectivity has been confirmed using molecular clones with selectively mutations in the rabbit model [118].

A Rex-deficient HTLV-1 full length viral clone was used to provide the first direct evidence that functional Rex expression is not required for *in vitro* immortalization by HTLV-1, but was critical for optimal viral transmission *in vivo* [114]. These results suggest that defects in the ability of Rex to promote unspliced and single spliced RNA to traffic are important for optimal viral spread.

1.5.3 p21 Rex

p21 Rex is a protein coded by the ORF III located in pX region and it is expressed from a singly-spliced mRNA which contains exon 1 and 3. p21 Rex is produced in HTLV-1 infected cells from alternatively spliced mRNAs and it is a truncated isoform of Rex lacking the N-terminal arginine-rich domain of the full-length protein (NLS, nuclear localizing sequence) and when expressed in cells inhibit RNA shuttling [119]. It was hypothesized that it might act as a repressor of full-length Rex, thereby inhibiting the expression of transcripts coding for structural proteins, enzymes and accessory proteins [120; 121] and playing a role as a latency-inducing factor in the HTLV-1 life cycle.

1.5.4 p12 and p8

HTLV-1 Orf-I encodes the 99 amino acid p12 protein which can be proteolytically cleaved at the amino terminus to generate the p8 protein

(Figure 3). Computational analysis of the amino acid sequence of p12 predict the existence of a non-canonical endoplasmic reticulum retention signal, two putative leucine zipper (LZ) motifs which form α helices in the protein, two transmembrane domains, a calcineurin-binding motif, four putative proline-rich (PXXP) Src homology 3 (SH3)-binding domains responsible for binding cellular signaling proteins, and a putative adaptin motif [122; 123].

These structural features may contribute to protein localization, homodimerization, and protein-protein interactions. The p12 protein undergoes complex post-translational modifications through proteolytic cleavage. The first cleavage occurs between amino acid positions 9nd 10 and is followed by a second cleavage between amino acids 29 and 30 [122]. The first proteolytic cleavage removes the ER retention signal at the amino terminus of p12, while the second cleavage generates the p8 protein [122]. The p12 protein localizes to cellular endomembranes, particularly within the ER and Golgi apparatus, while p8 traffics to lipid rafts at the cell surface and is recruited to the immunological synapse upon T-cell receptor (TCR) ligation [122; 124; 125].

The singly spliced mRNA encoding p12/p8 has been detected *in vitro* and in *ex vivo* HTLV-1-infected T-cells and macrophages [126]. The p12 recombinant protein is recognized in serum from humans infected with HTLV-1 and rabbits experimentally infected with HTLV-1 [127]. In addition, a cytotoxic T-lymphocyte (CTL) response to Orf-I products can be detected in HTLV-1-infected individuals [128].

Two natural variants of the p12 protein have been identified; one variant carries a lysine residue at position 88 and is commonly found in HTLV-1 strains from TSP/HAM patients while the second variant carries an arginine residue at position 88 and is found in HTLV-1 strains from all ATL patients and healthy carriers studied. One of the most important domains of p8/p12 is the conserved PSLP(I/L)T motif that is homologous to the calcineurin

binding motif present in Nuclear Factor of Activated T-cells (NFAT) [129]. This sequence has the capacity for calcineurin binding in the cell. Calcineurin and NFAT represent a few of the many $Ca2^{+}$ signaling proteins, transcription factors, and ER/cis-Golgi associated proteins in the cytoplasm that p12 interacts with to influence gene expression infected T lymphocytes (Figure 7(2)).

In addition to calcineurin and NFAT, calreticulin and calnexin bind p12 and mediate Ca^{2+} regulated pathways in the cell. These ER associated proteins are involved in regulating Ca^{2+} storage and signaling, respectively. Calreticulin is a chaperone protein responsible for retaining the MHC class I molecule folded in the ER along its maturation pathway. Calnexin assists in proper protein folding of glycoproteins that enter the secretory pathway. The modulation of NFAT activation and thus T-cell signaling is accomplished through interactions between Ras/MAP kinase by p12. When stimulated by phorbol ester and PMA, p12 can activate NFAT [130]. p12 accomplishes this through releasing intracellular Ca^{2+} from ER storage and by increasing Ca^{2+} entry into the cell (Figure 7(1)) [131].

HTLV-1 p12 has numerous effects on cell signaling when expressed exogenously. It can affect T-cell signaling or bind IL-2R β and γ subunits [132]. IL-2 expression is increased via NFAT activation in a Ca^{2+} dependent manner in Jurkat and primary T lymphocytes. In turn, this leads to a reduced dependency on IL-2 for T-cell activation in the presence of p12 [133]. In addition to Ca^{2+} signaling related proteins, p12 can also bind vacuolar H^{+}-ATPase and immature peptides of MHC class I, which leads to their proteosomal degradation [134;135]. In the ER, p12 binds to newly synthesized MHC-I heavy chains and prevents them from associating with the β_2-microglobulin, a component of the mature MHC-I complex (Figure 7(4)). Since improperly assembled proteins are removed from the ER for degradation, the p12-mediated inhibition of MHC-I heavy chain association with the β_2-microglobulin leads to its degradation by the proteasome and

results in decreased MHC-I cell surface expression. By decreasing antigen presentation through degradation of the MHC-I, p12 may diminish presentation of viral peptides and decrease recognition by cytotoxic T-lymphocytes [136]. Furthermore we know that NK cells recognize and destroy cells that express low levels of MHC-I at the cell surface. ORF-I decreases MHC-I expression to inhibit presentation of viral proteins to cytotoxic T-lymphocytes, which could make HTLV-1-infected cells susceptible to NK cell cytotoxicity [137]. In contrast, HTLV-1-infected T-cells are resistant to NK cell-mediated killing [137]. Early data demonstrated that several ATL cells lines had altered expression of intercellular cell adhesion molecule 1 (ICAM-1), a glycoprotein that facilitates the interaction between NK cells and T-cells [138]. Furthermore, the majority of HTLV-1-infected primary $CD4^+$ T-cells do not express ligands for the NK cell activating receptors, natural cytotoxicity receptors, and NKG2D [138].

IL-2 stimulation and p12 expression significantly increased the rate of syncytium formation, suggesting a novel role for IL-2 signaling and Jak activation in HTLV-1 virus transmission [139].

HTLV-1 p12 can be proteolytically cleaved to create a smaller protein, p8. This protein apparently serves a different role and acts to increase T-cell contact through LFA-1 clustering thereby enhancing the cellular contacts among T-cells to enhance viral transmission, while anergizing T-cell signaling (Figure 6(5)) [140]. The ability of p8 to decrease T-cell activation is likely mediated through inhibiting proximal T-cell receptor signaling at the immunological synapse where it decreases phosphorylation of key signaling proteins in a LAT-dependent mechanism (Figure 7(3)) [140].

The ability of p12 to induce LFA clustering on infected T lymphocytes was previously demonstrated and hypothesized to increase the efficiency of cell-to-cell spread of the virus [141]. The processing of p12 into p8 may account for the influence of the ORF1 encoded proteins on LFA-1

clustering on the cell surface and the formation of cellular conduits [140]. Equally as plausible is the ability of p12 to induce calcium-mediated LFA-1 clustering on the surface of T-cells, a known mechanism of LFA-1 functional activation [142].

The transmembrane domains of ORF-I appear to be dispensable for binding to the V-ATPase, while conservation of the proline-rich domains between amino acids 36 and 48 contributes to the strength of this interaction [122]. The 16 kDa protein is a membrane component of the V-ATPase, which is also found in clathrin coated vesicles, lysosomes, endosomes, Golgi vesicles, endoplasmic reticulum, and synaptic vesicles. This proton pump is responsible for the acidification of these intracellular vesicles [143]. The atypical function of the proton pump through binding of viral proteins such as HTLV-1 p12 may interfere in functions like the dissociation of receptor-ligand complexes and trafficking within the endosomal/lysosomal compartment. In addition, the acidification is essential for the formation of endosome carrier vesicles, which are intermediates between early and late endosomes [144]. HTLV-1 is known to infect dendritic cells and the acidification of lysosomes could play an important role in virus entry [145]. Indeed, the ablation of ORF-I expression impairs HTLV-1 replication in dendritic cells [7].

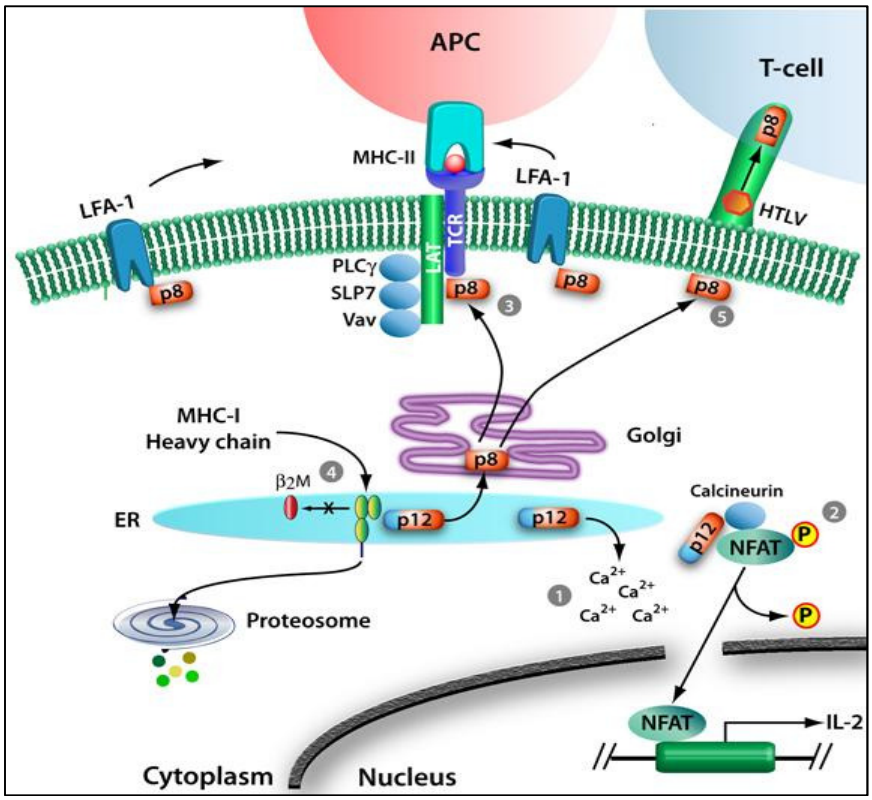


Figure 7: Functions of p12 and p8. In the ER, p12 is proteolytically cleaved at the amino terminus to generate p8, which traffics to the cell surface through the secretory pathway. (1) In the ER, p12 mediates Ca^{2+} release, which enables (2) either calcineurin binding of NFAT and subsequent dephosphorylation, nuclear translocation, and upregulation of the IL-2 gene, or p12 binding to calcineurin and inhibition of NFAT activation. (3) Upon trafficking through the secretory pathway, p8 localizes at the immunological synapse where it interacts with LAT and inhibits proximal TCR signaling. (4) In the ER, p12 binds the immature heavy chains of the MHC-I and prevents their interactions with the β_2-microglobulin, leading MHC-I degradation by the *proteasome*. (5) At the cell surface, p8 increases the clustering of LFA-1 and the formation of intracellular conduits and facilitates viral transmission to target cells. (Edwards *et al.*, 2011)

The first evidence that pX ORF-I was important for viral transmission was demonstrated in the rabbit model using a splice acceptor site mutant of the ACH infectious molecular clone [146]. Ablation of the acceptor splice site through the deletion of four nucleotides was associated with a reduction of viral infectivity *in vivo* [146] and *in vitro* in non-stimulated T-cells. The deletion of this p12 acceptor splice site would also introduce a frame shift in the HBZ antisense ORF, resulting in the deletion of the last 24 amino acids of HBZ [147].

However the replication capacity of subsequent specific HBZ mutants in context of molecular clones did not result in complete reduction of infectivity as observed in ORF1 splice mutants. Thus, the rabbit model was able to detect selected HBZ mutations and demonstrated that these were different in viral spread compared to ORF-I mutants. A recent study tested a variety of HTLV-1 mutant molecular clones for their ability to replicate in dendritic cells and *in vivo* in rabbits and macaques [82]. In this study, mutations to ablate p12, p30, and HBZ were introduced in the ClaI/SalI cassette from the HTLV-1 molecular clone pBST that encompasses the orf-I and the orf-II. Rabbits inoculated intravenously with these mutant clones had reduced viral loads at 16 weeks post inoculation before recovering to wild type control level. Dendritic cell cultures from macaques infected with these mutant clones had reduced viral replication parameters suggesting the importance of this cell type in early viral transmission [82]. Small groups of macaques inoculated with these same mutant molecular clones also exhibited limited viral expression [82] confirming the importance of p12/p8 and p30 expression for viral transmission.

1.5.5 p13

Differential splicing of mRNA from HTLV-1 orf-II results in production of the p13 protein [148; 149; 150]. p13 is translated from singly spliced monocistronic mRNA to form a highly basic 87 amino acid protein that corresponds to the carboxyl terminus of p30 (Figure 3) [148]. The p13

protein has been predicted to contain a short hydrophobic leader sequence, an amino terminus mitochondrial targeting signal (MTS) which allows the viral protein to target the mitochondria and is a predicted α-helix that is arginine rich and amphiphatic [151; 152], a transmembrane domain, a flexible hinge region and a carboxyl terminus β-sheet hairpin structure [153; 152]. The carboxyl terminus region contains multiple PXXP motifs that may mediate Src homology 3 (SH3) ligand binding. In addition, the carboxyl terminus region contains a cryptic nuclear localization sequence (NLS) [154]. The viral protein is predominantly localized within the inner membrane of mitochondria of transfected cells [151]. However, when expressed at high levels, p13 is able to localize within the nucleus and, when coexpressed with Tax, is directed to nuclear speckles [155]. Localization of the p13 protein within mitochondria and the nucleus suggests that this protein may modulate effects on apoptosis and transcriptional regulation (Figure 8). The incorporation of p13 into the inner mitochondrial membrane causes morphological changes such as swelling and a loss of inner membrane potential (Δψ) changing K^+ permeability of these organelles. The effects of p13 on K^+ permeability are dose-dependent [152]. p13-induced K^+ influx and mitochondrial membrane depolarization stimulates electron transport and mitochondrial respiration, which increases O_2 consumption and change energy production, redox status and induce apoptosis in cells [152]. Modulation of respiratory chain activity by p13 is accompanied by increased mitochondrial reactive oxygen species (ROS) production. Changes in mitochondrial Δψ also regulate intracellular Ca^{2+} homeostasis (Figure 8(2)). A p13 peptide was found to induce rapid efflux of Ca^{2+} from preloaded mitochondria. Though p13 reduces mitochondrial Ca^{2+} uptake, it does not significantly affect overall change in cytosolic Ca^{2+} concentration, suggesting that p13-mediated mitochondrial depolarization may alter Ca^{2+} concentration only locally [156].

Ectopic expression of p13 affects structure and disrupts the inner

membrane potential of mitochondria [152]. The ectopic expression of p13 causes HeLa and Jurkat T-cells to be sensitive to caspase-dependent, ceramide- and FasL-induced apoptosis. A farnesyl transferase inhibitor that prevents post-translational modification of Ras blocks this suppressive effect of p13 [157].

Interestingly, primary T-cells that express p13 are activated, while causing transformed cells to be sensitive to reactive oxygen species. Studies of a p13-knockout virus showed that although the protein is dispensable for viral replication in cultered cells, it is required for establishing a persistent infection in a rabbit experimental model [157].

Collectively these studies indicated that p13 has the ability to modulate cell survival via Ras-mediated cell signaling and has an essential role early virus transmission and in virus persistence. p13 interacts with farnesyl pyrophosphate synthase (FPPS), which is involved with synthesis of FPP substrate and is required for prenylation of Ras and subsequent activation of Ras [158]. In cells expressing Tax, p13 becomes ubiquitinated and is partially localized within the nucleus (Figure 8(4)) [159]. Interestingly, Tax mediates ubiquitination of p13 though this protein contains no lysine residues. Instead, p13 is likely ubiquitinated on serine and threonine residues and this modification increases the stability of the protein. Within the nucleus, ubiquitinated p13 associates with Tax to inhibit its binding to the CBP/p300 transcriptional coactivator [159]. A decrease in Tax-CBP/p300 complex formation results in decreased Tax-mediated viral gene transcription [159]. Thus, intracellular localization of p13 may be an additional regulatory switch between viral replication and latency.

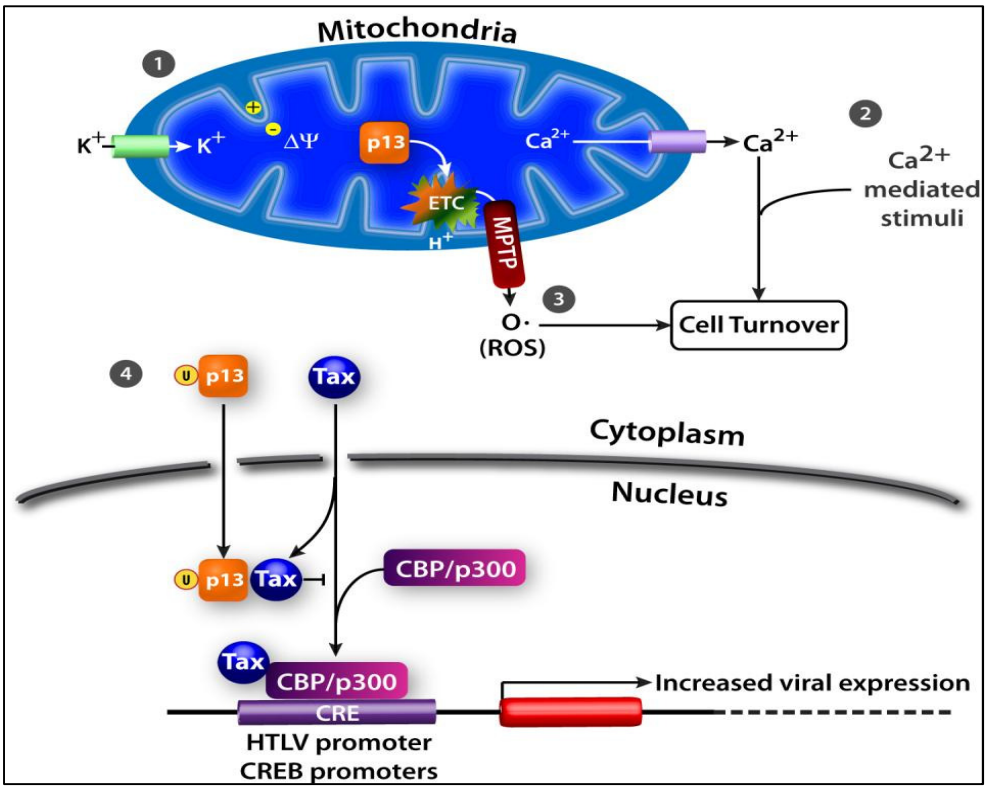


Figure 8. Functions of p13. In mitochondria, p13 mediates (1) K+ influx, inner mitochondrial membrane potential, and electron transport chain activity to affect (2) Ca2+ signaling and (3) ROS production. (4) In the presence of Tax, p13 is ubiquitinated and translocates to the nucleus. In the nucleus, p13 inhibits Tax-CBP/p300 complex formation to decrease transcription of cellular and viral genes. (Edwards *et al.*, 2011)

1.5.6 p30

In 1992, two groups independently reported a new doubly spliced mRNA which potentially encodes for two viral proteins. The first one shares the Env/Tax AUG and encodes for the nuclear/nucleolar p30. The second one is shorter and encodes for p13. Only indirect evidence of p30 protein expression in patients infected with HTLV-1 exists. However, this is also true for most other viral proteins, including Tax and Rex, and this possibly reflects the latent state of the *virus in vivo*. p30-encoded mRNAs can be

readily detected in HTLV-1-infected cell lines *in vitro* and in some freshly isolated samples from HTLV-1-infected subjects, and ATL and HAM/TSP patients. Antibodies directed to p30 and cytotoxic T cells that recognize peptides from p30 have been found in HAM/TSP patients and HTLV-1 carriers [160]. p30 is dispensable for T-cell immortalization *in vitro* but is required for efficient viral propagation *in vivo* [161].

The p30 protein is translated from doubly spliced monocistronic mRNA, containing exons 1, 2, and B, transcribed from HTLV-1 orf-II (Figure 3) [162; 163]. p30 is a highly basic protein with a net positive charge that contains three nuclear localization signals (NLS_1, NLS_2, and NLS_3) and an arginine-rich nucleolar localization and/or retention (NoRS) domain [164]. p30 also contains a Rex-binding domain (RexBD), a p300-binding domain, and a DNA-binding domain [165] which repress LTR-mediated transcription. Notably, HTLV-1 p30 has low genetic variability and is similar to HTLV-2 p28, suggesting a conserved mechanism for negative modulation of virus replication [164; 166]. Live cell imaging technologies, indicated a high mobility of GFP-p30 in the nucleus. In contrast, a much slower kinetic was consistently detected in the nucleolus, suggesting that p30 is strongly retained in the nucleolus [164]. Specifically, p30 is located in a granular component of the nucleolus where ribosome subunits are assembled and *de novo* mRNA is produced. In fact, p30 was found to interact with the large ribosomal subunit L18a18. These interactions were functionally relevant and played a role in p30 nucleolar retention along with nascent mRNA. Several studies have shown that the ribosomal protein L18a interacts with eukaryotic initiation factor-3 and facilitates internal re-initiation of translation, it is possible that p30 translocates from the nucleoli to the cytoplasm (Figure 9(1)) [165]. Similarly, p30 is specifically delocalized from the nucleoli to the nucleoplasm upon DNA damage to interfere with DNA repair processes [167]. In addition, p30 nucleolar retention signal mutants have similar functionality as wildtype p30, which

raises questions about the function of nucleolar localization. A recent hypothesis suggests that p30 retention within nucleoli may serve as a reservoir for when the protein is needed in the nucleus [167]. Interestingly, it has recently been shown that HBZ mRNA can regulate the production of p30, probably acting as an antisense RNA to silence its expression (Figure 9(4)) [168]. Localization of p30 within the nucleus and nucleolus suggest that this protein may mediate critical cellular processes such as cell cycle progression, DNA repair, and mRNA export [169]. Intriguingly, recent studies have shown that in a macaque model, ablation of p30 within the HTLV-1 provirus severely affects infectivity and leads to reversion of the virus to the wild type genotype [82]. This observation has been confirmed in vitro by p30 knockout in HTLV-1-infected human primary and monocyte-derived dendritic cells, in which infection is not sustained over time. In T-cells, p30 is not required *in vitro* for efficient viral replication [82].

p30 has different functions:

- inhibition of Nuclear Export of Tax/Rex mRNA
- repression of the CRE Pathway
- transcriptional and posttranscriptional Regulation of different pathways
- control on cell cycle and DNA Repair
- viral persistence in animal models

In contrast to HTLV-1 Tax and Rex, which enhance viral replication, p30 blocks the nuclear export of *tax/rex* mRNA to the cytoplasm, resulting in down-regulation of both positive regulators Tax and Rex, suppression of virus replication and latency to escape host immune surveillance and to favor propagation through cell division and clonal expansion of infected cells (Figure 9(5)) [170]. p30 can perform all these actions interacting with the p30 mRNA-responsive element (p30RE) of Tax/Rex mRNA and with Rex at the RexBD. Interestingly, p30RE spans the exon junction created after Env mRNA is spliced, hence p30 binds spliced Tax/Rex mRNA but not

the unspliced and singly spliced viral RNA.

The interplay between Rex and p30 is a regulatory switch between viral replication and latency. p30 specifically forms complexes with Rex and p30-Rex interactions are markedly increased by the presence of viral mRNAs. Rex binds with high affinity to the Rex-responsive element (RexRE) at the 3' end of viral mRNA and, together with CRM1, shuttles unspliced gag/pol and singly spliced env transcripts to the cytoplasm (Figure 8(3)). Once bound to mRNA, Rex is no longer accessible to binding by p30 and is able to shuttle transcripts to the cytoplasm. However, viral mRNA-bound p30 efficiently interacts with Rex, but tax/rex transcripts are still retained in the nucleus (Figure 9(5)) [171]. An excess of Rex, which displaces p30 from the p30RE, reverses nuclear retention of viral mRNA. A further spliced version of Tax/Rex has been found in HTLV-1 infected cells, p21rex. During splicing of p21rex, the p30RE is removed from Rex mRNA, which allows the transcript to escape p30-mediated nuclear retention.

In addition to its post-transcriptional activity, p30 has been reported to have transcriptional activities both as activator or repressor. When fused to the Gal4 DNA-binding domain, p30 shows strong transcriptional activation. The ability of p30 to induce transcriptional activation *in vitro* is CBP/p300-dependent [172].

Additional studies showed that p30 repressed the cellular CREB-responsive element-driven reporter gene activity in a dose-dependent manner. When expressed at low levels, p30 was shown to activate the HTLV-1 LTR reporter gene. However, at high levels, p30 repressed the LTR reporter activity [172]. Several other proteins bind CBP/p300, including members of the Jun-family, c-Myb, c-Fos, STAT1/2, NF-κB, p53, and TATA-binding protein (TBP) [82]. p30 disrupts CREB-Tax-p300 complex formation on the TRE (Tax-responsive element) of the viral LTR, resulting in repression of HTLV-1 transcription [173].

Therefore, the expression level of p30 may play an important role in its

function in the cell. Because p30 suppresses Tax production by retaining tax/rex mRNA in the nucleus, it affects CRE- and TRE-mediated transcriptional activation [88]. Interestingly, histone acetyltransferase (HAT) activity of p300 modulates p30-dependent transcriptional downregulation, whereas p30-dependent LTR repression is enhanced by deacetylation and inhibited by acetylation [173]. Taken together, these data suggest that p30 might decrease transcription of the viral genome, thereby facilitating viral latency.

Microarray gene expression analyses of human T-cells showed that HTLV-1 p30 affects a number of cellular genes at the transcriptional level. p30 represses many cellular genes, particularly adhesion molecules, such as integrins and cadherins, but increases the expression of certain target genes involved in T-cell activation and apoptosis [174]. Furthermore, p30 is able to retain some cellular transcripts within the nucleus, similarly to viral tax/rex mRNA. Included among these transcripts are MDM4, which is a regulator of p53, and HDAC3, which is a histone deacetylases involved in transcriptional repression.

Moreover, p30 has been shown to interact with the cellular transcription factor PU.1 in human macrophages. PU.1, also known as Spi-1, is a transcriptional factor comprised in the ETS family. Its expression is cell-specific and it has been found in macrophages, dendritic cells, B-cells and neutrophils. PU.1 plays a critical role in cell activation and differentiation. Interestingly, PU.1 can interact with a variety of factors, including IRF4, ICSBP/IRF8, JUN and NFkB, and promotes transcription of type 1 interferons (IFNs) [23-30]. Therefore, PU.1 is involved in the signal transduction upon activation of different toll like receptors (TLRs) including signal transduction by Toll-like receptor-4 (TLR-4) [175]. The interaction of p30 and PU.1 leads to inhibition of the DNA binding and transcriptional activity of PU.1. This, together with the p30-mediated inhibition of GSK-3β, yields decreased expression of TLR-4 at the cell surface, resulting in

decreased secretion of *pro-inflammatory* cytokines, such as MCP-1, TNF-α and IL-8, and an increase of the anti-inflammatory cytokine IL-10 [175]. This finding is of interest because 80% of ATL patients have high levels of IL-10 in serum. Interference of TLR4 signaling by p30 and reduction of released amounts of *pro-inflammatory* cytokine IL-10 may impair the ability of dendritic cells to activate adaptive immunity in ATL patients and thereby explain the limited proliferation of virus-specific CTL reported in ATL patients. These data indicate a global effect of p30 on numerous genes in infected cells, suggesting that p30 is involved in more than just virus replication control. Until now, no consistent DNA response element has been reported, so the function of p30 in transcription of cellular genes remains to be determined [176].

Expression of p30 results also in the accumulation of T-cells in the G2 phase of the cell cycle. p30 is able to enhance phosphorylation and activation of check point kinase 1 (Chk1). Chk1 is activated by ATM/ATR kinase following single strand DNA damage and results in a G2 arrest of the cell cycle [177]. p30 specifically binds to ataxia-telangiectasia mutated (ATM) and regulator of 20S proteasome activators γ (REGγ) in multiprotein high molecular weight complexes. By binding to ATM, p30 prolongs cell survival following DNA damage by inhibiting ATM autophosphorylation and subsequent activation of proteins involved in DNA repair, cell cycle check points, and apoptosis [178]. The effect of p30 interactions with REGγ remains to be determined. REGγ is localized in the nucleus where it interacts with and stabilizes p30, thereby altering p30 turnover. In addition, p30 is able to bind to and prevent complex formation of cyclin E and cyclin-dependent kinase 2 (CDK2). Disruption of this complex prevents phosphorylation of retinoblastoma and subsequent E2F-mediated transcription, two key steps for G1/S transition [179]. By inhibiting cyclin E-CDK2 complex formation, p30 delays entry of cells into the S phase of the cell cycle. The inhibition of cell cycle progression is consistent with the

observation that dendritic cells isolated from peripheral blood of ATL patients are unable to stimulate proliferation of $CD4^+$ and $CD8^+$ T-cells [180].

p30 is also able to interact with multiple proteins involved in DNA repair processes. Upon DNA damage, p30 specifically delocalizes from the nucleolus to interact with and affect correct assembly of MRN complexes. MRN complexes are a key factor of DNA repair and contribute to homologous recombination (HR) during the S phase of the cell cycle. In contrast, during G1 or M phases, DNA damage is preferentially repaired by the nonconservative nonhomologous end joining (NEHJ) pathway. By interacting with these complexes, p30 activates a shift from conservative HR to the error-prone NEHJ pathway, thus favoring an accumulation of genomic alterations. Overall, these observations suggest that p30 could contribute to the accumulation of mutations that are characteristic of transformed HTLV-1-infected T-cells.

HTLV-1 p30 exact function and relevance *in vivo* still remains elusive. It has been previously shown *in vitro* that the ablation of p30 expression does not affect viral infectivity of HTLV-1 in human primary cells and that p30 is dispensable for viral replication and immortalization of primary human T-lymphocytes [181]. It should be noted that the *in vitro* infectivity of p30-ablated HTLV-1 was not sustained over time in primary dendritic cell [82]. Other observations, such as the presence of antibodies to p30 during infection may provide evidence of the importance of p30 *in vivo* [182]. It was first shown that the ablation of p30 expression *in vivo* result is a dramatic decrease of HTLV-1 viral load in a rabbit model [183]. However, the mutation introduced to prematurely stop the translation of p30 affected hbz as well, whose ablation alone decreases viral replication [184]. Later studies have used different mutations to ablate p30 expression while preserving hbz [82]. Valeri et al showed that in the macaque model, but not in the rabbit model, the ablation of p30 expression within a biologically

active HTLV-1 molecular clone (p30-KO) or p12/p8 (p12-KO) severely affects its infectivity. When infection occurs in the case of p30-KO, it is associated with early reversion of the virus to the wildtype genotype, where in the case of p12-KO, neither infection non-genetic reversion is observed underscoring the importance of these viral genes [82]. Furthermore, the infectivity of p30-KO and p12-KO in human primary monocyte-derived dendritic cells (*Mo-mDCs*) is also severely impaired. In contrast, the lack of expression of p30 or p12/p8 in human B-cells [82] or primary human $CD4^+$ T-cells (our unpublished data) does not affect viral replication *in vitro.*

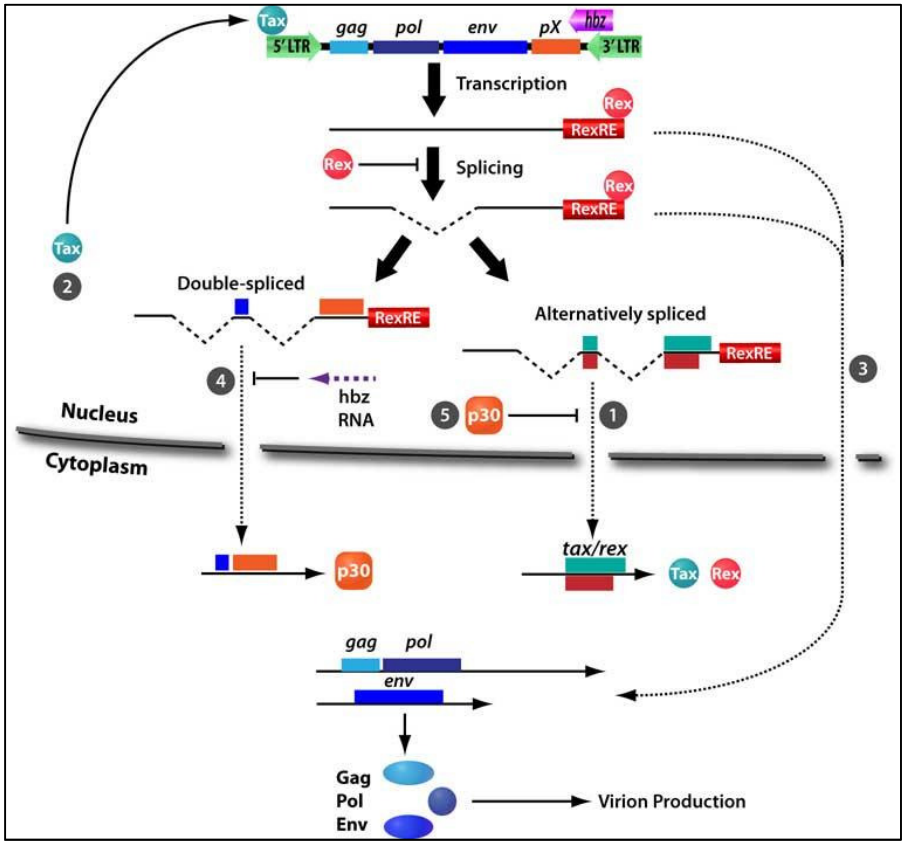


Figure 9. Functions of p30. (1) Alternatively double spliced mRNA is translated to form the Tax and Rex regulatory proteins. (2) Tax protein localizes to the nucleus to exert its function on the LTR as a positive

regulator of viral transcription. (3) Within the nucleus, Rex recognizes the Rex-responsive elements (RexRE) of viral mRNA and shuttles these transcripts to the cytoplasm while inhibiting splicing processes. However, some of the viral RNA is processed in the spliced env mRNA, the double spliced p30, (4) and the alternatively spliced tax/rex mRNA. p30 mRNA is subject to negative regulation by hbz mRNA. Once p30 protein is produced, it translocates to the nucleus and (5) interacts with p30-responsive elements (p30RE) created by the double splicing and therefore is present on tax/rex mRNA only. Moreover, p30 interacts with Rex to inhibit Rex-mediated nuclear export of double spliced viral mRNA, including tax transcripts. By preventing Tax production, p30 decreases viral transcription. (Edwards *et al.*, 2011)

1.5.7 HBZ

The presence of an anti-sense transcript of HTLV-1 was first detected by Northern blot in HTLV-1-infected cell lines [185]. However, the function and exact structure of this gene product remained unknown. In 2002, a viral protein that binds to CREB-2 was found by yeast two-hybrid screening, and named HTLV-1 bZIP factor (HBZ). HBZ was first found to inhibit viral gene transcription of the sense strand [186]. The negative strand of the HTLV-1 genome contains one ORF located in the pX region (antisense orientation) (Figure 3) which generates at least 2 different transcripts, one spliced (hbz sp1) and the other unspliced (hbz us) [187; 188; 189]. The first exon of the *HBZsp1* gene transcript is present in U3 and R regions of the 3′LTR. The difference between HBZ sp1 and HBZ us is the presence of the first exon in the former transcript so only 7 amino acids in the N- terminus. Hbz sp1 has multiple transcriptional initiation sites in the U5 and R regions of the 3' LTR, whereas the hbz us gene initiates within the tax gene. Both hbz sp1 and hbz us have TATA-less promoters [190]. It has been reported that the basal transcription factor Sp1 is critical for many TATA-less promoters [191; 192]. Since Sp1 is a well-known regulator of housekeeping genes, transcription

of the *HBZ* gene may be relatively constant. The TRE sequence in the 3' LTR also functions to enhance transcription of the hbz anti-sense transcripts. However, the enhancing activity for anti-sense transcription is relatively weak when compared with sense transcription. It is known that the *HBZ* gene transcript is better correlated with proviral load than the *tax* gene transcript [193], indicating that the *HBZ* gene is constantly expressed in HTLV-1 infected cells particularly in ATLL cases.

Quantitative analyses of the HBZ gene transcripts showed that The HBZ SP1 protein is fourfold more abundant and has a longer half-life than HBZ US isoform [194], and the hbz sp1 mRNA is expressed at higher levels compared to the hbz us mRNA [195]. This observation correlates with the finding that the promoter activity of the HBZsp1 gene was much higher than that of the HBZ us gene.

Relative expression level of the HBZ gene, adjusted by proviral load, was almost equivalent among HTLV-1 carriers, HAM/TSP patients, and ATL patients [196]. A previous study reported that HTLV-1 proviral load was correlated with the expression level of the *tax* gene [197]. However, the HBZ gene transcript was more closely correlated with proviral load than was the level of the *tax* gene transcripts [196]. Kinetic study of the HBZ sp1 gene transcripts in rabbits shows that HBZ sp1 gene transcription was detected one week post-infection and increased and stabilized, while other viral genes were at or below the limit of detection [198]. This finding supports a correlation between HBZ sp1 gene expression, proviral load, and survival of HTLV-1 infected cells. HBZ gene expression is closely linked with disease severity of HAM/TSP, suggesting that the HBZ gene expression plays critical roles in proliferation of HTLV-1 infected cells and pathogenesis.

The spliced transcript of HBZ is translated into a polypeptide of 206 amino acids, while the protein product of unspliced HBZ is a polypeptide of 209 amino acids. The HBZ protein contains 3 domains: an N-terminal

transcriptional activation domain (AD), a central domain (CD) and a C-terminal basic ZIP domain (bZIP). It localizes in the nucleus with a speckled pattern and contains three regions associated with its nuclear localization: two regions rich in basic amino acids and a DNA binding domain [199].

HBZ is not necessary for viral replication or immortalization *in vitro* of T cells, although mutation of the HBZ gene resulted in decreased proliferation of infected cells *in vivo* [200], implicating HBZ in infectivity and viral persistence.

HBZ interacts with a number of transcription factors, including CREB-2, p300/CBP, Jun family members, and NF-kB. Through interactions mediated by its bZIP domain, HBZ abolishes the ability of CREB-2 to bind to theTax responsive element (TxRE) in HTLV-1 LTR, resulting in the suppression of transcription from the 5' LTR by Tax (Figure 8) [201]. HBZ interacts with cellular coactivator CBP/p300 in its N-terminal region, leading to suppression of viral transcription by inhibiting the recruitment of CBP/p300 to the HTLV-1 promoter [202].

So far, there is no report that HBZ has the capacity to associate with DNA directly. It is apparent that HBZ exerts its suppressive effect on HTLV-1 transcription mainly by interacting with cellular proteins on the HTLV-1 promoter. HBZ's bZIP domain also mediates formation of heterodimers with several AP-1 transcriptional family members, such as c-Jun, JunB, and JunD, but not c-Fos and modulates their activity (Figure 10) [203; 204]. Binding of HBZ to JunB and c- Jun decreases their DNA binding capability by preventing their interaction with Fos, leading to repression of the AP-1 complex. Additional AP-1 transcriptional repression is explained by HBZ inhibition of c-Jun by promoting the degradation of its protein product [205] (Figure 10) and sequestration of JunB by HBZ within nuclear bodies. In contrast to JunB and c- Jun, the interaction of HBZ with Jun-D, another member of AP-1 family, stimulates its transcriptional activity, and results in the activation of JunD-dependent cellular genes (such as Sp1) including

human telomerase reverse transcriptase (hTERT) (Figure 8) [204;206].

NF-κB is activated in HTLV-1 infected cells and ATL cells. Activated NF-κB plays important roles in the proliferation of ATL cells and inhibition of apoptosis [207]. HBZ has been shown to inhibit the activation of the classical NF-kB pathway by two different mechanisms (Figure 9): by inhibiting the DNA binding of the NF-kB subunit p65 or by increasing the expression of PDLIM2, the E3 ubiquitin ligase of p65, leading to enhanced ubiquitination and degradation of p65 [208]. NF-κB suppressive activities are common among different viruses, suggesting that these suppressive activities are important for viral infection. A virus might escape from the host immune system by suppressing the classical NF-κB pathway.

Suppressed expression of the HBZ gene by shRNA leads to decreased proliferation of ATL cell lines so HBZ expression is associated with proliferation of ATLL cells *in vivo* and *in vitro* [209; 210]. Mutational analyses of the hbz gene showed that hbz mRNA, rather than HBZ protein, has a growth-promoting effect on T-cells [210]. The coding sequence of the HBZ gene was replaced with silent mutations, which could produce the same protein while the RNA structure was completely altered. This mutant did not have a growth-promoting activity. Only HBZ sp1 RNA, not HBZ us RNA, promotes proliferation of T-cells, indicating that the first exon of the HBZ sp1 transcript is critical for this activity [211]. Further details of how HBZ sp1 RNA promotes proliferation remain to be elucidated.

An intriguing aspect of ATLL pathogenesis is represented by the fact that Tax expression is not detected in about 60% of leukemia cases despite its proven central role in leukemogenesis; i.e. it immortalizes T-lymphocytes *in vitro* and induces cancer in transgenic animals [212]. Three mechanisms for inactivating Tax expression have been described:

- genetic changes (nonsense mutation, deletion, and insertion) of the *tax* gene [212];
- deletion of the 5' LTR (which is a promoter/enhancer of viral gene

transcription of the plus strand) [213];

- CpG sites of the 5' LTR hypermethylation [214].

These findings suggest that Tax expression is not necessary in the late steps of the leukemogenic process although its expression is required at an early stage of ATL or in the carrier state. One possible scenario is that since Tax is the major target of cytotoxic T-lymphocytes (CTL) *in vivo*, these mechanisms to disrupt or decrease 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 [215], suggesting that hbz gene expression plays a critical role in the development of this disease.

HBZ gene is responsible for leukemogenesis by HTLV-1. Further, HBZ might be a novel target for prevention of, and therapies for, HTLV-1-associated diseases.

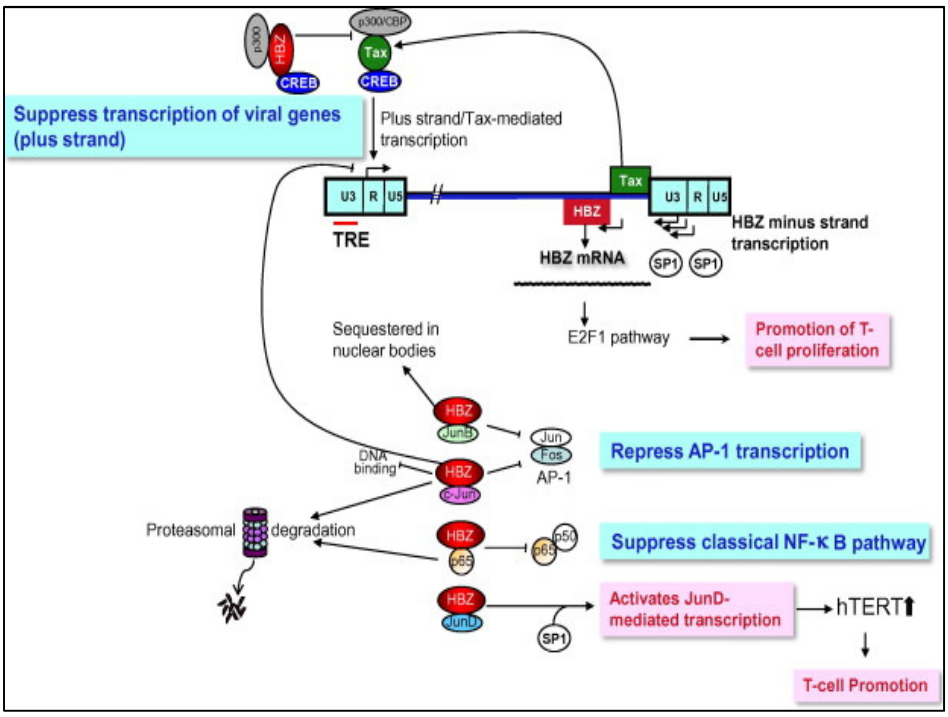


Figure 10. Functions of HBZ. HBZ protein interacts with c-Jun or CREB and suppresses viral transcription from the 5′LTR. HBZ mRNA promotes T-cell proliferation. A transcription factor, SP1, plays a critical role in the HBZ gene transcription. (Masao Matsuoka, 2010).

1.6 Type 1 Interferons and the Virus Host Relationship

Viruses have adapted strategies to evade or even inhibit key elements of host immune responses. Because particular arms of the responses are susceptible to microbes escape mechanisms, the host has evolved several ways to activate a broad range of defense mechanisms to ensure effective protection. An example of these means could be type 1 IFNs that are important molecules for antiviral defense and immune regulation. The host has a variety of pathways to elicit the expression of the IFN-α/β cytokines. Cellular sensors fall into two functional classes that differ fundamentally with respect to localization, associated with either the cell membrane or the cytoplasm (Figure 11). An important biological consequence of this differential localization is the flexibility of the host in triggering type 1 IFN production, either in infected cells and/or before cells are exposed to viral products capable of blocking induction. There are two other types of interferon, which can be differentiated from type 1 based on the type of receptor through which they signal: IFN type 2 binds to IFNGR (IFNγ) and IFN type 3 which signal trough a receptor complex (IL10R2 and IFNLR1). These IFNs directly activate macrophages and natural killer cells.

1.6.1 Extracytoplasmic pathways for pathogen sensing: Toll-like receptors

Toll-like receptors (TLRs) are an essential arm of the innate immune response to bacteria, viruses and fungi and link recognition of distinct features of these microbes to the induction of *pro-inflammatory* signaling pathways. The Toll-like receptor (TLR) family is composed of membrane proteins with domains designed to sample the environment for pathogen-

associated molecular patterns (PAMPs) [216]. Different TLR molecules recognize specific PAMPs: multiple microbial products can serve as ligands, including LPS (TLR4), lipopeptides (TLR2/1 and TLR2/6), flagellin (TLR5), and TLR3, TLR7, TLR8, and TLR9 appear to play important roles in identifying viral products. Particularly TLR9 recognize unmethylated CpG motifs in DNA, and TLR7 and 8 single-stranded RNA. In contrast, TLR3 appears to represent a more general sensor of viral infection through detection of double-stranded RNA (dsRNA), a product of viral replication and transcription for both RNA and DNA viruses. Accordingly, TLR9 becomes activated in response to infection with DNA viruses such as herpesviruses, and TLR7 and TLR8 respond to RNA viruses such as influenza viruses and HIV. TLRs can also be subdivided into two groups based on their localization within the cell. While TLR1, TLR2, TLR4, TLR5 and TLR6 are localized at the cell surface, the nucleic acid-sensing TLRs, TLR3, TLR7, TLR8 and TLR9 are localized intracellularly within endolysosomes. This intracellular localization facilitates recognition of nucleic acids released from microbes degraded within endolysosomes.

After recognition of pathogens, TLRs become activated and transmit signals through their cytoplasmic Toll/ interleukin-1 receptor (TIR) domains, resulting in the transcriptional induction of multiple genes involved in innate and adaptive immunity, including type 1 IFN.

TLR3 induction of type 1 IFN is mediated through the TIR domain–containing adaptor- inducing IFN-β (TRIF). TRIF mediates the activation of IkB kinase ε (IKKε) and tank binding kinase 1 (TBK1), which phosphorylate IFN regulatory factor 3 (IRF3), resulting in its dimerization and nuclear translocation where it promotes gene transcription. TRIF also mediates the activation of neural factor kB (NF-kB) and activating protein 1 (AP1) through the kinase complex IKK α/β/γ and the mitogen-activated protein kinase (MAPK) cascade, respectively [217]. These three transcription factors (IRF3, NF-kB, and AP1) coordinate the transcriptional regulation of

the IFN β gene. Induction of type 1 IFN by TLR7/8/9 is mediated by the adaptor molecule myeloid differentiation primary response protein 88 (MyD88), which associates with the TIR domain of the TLRs, the interleukin-1 receptor–associated kinases IRAK1 and IRAK4, and the tumor necrosis factor receptor–associated factor 6 (TRAF6). This results in downstream activation of IRF7, and of the IKK α/β/γ and the MAPK cascades, leading to NF-kB and AP-1 activation [217]. Both TLR3 and TLR7 and 8 are able also to activate *via* IRFs a transcription factor called PU.1, which is responsible of the transcription of different interferon stimulated genes (ISGs).

1.6.2 IFN-Mediated Effects On Defense

As secreted factors, the type 1 IFNs regulate a range of immune responses through the type 1 IFN receptor, a cell surface transmembrane receptor composed of two subunits, IFN-α receptor 1 (IFNAR1) and IFNAR2 [218; 219]. Binding of the IFNAR results in receptor subunit dimerization and activation of kinases that associate with their cytoplasmic tails: the Janus-activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). In turn, tyrosine phosphorylation activates the signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2), to form a trimeric STAT1-STAT2-IRF9 complex, also known as IFN-stimulated gene factor 3 (ISGF3), as well as a STAT1 homodimer complex, known as the IFN-γ–activated factor (GAF). Both complexes translocate to the nucleus and bind to DNA regulatory sequences containing IFN-stimulated response elements (ISREs) and IFN-γ–activated sites (GAS), respectively. The subsequent stimulation leads to the transcription of more than 100 IFN-stimulated genes (ISGs), whose concerted action leads to the generation of an ‘‘antiviral state.’’ Although signaling through STAT1/STAT2 and STAT1/STAT1 dimers are the best characterized of the type 1 IFN-induced intracellular pathways to gene expression, cells can differ greatly in their signaling response to type 1 IFN, and a variety of other signaling pathways

can also be activated [218; 219]. The flexibility that receptors for type 1 IFNs have for accessing downstream signaling molecules may be regulated by the relative abundance of STATs themselves [220].

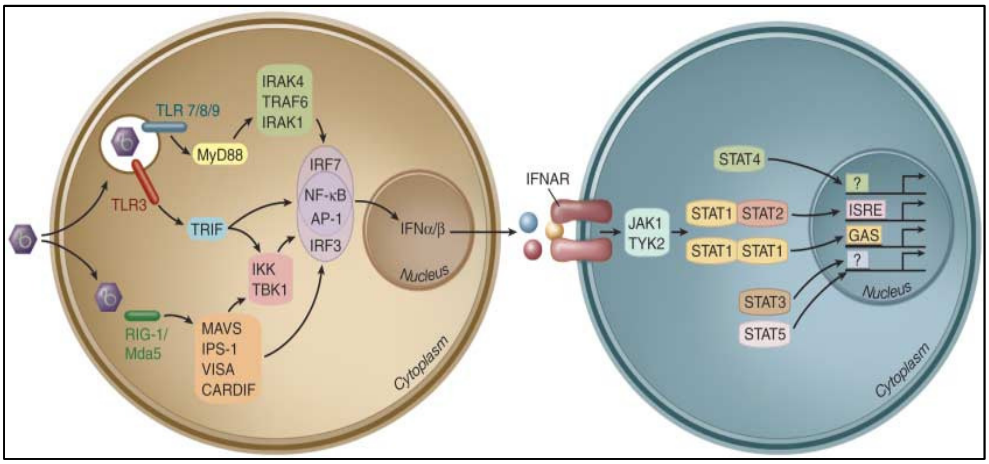


Figure 11. The pathways to and from type 1 IFN are flexible. Families of sensors are available to detect viral products and induce expression of the cytokines. One set senses components in the cytoplasm. Another set is localized in cell membranes. There is also flexibility in the signaling pathways used by type 1 IFNs, with the potential to induce the activation of multiple STAT molecules and their downstream targets of transcription. (Sastre *et al.,* 2006)

1.6.2.1 Antiviral activities of ISGs

The myxovirus resistance GTPase protein A (MxA), the protein kinase stimulated by dsRNA (PKR), the apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (APOBEC3G), and the oligoadenylate synthetases (OAS) are among the best characterized ISGs with antiviral activity. MxA is a guanosine triphosphatase (GTPase) belonging to the dynamin family that sequesters viral ribonucleoproteins to specific subcellular compartments. PKR is a serine-threonine kinase that phosphorylates downstream substrates upon recognition of dsRNA, including the elongation initiation factor eIF2α, resulting in the inhibition of

protein translation. APOBEC3G is a cytidine deaminase enzyme that exerts innate antiretroviral immune activity against retroviruses. The OAS proteins are also activated by dsRNA, leading to the generation of oligoadenylates, which activate ribonuclease L (Rnase L) that degrades cellular and viral RNA. Both PKR and the OAS/RNaseL systems have profound inhibitory effects on basal cellular processes that eliminate virus-infected cells by suicide [221]. Even in the absence of these IFN-regulated antiviral pathways, however, IFN can still induce an effective antiviral response [222]. The existence of other multiple ISGs with antiviral activity, multiple IFN genes, and multiple pathways leading to the production of IFN raises the intriguing question of whether hosts have evolved redundant pathways to make it generally difficult for viruses to use any single mechanisms to inhibit the IFN antiviral response or whether different factors of the IFN system mediate inhibition of specific virus families.

1.6.2.2 Regulation of immune responses

Type 1 IFNs extend their antiviral defense functions to a number of other immune response components. They amplify their own expression through two independent mechanisms: the induction of IRF7 and the accumulation of pDCs. These cytokines also activate natural killer (NK) cells to mediate elevated cytotoxicity [223] and induce interleukin-15 (IL-15) to promote NK cell proliferation [224]. At high concentrations, type 1 IFNs inhibit IL-12 [224] and NK cell responsiveness for IFN-γ expression. All of these effects on innate immune responses depend on STAT1. Type 1 IFNs also regulate adaptive immunity. The early STAT1-dependent induction of IL-15 contributes to short-term proliferation of memory $CD8^+$ T cells [225]. However, only certain subsets of cells are equipped to respond to IL-15 and, consistent with the anti-proliferative effects of type 1 IFNs, STAT1 acts to limit nonspecific $CD8^+$ T cell expansion, at times overlapping with the induction of innate responses [226]. Hence, early during infections, the type 1 IFNs work to enhance proliferation of certain cell subsets and inhibit

others through STAT1-dependent mechanisms.

There are many other paradoxical effects assigned to type 1 IFNs, and some of these may be explained by regulation of accessibility to different signaling pathways. STAT1 is induced by the cytokines, and the protein concentrations of this molecule are elevated at times of type 1 IFN induction during viral infection [221]. These conditions may direct responses to STAT1/STAT2 and STAT1/STAT1 with induction of the antiviral defenses and general inhibition of proliferation as described above, but they also limit the ability of type 1 IFNs to activate STAT4 [221]. To be effective in defense, antigen-specific $CD8^+$ T cells must expand in the presence of type 1 IFNs, and their IFN-γ production is aided by type 1 IFN- and STAT4- dependent events [221]. Thus, the elevated STAT1 levels, induced early during infection, and the consequences for proliferation and STAT4 accessibility, present inherent obstacles for generating protective T cell–mediated immunity. To overcome this, antigen-specific $CD8^+$ T cells with lower relative levels of STAT1 are induced and preferentially undergo proliferation at times when STAT1 levels are elevated in most other cells [226]. Linking cytokine signaling to different pathways, depending on transcription factor levels, is a sophisticated means of providing cells with a variety of downstream options using only a limited number of genes.

In addition to these effects during acute responses, the type 1 IFN receptor helps with long-term maintenance of the CD8 T^+ cell pool [227]. Although the role for STAT1 in this effect has yet to be defined, its ability to inhibit proliferation may serve to protect CD8 T^+ cells from chronic stimulation and eventual depletion through clonal ‘‘exhaustion’’ [228]. In the absence of STAT1, type 1 IFNs can induce growth as well as have anti-apoptotic effects on T cells through possible STAT3- and/or STAT5-dependent events [229; 230].

1.6.3. Viral Evasion of IFN Responses

Many viruses dedicate a substantial part of their genomes to down-

modulating the IFN pathways. A general mechanism used by several viruses is inhibition of cellular gene expression by inhibiting transcription, RNA processing, and/or translation [220]. Virus-induced shutoff of cellular protein expression not only favors the diversion of cellular resources for viral protein expression but also prevents the synthesis of IFN and of ISG products. Most viruses also encode viral products that specifically target pathways involved in the response to IFN, and such products are generically known as viral IFN antagonists. Typically, different virus families are characterized by the presence of specific viral IFN antagonists lacking homology with those from other families. Nevertheless, viral IFN antagonists focus inhibition on at least one of three key pathways: the IRF3, the JAK- STAT, and the PKR pathways.

1.6.4. Virus and host relationship

As we've described above, multiple offensive/defensive mechanisms have evolved to result in a balance between hosts and viruses, and type 1 IFNs have emerged as pivotal in this conflict (Figure 12). Antagonism of these cytokines appears to be a common feature shared by all viruses, and the variety of mechanisms for ‘‘hiding’’ from the direct antiviral effects of type 1 IFNs provides the virus with opportunities to replicate and infect a new host before being eliminated by secondary innate and adaptive immune responses in the case of acute viral infections. In chronic infections, this can emerge as a means to establish viral persistence in the host. The diverse and multiple viral approaches to avoiding IFN induction and function provide persuasive evidence for the potency of these mediators in early defense. A direct consequence of disrupting the function of viral IFN antagonists is a decrease in viral replication and pathogenicity in the host. On the far side of the spectrum, however, viruses equipped with highly refined means of disrupting innate mechanisms activated by type 1 IFN are likely to be in the highest order of pathogenicity. High pathogenicity, however, is not necessarily advantageous for the virus: conditions that

result in too rapid a destruction of the host may have deleterious effects on long-term survival of an infectious agent that requires the host for further propagation.

Although viruses evade the direct antiviral defense mechanisms activated by type 1 IFNs, hosts take advantage of these cytokines to elicit a wide range of responses. The classical signaling pathways used to induce direct antiviral defenses also activate some of these, particularly the ones elicited during innate periods of responses. The importance for protection is clearly shown by the fact that deficiencies in STAT1, and therefore in innate responses activated by type 1 IFNs, result in extreme sensitivities to viral infections [231; 232]. The host, however, has adapted to also use other signaling pathways to activate additional defense mechanisms, most clearly demonstrated for STAT4-dependent induction of T cell IFN-γ production. Thus, new complex and important defense responses have been attached to the critical early antiviral cytokines. One consequence of using these factors to promote downstream innate and adaptive responses is that, even if viruses have escaped their direct antiviral effects, the host can limit the window of opportunity for pathogen advancement.

Much progress has been made, but there is still much to be learned about the pathways regulating IFN induction and function during viral infections. Nevertheless, there is evidence to conclude that, as in many difficult relationships, viruses and their hosts are learning to live together and that type 1 IFNs are important players in this compromise.

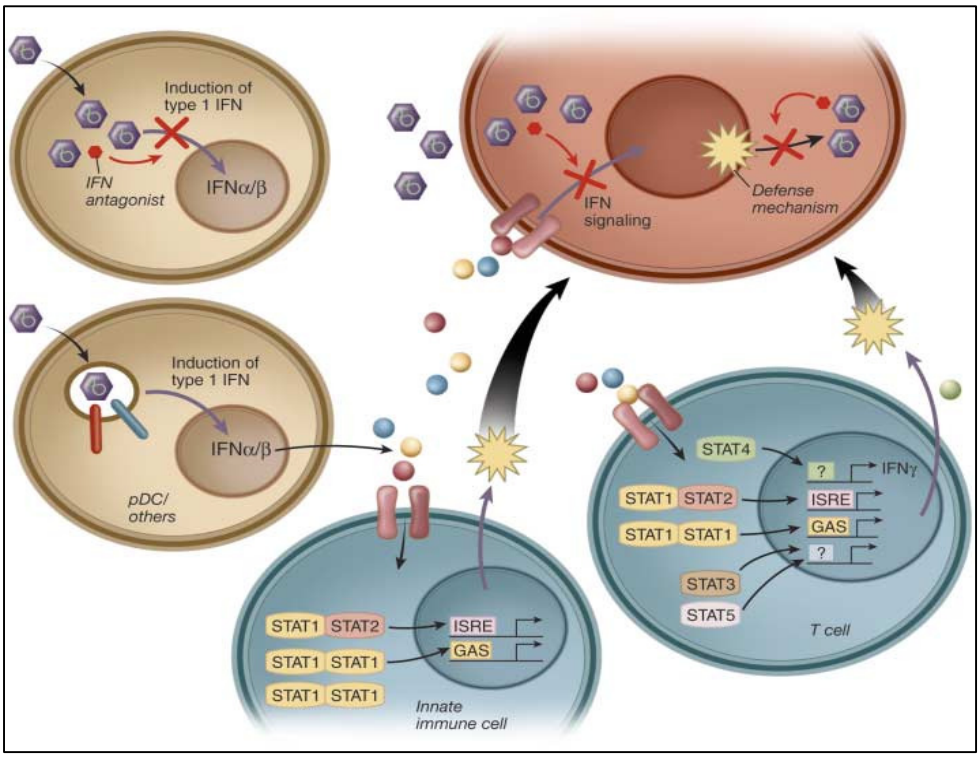


Figure 12. The host uses type 1 IFNs to its advantage despite viral evasion mechanisms. The sensors for detecting viral products include components in the cytoplasm that are particularly sensitive to viral blocks. The set localized in cell membranes is available for sensing viral products before cells are infected. Once induced, the cytokines enhance innate and adaptive antiviral defense mechanisms as well as direct antiviral pathways. The intracellular signaling pathways used by type 1 IFNs appear to be modified to access a variety of downstream target effects in different immune cell subsets. The concentration of the various STAT molecules may act to shape accessibility to different signaling pathways. As a result of these events, an infectious agent overcoming the direct antiviral effects of type 1 IFNs has to deal with additional immune mechanisms of defense. (Sastre *et al.,* 2006)

1.7 HTLV-1 and innate immunity

As mentioned before, upon viral infection, several defense mechanisms cooperate to limit propagation of the pathogen within the organism. Classically, two types of immune responses have been defined: the innate response, rapidly engaged but transient and poorly specific, and the adaptive response, delayed but specific which allows the development of immune memory.

All cells express receptors that are able to be viral sensors and to recognize structural motifs specifically present in pathogens (PAMPs). These sensors such as TLRs (Toll-Like Receptors) and RLHs (RIG-I-Like Helicases) are referred to as PRRs (Pattern Recognition Receptors). Interaction of the PAMPs with the PRRs triggers signaling pathways that lead to activation of IRF3 (Interferon Regulatory Factor 3) and IRF7 transcription factors, eventually allowing transcription from the IFN-I promoters [233; 234].

IFN-I displays both autocrine and paracrine effects. Engagement of the IFN-I receptors (IFNAR-1 and IFNAR-2) at the cell surface induces activation of Jak1/Tyk2 and STAT1/STAT2 signaling cascade that subsequently promotes the expression of a large number of anti-viral effectors known as interferon-stimulated genes (ISGs) [235].

IFN-I can be produced by all cells upon sensing virus infection, and in particular by innate immune cells such as macrophages and dendritic cells (DCs). These cells belong to the family of professional antigen-presenting cells. They have the capacity to capture antigens, process them and display them to lymphocytes [220]. Antigen presentation is therefore a central process in the immune response, since it allows priming of the adaptive response, thus bridging the innate and adaptive responses. For this reason, innate immunity is also essential for the activation of adaptive immunity.

Plasmacytoid dendritic cells (pDCs) are the major IFN-I producers and they are found in an immature stage in the peripheral blood, and more

predominantly in inflamed tissues [236]. Myeloid dendritic cells (myDCs) circulate in the blood and constantly migrate into secondary lymphoid organs [220]. Many *in vitro* studies of DCs use myeloid-like DCs generated from $CD14^+$ monocytes (*Mo-mDCs*), which unluckily are not identical to *in vivo* or *ex vivo*-purified DCs. Therefore, those results should sometimes be handled with caution [220].

In addition, other effectors of innate immunity such as restriction factors are present in cells before infection takes place and can act as immediate inhibitors of a given infectious agent [237]. When these proteins are not able to block viral spread, another efficient defense strategy consists of killing the infected cells. Members of both the innate system (natural killer cells) and the adaptive system (cytotoxic T lymphocytes) have the ability to kill infected cells. Invariant NKT (iNKT) cells are a small population of T cells that express cell surface antigens associated with the NK lineage. These cells display attributes of both innate and adaptive immunity, and modulate the immune response by secreting large amounts of cytokines.

1.7.1 HTLV-1 infects cells that play a major role in the innate immune response

As mentioned before, *in vivo* the HTLV-1 provirus is predominantly detected in $CD4^+$ T lymphocytes [238], even if infection of $CD8^+$ T lymphocytes [239; 240], and of B lymphocytes has also been documented. It thus appears that the main *in vivo* cellular targets of HTLV-1 are cells from the adaptive immune system.

However, innate immune cells (monocytes, macrophages, DCs) are permissive to the virus *in vitro* and/or are infected *in vivo* [223; 241; 242] (Table 3). *In vivo* infection of DCs was demonstrated almost 20 years ago in HTLV-1-infected individuals [243]. Although myDCs and pDCs represent less than 1% of the cells in the peripheral blood, thus not significantly contributing to the total proviral load (PVL), their infection could greatly affect immune system function. The determinants of HTLV-1 proviral load

(the number of integrated copies of HTLV-1 expressed as a proportion of PBMCs) remains approximately stable in one individual over years, but may vary 1000- fold between individuals. A high proviral load is one of the best predictors of HAM/ TSP and ATL, although many patients with a high load will remain lifelong asymptomatic carriers. However, there is little longitudinal data to confirm that a high proviral load is the cause of disease rather than the consequence. Nevertheless, given the association between proviral load and disease a number of studies have attempted to elucidate the determinants of proviral load.

1.7.1.1. Dendritic cells

Until recently, the paradigm for HTLV-1 infection was a model in which viral transmission required cell-to-cell contact. Unlike most retroviruses, cell-free virions of HTLV-1 and other deltaretroviruses are very poorly infectious *in vitro* [244]. Cell-free HTLV-1 does not stably infect its primary target cells, $CD4^+$ T cells, *in vitro*, although transient infection has been reported [245]. In 2008, Jones *et al.* demonstrated that both myDCs and pDCs could be productively infected *in vitro* by cell-free HTLV virions [7; 229]. Infected DCs rapidly and reproducibly transfer HTLV-1 to autologous primary $CD4^+$ T cells, resulting in chronic productive infection of $CD4^+$ T cells and generation of interleukin-2 (IL-2)-independent infected and immortalized transformed cells. Paralleling recent observations showed that HIV-1, although highly infectious as a cell-free particle, is more efficiently transmitted by DC–T cell interactions [246]. This led to the establishment of a model in which DCs that are present at the site of infection could be the primary target cells in a newly infected individual, allowing subsequent cell-to-cell transmission of the virus to T cells (Figure 13). Whether these DCs are infected through cell-to-cell contact or by cell-free virus *in vivo* remains to be investigated. Factors that contribute to HTLV-1 dissemination, persistence and pathogenesis remain poorly understood.

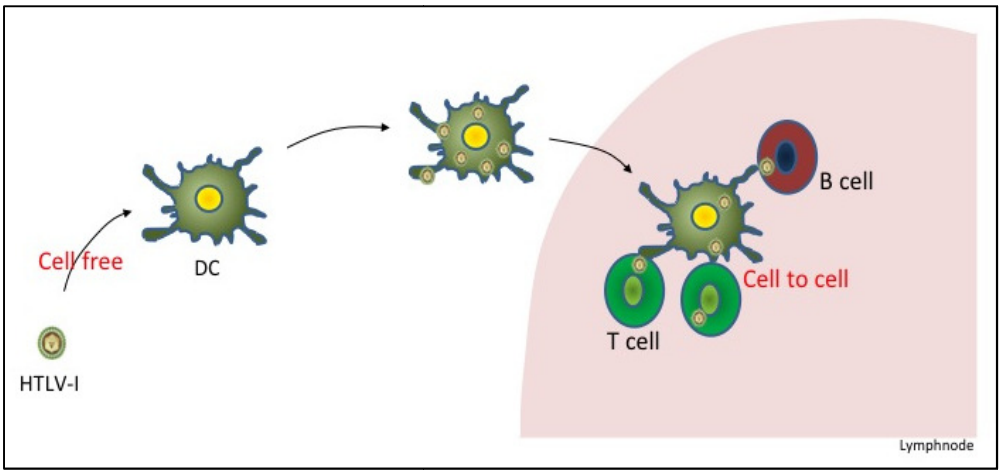


Figure 13. Recently, the model of infection of HTLV-1 has been re-thought based on the observation of Jones *et al.*, who showed that DCs could be efficiently infected by cell free virus *in vitro*, contrarily to how observed in the past, when the accepted model was based exclusively on cell-to-cell infection. Therefore, a new important role is prospected for DCs, which now could be the primary target of cell-free HTLV-1 during new infection, then subsequently transmit the virus to main target T-cell based on cell-to-cell manner.

Many viruses infect DCs to facilitate their transmission, including the retroviruses HIV-1 and mouse mammary tumor virus [247; 248]. Some viruses do this directly, hijacking the trafficking properties of DCs to facilitate their transport from the periphery to lymphnodes, where they infect target cells. Viral infection of DCs can also indirectly facilitate spread by impairing the ability of DCs to mount an appropriate immune response [232].

These *in vitro* observations are supported by earlier *in vivo* studies [249; 250], indicating that they are directly relevant to HTLV-1 transmission *in vivo*. Studies of DCs isolated from HTLV-1–infected individuals, along with observations of efficient *ex vivo* infection of pDCs and myDCs, suggest that cell-free HTLV-1 can also infect DCs *in vivo.* For example, Jones et al

observed also that HTLV-1 productively infects DCs and pDCs from an individual with HAM-TSP express HTLV-1 proteins, these findings confirm and extend earlier reports that *DCs* generated from monocytes from some individuals with HAM-TSP express viral proteins [233] and that DCs in infected individuals contain viral sequences [234]. High HTLV-1 proviral loads (4–37 copies per 100 cells) have been observed in pDCs isolated from asymptomatic individuals.

For many viruses, infection of DCs affects the host's ability to mount an appropriate immune response by interfering with DC development, maturation, function and/or viability [232]. A number of aspects of HTLV-1–induced immunosuppression are consistent with alterations in DC function. Adaptive immunity is impaired in asymptomatic HTLV-1–infected individuals. Both *Mo-mDCs* generated from monocytes of HTLV-1–infected individuals and *Mo-mDCs* infected *in vitro* are poor stimulators of autologous T cell differentiation. HTLV-1 infection of DCs also interferes with innate immunity: pDCs isolated from HTLV-1– infected individuals are impaired in their ability to produce type I interferon [251], an important antiviral mechanism of the innate immune system.

HTLV-1 infection of DCs may have an important role in the development of ATL and HAM-TSP. Individuals with ATL, like asymptomatic carriers, have impaired immune systems and development of ATL is associated with an increase in the number of HTLV-1– infected cells not recognized by $CD8^+$ CTLs. In individuals with HAM/TSP, high viral loads are associated with decreased degeneracy of T cell recognition [252]. These observations suggest that, in both of these diseases, infection of DCs modifies the presentation of HTLV-1 antigens and thus generates a repertoire of $CD8^+$ T cells that do not efficiently recognize the virally infected cells. Development of ATL is also associated with the loss of pDCs and myDCs [235], consistent with observations from other viruses that infection of DCs can induce cytopathic effects.

It was demonstrated also that infection of DCs is required to establish and maintain HTLV-1 infection in macaques model [82].

1.7.1.2. Monocytes and macrophages

Monocytes and macrophages might also represent a putative virus reservoir *in vivo*. Koyanagi *et al.* showed that the PVL ranged between 0 and 140 copies per 104 monocytes in a group of 22 HTLV-1- infected individuals [253]. Several studies hypothesized a latent infection of monocytes, which would allow them to escape immune recognition [254]. Viral reactivation could then be induced upon monocyte-to-macrophage differentiation. Infection of monocytes and monocyte-derived cells could be of particular interest in the context of mother-to-child transmission. Indeed, breast milk predominantly contains macrophages, rather than T cells. This could explain how prolonged breastfeeding leads to viral transmission. A recent article showed that an *in vitro*-infected breast milk macrophage cell line could transmit the virus to peripheral blood lymphocytes [255], supporting the hypothesis that these cells may be a viral reservoir.

1.7.1.3. NK and iNKT cells

Finally, HTLV-1 infection of activated NK cells was documented *in vitro* [256], but has not been demonstrated *in vivo*. Infection of iNKT cells was however recently demonstrated *in vivo*, in ACs and HAM/TSP patients [240]. The physiopathological consequences of putative NK infection and of iNKT infection have not been understood yet.

	Infection by HTLV-1	**Control of HTLV-1 Replication**	**Infection-Induced Alteration of Cell Numbers in vivo**	**Infection-Induced Alteration of Function**
Monocytes / Macrophages	Demonstrated *in vitro* and *in vivo*	ND	ND	Alteration of *in vitro* differentiation into functional

				DCs
Myeloid Dendritic Cells	Demonstrated *in vitro* and *in vivo*	Secretion of IFN-α upon *in vitro* HTLV-1 sensing	Decreased (ATLL and HAM/TSP patients)	ND
Plasmacytoid dendritic cells	Demonstrated *in vitro* and *in vivo*	- Secretion of IFN-α and maturation into killer pDCs - Inverse correlation between the PVL and the ability of *ex vivo*-stimulated cells to produce IFN-α	Decreased (ATLL and HAM/TSP patients)	Alteration of the ability to secrete IFN-α upon *ex vivo* stimulation
Natural killer cells	Demonstrated only *in vitro*, on activated cells	Inverse correlation between infected cell lines sensitivity to NK-mediated cell lysis and tumorigenicity	Decreased (ATLL and HAM/TSP patients)	High rate of *ex vivo* proliferation (ACs and HAM/TSP patients)
Invariant Natural Killer T Cells	Demonstrated *in vivo*	Inverse correlation between the PVL and iNKT cells frequency (ATLL and HAM/TSP patients)	Decreased ATLL and HAM/TSP patients)	Low rate of *ex vivo* proliferation and perforin production in infected individuals

Table 3. Interplay between innate immune cells and HTLV-1. ND, not determined.

1.8 Control of HTLV-1 replication by innate immune cells

A recent *in vitro* study suggested that normal human pDCs are activated and able to secrete IFN-α by a TLR-7-dependent mechanism when put in contact with concentrated purified HTLV-1 virions [257] (Figure 14). It is therefore possible that HTLV-1 activates pDCs and triggers their differentiation into killer pDCs [241]. These results suggested that infected human pDCs have an intact IFN-I induction pathway.

Hishizawa *et al*., however, demonstrated that pDCs isolated from a small number of HTLV-1 ACs had impaired IFN-α production, consistent with previous observations reporting impaired immune function in some ACs [258; 259].

Finally, Hishizawa *et al.* also found a negative correlation (Table 3) between the PVL in ACs and the ability of pDCs isolated from these individuals to secrete IFN-α *ex vivo* [235]. Although further investigation is needed to demonstrate the causative relationship between both parameters, these results may indicate that efficient IFN-I production could limit viral replication and hence protect against a high PVL. Alternatively, another interpretation of this study is that when PVL increases, the number of infected pDCs also increases and therefore their ability to secrete IFN-I is lowered.

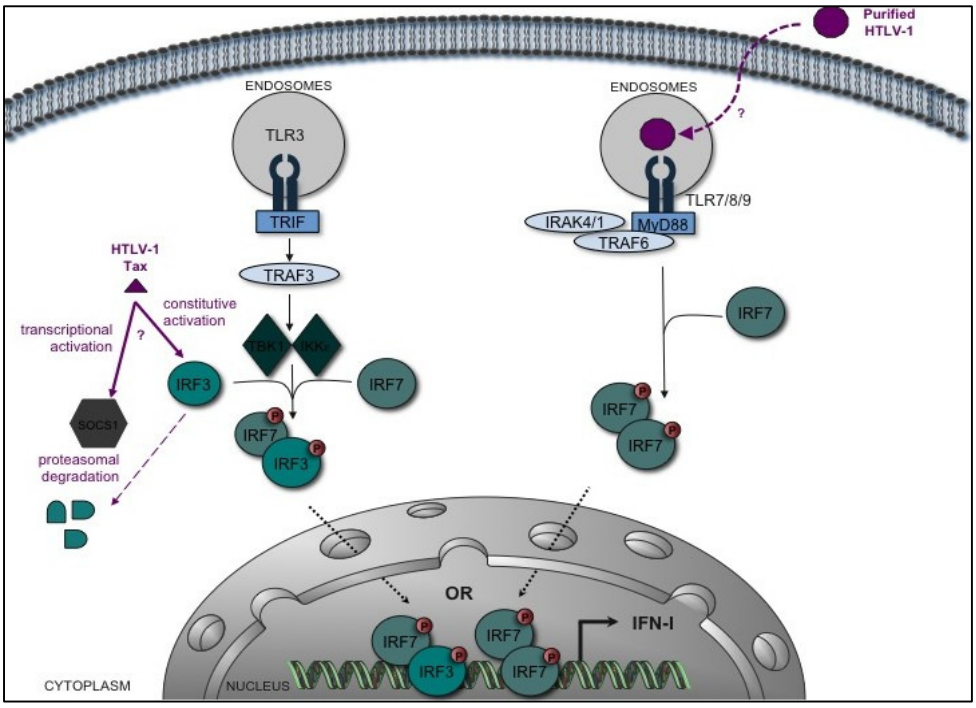


Figure 14. Interplay between HTLV-1 and the type-I interferon (IFN-I) induction pathway. Here we show simplified schematic of the TLR-induced IFN-I induction pathway. Recognition of PAMPs by TLR3 induces the recruitment of the adaptor molecule TRIF. TRIF activates via TRAF3 the kinases TBK1 and IKKε that phosphorylate IFR3 and IRF7 transcription factors. IRF3/7 heterodimers activate transcription of IFN-I (especially IFN-β). This initial transcription and synthesis wave is followed by a second wave of IFN-α resulting from an amplification loop that involves IRF7. TLR7, 8 and 9, specifically expressed in pDCs, activate IRF7, which stimulates synthesis of IFN-α via MyD88, IRAK1/4 and TRAF6. (Sastre *et al.* 2006)

1.9 Alteration of Innate Immune Cell Phenotype, Function and Abundance in the Context of HTLV-1 Infection

Several studies were designed to monitor a possible functional alteration of innate immune cells, in the context of HTLV-1 infection. In a physiological situation, these cells are fully efficient if they are able to arise from

precursors in an adequate environment, become activated upon stimulation and differentiate into effectors cells.

Some studies had shown that HTLV-1-infected macrophages and DCs could favor T cell proliferation [260]. This suggested that infection enhanced the antigen presentation and T cell-stimulation functions of DCs, potentially participating in the spontaneous T cell proliferation that has been observed in patients. However, this model was not supported in recent results, which demonstrated that monocytes isolated either from HTLV-1-infected ACs, or individuals with ATLL or HAM/TSP, are deficient in their ability to differentiate into functional DCs [261]. Physiologically, activated DCs express high levels of adhesion molecules, MHC antigens and co-stimulatory molecules, which all cooperate to potentiate the antigen-presenting function of these cells, and their ability to induce activation of T lymphocytes. In addition, DCs derived from *in vitro* cultured infected monocytes express abnormal levels of surface activation markers, and are poorly able to activate autologous T lymphocytes.

The impact of HTLV-1 infection on the number of DCs was assessed *in vivo* [235]. ATLL and HAM/TSP patients had a lower absolute number of myDCs and pDCs than uninfected individuals, while this was not the case for ACs. The correlation between DC number and clinical status suggests that the depletion of DCs could play a role in HTLV-1 physiopathology. It should however be emphasized that measurements of cell numbers were performed on peripheral blood samples. The observed depletion of DCs in the blood could be the consequence of a massive migration of DCs into tissues.

1.10 Alteration of molecular pathways by HTLV-1

1.10.1. Modulation of IFN-I Production

IRF3 and IRF7 play essential roles in the early phase of IFN-I gene activation. IRF3 is constitutively expressed and activated through

phosphorylation by IKK and TBK1. This promotes the transactivation of downstream genes such as IFN-β. In contrast, IRF7 protein is synthesized *de novo* upon IFN stimulation and contributes to amplification of the IFN response, by inducing expression of IFN-α [262]. A very recent study of *ex vivo* $CD4^+$ cells isolated from 30 HTLV-1-infected individuals (ACs, HAM/TSP, ATLL) demonstrated that SOCS1 (Suppressor Of Cytokine Signaling 1) is strongly up-regulated in ACs and patients with HAM/TSP but not in those with ATLL. This protein was previously shown to suppress IFN signaling by preventing STAT1 phosphorylation [263]. Interestingly, SOCS1 expression correlated with HTLV-1 PVL in $CD4^+$ cells of HAM/TSP patients. Tax was recently shown to promote the expression of SOCS1 *in vitro*. It was shown also that HTLV-1-infected cells that express viral mRNAs (and in particular tax) are likely to be impaired for early IFN induction signaling.

The viral p30 protein was also shown to modulate innate cell activation [264]. Expression of p30 in human macrophages (i.e., the THP-1 cell line) alters TLR4 signaling, a critical pathway in the innate response to bacterial infection, and inhibits the production of *pro-inflammatory* cytokines normally secreted in response to TLR4 stimulation [248]. This altered TLR4 signaling is due to down-regulation of TLR4 expression and is mediated by an interaction-dependent inhibition of the transcriptional factor PU.1. p30-mediated inhibition of *pro-inflammatory* signaling is accompanied by a stimulation of anti-inflammatory cytokine secretion such as IL-10, suggesting that p30 might interfere with the balance of pro- and anti-inflammatory responses during bacterial infection.

1.10.2. Modulation of IFN-I signaling

Several reports showed that HTLV-1-infected cells still express IFNAR receptors [265]. However interestingly, HTLV-1 expression led to decreased Tyk2 and STAT2 phosphorylation, two major players in the IFN-I signaling pathways. This effect might be mediated through Gag or Protease expression. Downstream of Tyk2 and STAT2 phosphorylation, the active

ISGF3 complex (Interferon Stimulated Gene Factor 3), containing phosphorylated STAT1, STAT2, IRF9, and the CBP/p300 transcription co-activators, triggers the expression of a number of genes. In addition to its effect on SOCS1, Tax overexpression also alters ISGF3 function by preventing interaction of the STAT2 component of ISGF3 with CBP/p300, therefore leading to modulation of the IFN-α transduction cascade [266].

Aim

2. AIM OF THE STUDY

In spite of over 30 years of study, several key features of the HTLV-1 infectivity, viral persistence and pathogenesis remain obscure.

It is well known that HTLV-1 primarily infects $CD4^+$ T-cells and has been detected in *ex vivo* $CD8^+$ T- cells, B-cells, monocytes and dendritic cells (DC) from infected individuals. DC and monocytes may contribute to viral persistence and pathogenesis. Several studies reported alteration in monocytes and DCs differentiation, activation and functions in HTLV-1 infected patients. For example, Journo et al [268] showed that DCs isolated from HTLV-1-positive patient are associated with a defect of IFN-α upon stimulation and their ability to activate T-cells is impaired; these cells express also abnormal level of activation marker on the cell surface.

p30 seems to play a key role during HTLV-1 infection of DCs. It was demonstrated that in the macaque model, the ablation of p30 expression severely affects infectivity. Furthermore, the infectivity of p30-KO in human primary monocyte-derived dendritic cells (Mo-mDCs) is severely impaired and is not persistent.

Prior data demonstrated also that p30, by interacting with the cellular transcription factor PU.1 in human monocytes, affects TLR4 signaling and the expression of several genes involved in apoptosis, cell cycle, and transcription, pointing to p30 as a regulator of innate response to HTLV-1 [264].

The work described in the present thesis was aimed at understanding the role of HTLV-1 p30 during infection of human monocytes and dendritic cells. In particular we wanted:

- ✓ to understand the mechanism by which p30 affects viral infectivity and persistence;
- ✓ to study the effect of p30 on monocytes and dendritic cells (DC).

Indeed we hypothesized that p30 may affect not only TLR4 but also TLR3, TLR7 and 8 signaling in virus infected monocytes and in dendritic cells, and that by affecting the type 1 IFN response p30 affects the activation and differentiation of these cells and ultimately the host response to the virus allowing viral replication and spread throughout the body.

Thus, understanding how HTLV-1 evades the innate host response and affects immune activation/inflammation is of importance to gain more understanding on its oncogenicity and ability to induce autoimmune manifestations.

Material and Methods

3. MATERIAL AND METHODS

3.1 Cell lines

The 729-6 B-cell lines infected with the pACH HTLV-1 wildtype (WT) virus and p30-KO viral mutants were maintained in RPMI 1640, 10% fetal bovine serum (FBS) and used as a source of virus to infect other cell types. In the case of leukemic monocyte-like THP-1 human cell line, the same media was supplemented with 50 μM β-mercapto-ethanol. To stimulate THP-1 cells we used 50 ng/ml phorbol myristyl acetate (PMA) for 3 hours (Sigma, St. Louis, MO, USA). We used TLR agonists to study ISGs expression in HTLV-1 infected THP-1 cells such as 20 ng/ml lipopolysaccharide (LPS) (List Biological Laboratories Inc., Campbell, CA, USA), 10 μg/ml poly(I:C) HMW (InvivoGen, San Diego, CA, USA) or Imiquimod (InvivoGen, San Diego, CA, USA).

3.2 Primary human cells

3.2.1 Separation of monocytes by adhesion

Primary human monocytes were obtained from heparinized human peripheral blood from healthy donors. Whole blood was stratified by gradient using Ficoll-Paque Plus (GE Healthcare, Chalfont St. Giles, UK) and centrifuged at 2300 rpm for 25 min. Cells were collected and washed with PBS (1900 rpm for 10 min). The number of viable leukocytes was determined by trypan blue exclusion. Human monocytes were separated by adhesion after 6 hours and resuspended in RPMI 1640, 10% FBS. We used TLR agonists to study ISGs expression in HTLV-1 infected primary monocytes such as 20 ng/ml lipopolysaccharide (LPS) (List Biological Laboratories Inc., Campbell, CA, USA), 10 μg/ml poly(I:C) HMW (InvivoGen, San Diego, CA, USA) or Imiquimod (InvivoGen, San Diego, CA, USA).

3.2.2 Separation of monocytes by elutriation

To obtain primary monocyte-derived dendritic cells (Mo-mDC), monocytes were alternatively separated from PBMCs by elutriation to keep them inactivated and left “untouched”. Elutriation is a process that allow to separate particles based on their size, shape and density, using a stream of gas or liquid flowing in a direction usually opposite to the direction of sedimentation (Figure 1).

The centrifuge was run at 20 °C and constant speed (1000 rpm). The pump was turned on and the system was washed first with 200 ml of 70% ethanol, then with 200 ml of PBS to remove traces of alcohol. PBS was then replaced by freshly made elutriation fluid at an initial flow rate used for the introduction of cells into the chamber. The further run serves to remove bubbles from the system. After instrument preparation, PBMCs were placed into a specially designed centrifuge rotor/chamber and subjected to centrifugal field. The cells were then sequentially washed out of the rotor based on their size, using a buffer stream that flows in the direction opposite the centrifugal field at permanent flow rate of 7ml/min. By balancing centrifugal force against the opposing buffer flow, we could collect lymphocytes in the first 4-5 tubes, which are about 6-8 µm. When effluent is clear, the dial of pump was increased by 0.05 unit increments for each of the next 3 fractions. Around tube 8 you are collecting monocytes, which are 8-10 µm. Then cells were checked for the purity (>98% $CD14^+$).

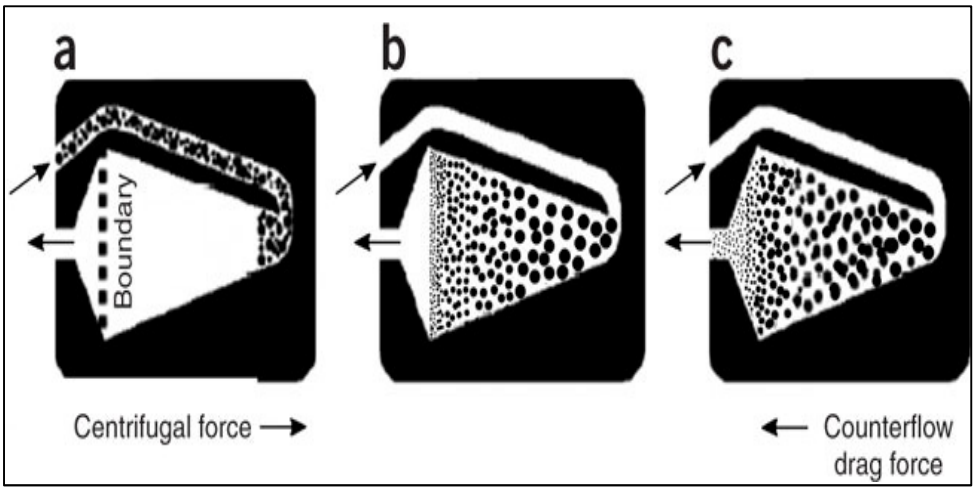


Figure 1. Schematic representation of elutriation process.

3.2.1.1. Differentiation of Mo-mDC

Elutriated monocytes were then differentiated in approximately seven days of culture in RPMI 1640, 20% BIT (Stem Cell Technologies, Vancouver, Canada) with 50 ng/ml IL-4 (Peprotech, Rock Hill, NJ, USA), 50 ng/ml GM-CSF (Peprotech, Rock Hill, NJ, USA) and 10 ng/ml Transforming growth factor- β (TGF-β) (R&D systems, Minneapolis, MN, USA). Mo-mDC purity was checked by phenotype using $CD14^-$, $CD3^-$, $CD19^-$, $CD1a^+$, and $CD11c^+$ Ab by flow cytometry. We used TLR agonists to study ISGs expression in HTLV-1 infected Mo-mDC such as 20 ng/ml lipopolysaccharide (LPS) (List Biological Laboratories Inc., Campbell, CA, USA), 10 µg/ml poly(I:C) HMW (InvivoGen, San Diego, CA, USA) or Imiquimod (InvivoGen, San Diego, CA, USA).

3.3 HTLV-1 mutagenesis in 729-6 cell line

Mutations to ablate p12 and p30 were introduced in the *Cla*I/*Sal*I cassette from the HTLV-1 molecular clone pBST that include the *orf-I* and the *orf-II*. The molecular clone pACH was cleaved at the *Cla*I/*Sal*I to generate the backbone for the construction of all viral mutants. The pBST *Cla*I/*Sal*I cassette was ligated to the pACH backbone to obtain the wild-type HTLV-1 clone. Mutations to ablate p30 were introduced into the pBST cassette. To

generate the p30 knockout (KO) mutant, we replaced the leucine in position 3 (CTA) of p30 with a termination codon (TAA). The mutant was generated with the use of complementary mutant site-specific primers, the Phusion enzyme, and mastermix (Finnzymes; New England Biolabs), where the reaction in the thermocycler was followed by *Dpn*I digestion before transformation of One Shot Max Efficiency DH5α (Invitrogen). The following DNA primers were used to generate the mutant clone:

- ✓ p30ko forward, 5'-CCTGCATTTTTTCTTTCCTAGCA*TAA*TGGTGT-TTCGCCTTAAAAGCCCCT-3'; p30ko reverse, 5'-AGGGGCTTTTAAG-GCGAAACACC*ATT*ATGCTAGGAAAGAAAAAATGCAG-3';
- ✓ WT-forward 5'-TGCTTTCTCCGGGCGACGTCAGCAGCCTTCTTCTC-3'; and WT-reverse, 5'-GCGGAGAAGAAGGC*T*GCTGACGTCGCC-3'.

The mutant clones were inserted into the pH6neo vector, and all the resulting clones were verified by DNA sequencing of the *Cla*I/*Sal*I fragment inserted in the provirus. 729-6 human lymphoblastoid B cells (5 x 10^6) were electroporated with 5 ug of WTneo or 30KOneo with the use of AMAXA (Amaxa Biosystems) according to the manufacturer's guidelines. Infected cells were selected by culture in neomycin. Stable 729 B-cell lines expressing each mutant, and the cell lines expressed equivalent levels of p19 Gag in the supernatant were thus generated and subsequently use as a source of virus to infect THP-1 cells.

3.4 Virus infection

HTLV-1-WT or –p30-KO producer 729-6 B-cell lines were used to harvest HTLV-1 virions. The supernatants of such cell lines were collected and ultra-centrifuged at 23000 rpm for two hours and thirty minutes to isolate the virions, which then were resuspended in PBS. In order to enhance the infectivity, as THP-1 cells are hard to infect, we spin-infected THP-1 cells at

3000 rpm for one hour in presence of 8 µg/ml polybrene (Sigma, St. Louis, MO, USA). Infected cells were then resuspended in RPMI 1640, 10% FBS supplemented with 50 µM β-mercapto-ethanol and kept in culture for several weeks.

3.5 Lentiviruses Transduction of THP-1 cells

In order to efficiently express p30 or p12/p8 proteins, THP-1 cells were transduced with lentiviral vectors based on Naldini's system. This method consists in the use of 3 different plasmid: two packaging plasmid that are pSAX (gag/pol) and pDM2.G (env) and one lenti construct (pSDM101 expressing GFP) with a capside-like sequence and a polylinker sequence were viral genes (p30 or p12/p8) were inserted. The lenti construct with p30 and p12/p8 plasmid expresses the fusion protein of HTLV-1 p30 or p12/p8 tagged with the influenza hemagglutinin (HA1) tag. In order to produce lentiviruses, pSDM101 (expressing GFP only) or pSDM101-p30 (co-expressing GFP and HTLV-1 p30) or pSDM101-p12/p8 (co-expressing GFP and HTLV-1 p12/p8), pDM2.G and pSAX were transduced 293T-cells with LipoD293 (Signagen, Rockville, MD, USA) according to manufacturer's instruction. The supernatant was harvested, 0.22µm filtered and used during spin-infection, as described above. Efficiency of transduction was checked by flow-cytometry and ranged between 80% and 95%.

3.6 Detection of virus productions by ELISA

The production of HTLV-1 in the supernatant of the infected cell cultures was assessed by measuring the amount of MA (p19 Gag) protein by ELISA (Zeptometrix, Buffalo, NY, USA). Supernatants were initially treated with Lysing Buffer (1:10). HTLV antigen standards were prepared diluting 1:2 the given solution obtaining concentrations from 800pg/ml to 0pg/ml. Standards and samples were distributed in duplicate and incubated for 2 hours at 37 °C. Then the microplate was washed 6 times with Wash Buffer 1X and then HTLV Detector Antibody was added to each well and

incubated for 1 hour at 37 ℃. After washing the plate, Peroxidase was added to the plate and incubated for 1 hour at 37 ℃. Then the plate was washed and Substrate Solution was added into each well and incubated uncovered for 30 min at room temperature (RT). A blue color developed in wells containing viral antigen. The reaction was stopped adding Stop Solution resulting in a color change from blue to yellow. The optical density of each well at 450nm was read using a microplate reader (Victor Wallac Perkin Elmer) within 15 min.

3.7 Detection of HTLV-1 proviral load

HTLV-1 proviral load was measured in the various cell lines. Real-time PCR was performed on genomic DNA extracted from different cell lines with the DNeasy tissue kit, according to the manufacturer's protocol. The QIAGEN method was used for the DNA elution stepin with 10mM Tris (tris(hydroxymethyl)aminomethane; pH8.0). The quantity and quality of the DNA were assessed by Nanodrop. Five hundred nanograms of genomic DNA was subjected to real-time PCR. The TaqMan probe and PCR primers for the real-time PCR were designed within the integrase gene of HTLV-1 x 1MT. The sequence of the TaqMan probe was: 5'-TGT CCACCTGCCATTAAGCCCGA-3', the DNA primers sequences used had the following sequence:

- ✓ forward 5'-GCAGAGGAGGAAATTACCCAGTAC-3'; reverse 5'-CAATTTTACCCAGGCATTTAATGT-3'.

Reaction conditions were as follows: the 25 µL of PCR mixture for HTLV-1 and cells albumin DNA consisted of 500 ng of DNA extracted from PBMCs; 200nM primers; 100nM probe; 2X TaqMan Universal PCR Mastermix (Applied Biosystems) which consists of 10mM Tris-HCl (pH 8.3); 50mM KCl; 5mM $MgCl_2$; 300µM each of deoxyadenosine triphosphate, deoxycytidine triphosphate, and deoxyguanosine triphosphate; 600µM deoxyuridine triphosphate; 0.625 U of AmpliTaq Gold DNA polymerase;

and 0.25 U of uracil N-glycosylase. Used for HTLV-1 and cells albumin DNA amplification, 1 cycle at 50°C for 2 minutes and 1 cycle at 95°C for 10 minutes were followed by a 2-step PCR procedure consisting of 15 seconds at 95°C and 1 minute at 60°C for 50 cycles. The amplification was performed with the ABI Prism 7500 Sequence Detector system (Applied Biosystems). The normalized value of the HTLV-1 proviral DNA load was calculated as HTLV-1 DNA copy number/albumin gene copy number and expressed as the number of HTLV-1 proviral DNA copies per 10^6 cells.

3.8 Flow cytometry

In order to determine the surface expansion of specific markers on the THP-1 cell lines, primary monocytes or Mo-mDC, phenotype was assessed by flow cytometry. Cells were incubated with the proper antibodies for fifteen minutes at room temperature, then washed in PBS and fixed in 1% paraformaldehyde, run on LSR II (BD Bioscience, San Jose, CA, USA) and analyzed with FlowJo (Tree Star Inc., Ashland, OR, USA). In the case of intracellular cytokine staining, 1µg/ml of the protein transport inhibitor Brefeldin A (BD, San Jose, CA, USA) was added to the culture 16 hours before the analysis. Then cells were stained for surface markers as described above and then permeabilized with Saponin 1% and stained with the proper intracellular cytokine antibody for 45 min at 2-8 °C. Then cells were washed in PBS and fixed in 1% paraformaldehyde, run on LSR II and analyzed with FlowJo. The antibodies used were CD14 (Biolegend, San Diego, CA, USA), CD80, CD83, CD86, TNF-α, CCR7 (BD Bioscience, San Jose, CA, USA), IL12 (eBioscience, San Diego, CA, USA), TLR4.

3.9 Quantification by real-time PCR of ISGs expression

For real-time PCR, total RNA was extracted from cells and retro-transcribed (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Reactions were performed using a SYBR Fast qPCR mix (KapaBiosystems, Woburn, MA, USA). The primer sequences were (5'-3'):

- 18s: forward 5'-GCCCGAAGCGTTTACTTTGA-3', reverse 5'-TCCATTATTCCTAGCTGCGGTATC-3';
- MxA: forward 5'-AGGAGTTGCCCTTCCCAGA-3', reverse 5'-TCGTTCACAAGTTTCTTCAGTTTCA-3';
- APOBEC3G: forward 5'-CCGTCTGGGTGTGCTACGAA-3', reverse 5'-GCTTCCTCCACTTGCTGAACCA-3';
- OAS: forward 5'-CAGTCCTGGTGAGTTTGCAGT-3', reverse 5'-GCCAGTGCTTTATCAAGAGGAT-3'.

Used for 18s and ISGs cDNA amplification, 1 cycle at 95°C for 10 minutes was followed by a 3-step PCR procedure consisting of 10 seconds at 95°C, 15 seconds at 59°C and 20 seconds at 72°C for 40 cycles. The amplification was performed with Rotor Gene Q (Quiagen).

Results were expressed as ΔΔCt and presented as ratios between the target gene and the 18s housekeeping mRNA.

3.10 Immunoblot analysis

To evaluate the expression of viral proteins or tubulin as a control, THP-1 or Mo-mDC cells were lysated with Lysis buffer. Proteins concentration was measured with Bradford method and 20 µg of total proteins for each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–12%) in MES SDS running buffer (Invitrogen, Carlsbad, CA, USA). The ratio charge/mass is constant for the presence of the SDS; the separation takes place on the basis of the effects of molecular size due to the size of the meshes of the gel. The smaller proteins pass through more easily, while frictional forces delay those of larger size. The run was performed for 1 hour at 150V. When the gel finished to run, proteins were transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA) for 1 hour at 230mA in buffer containing 25 mM Tris, 192 mM glycin and 15% (v/v) methanol. The membranes were blocked with PBS, 0.1% Tween and 4% nonfat dried milk, for 30 min at room temperature and then they were

probed with primary antibody directed to the HA-tag or the tubulin overnight at 2-8°C and the proper horseradish peroxidase (HRP)-conjugated secondary antibodies was used for detection for 1 hour at room temperature. As HRP substrare SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) was used.

3.11 ChIP assay

ChIPs were performed as follows. Briefly, for each chromatin preparation, $5x10^7$ cells were resuspended in 50 ml of medium without FBS and cross-linked in 1% formaldehyde for 10' at RT'. Cross-linking was quenched by 125mM glycine, 5' at RT. Cross-linked cells were then washed with ice-cold PBS and resuspended in ice cold RIPA buffer (10mM Tris-HCl pH8, 140 mM NaCl, 1mM EDTA pH 8, 0.5mM EGTA, 1% Triton X-100, 0.3% SDS and 0.1% Sodium Deoxycholate) to a final concentration of $5x10^6$ cell/ml. DNA was sheared with a Misonix XL sonicator, by performing 12 x 30 seconds sonication cycles at power setting of 5. For each single immunoprecipitation, 5×10^6 chromatin cell equivalents in the 200-500 bp range were immunoprecipitated with 5µg of the following antibodies: anti-HA, anti-RNA-Polymerase II (Pol II), anti-RNA-Polymerase II phosphoSerine5 (Pol II-S5) (Abcam), anti-RNA-Polymerase II phosphoSerine2 (Pol II-S2) (Covance) and anti-PU.1 (Cell Signalling. Parallel ChIPs were run with a Flag M2 antibody used as negative control (Sigma). Immunoprecipitation-enriched DNAs were used to perform real-time PCR.

Oligonucleotides were designed to specifically target and quantify the promoter region of:

- interferonα1 (IFNα1) (forward 5'-GGAACAAGATGGGGAAGACA-3', reverse 5'-GCAGATACTTCTGGGCTTGC-3'),
- IFNα2 (forward 5'-AAGGCTCTGGGGTAAAAGA-3', reverse 5'-GACCTTGCTTTGTGCCTAGC-3'),

- IFNβ (forward 5'-AGGACCATCTCATATAAATAGGCCATACCC-3', reverse 5'-ACTGAAAATTGCTGCTTCTTTGTAGGAATC-3'),
- TLR4 (forward 5'-GCCAACTAGCTTCCTCTTGCTG-3', reverse 5'-CACCGTCTGACCGAGCAGTT-3')
- Ubiquitin B (forward 5'-GAAGGAAGAGAAGCGCATAGAGGAGAA-3', reverse 5'-CTCATAGCCGTAAGAAAGGCTCCTAAA-3').

The ΔCt method was used to calculate ChIP Q-PCR enrichments.

3.12 Statistical analysis

Anova tests with Bonferroni post-tests were performed with the informatics support of Graphpad Prism 5.

Results and discussion

4. RESULTS AND DISCUSSION

4.1 THP-1 lentivirus transduction

Since the cells we want to work with (THP-1 monocytic cell line) are hard to transfect, we generated lentiviruses to transduce HTLV-1 regulatory proteins (p30 or p12/p8) in the target cells. Using a third generation Naldini's systems we produced in 293T lentivirus coexpressing both p30 and GFP (p30 LV), p12/p8 and GFP (p12/p8 LV) or GFP only (CTRL LV) to monitor the infection.

We transduced THP-1 cells with these lentiviruses: CTRL LV, p30 LV or p12/p8 LV. To control the efficiency of the transduction after 72 hours we observed the cells with fluorescence microscopy. As we can see in the figure below most of the cells are green compared to the imagine in bright field (Figure 1, left panel) proving a good efficiency of transduction. Then, using flow cytometry we measured the expression of GFP in THP-1 lentivirus transduced cells. As we can see in the histogram below these LV are able to infect THP-1 cells with an efficiency spanning from 70 to over 90% (Figure 1, right panel).

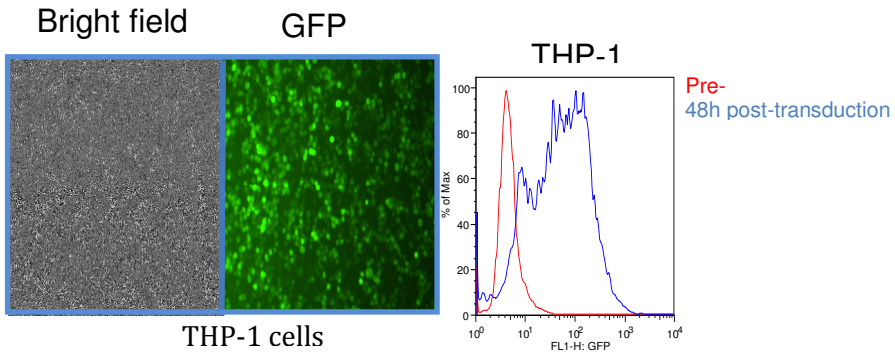


Figure 1. Flurescence microscopy of LV transduced THP-1 cells (left panel). Flow cytometric analysis of THP-1 cells expressing lentiviruses. GFP expression in the cells was assessed before (red line) and after 72

hours of transduction (blue line) (right panel).

4.1.1 Cellular localization of p30 in LV transduced THP-1 cells

After that we wanted to confirm the appropriate localization of p30 in THP-1 cells transduced with p30 expressing lentivirus. The p30 plasmid expresses the fusion protein of HTLV-1 p30 tagged with the influenza hemagglutinin (HA) tag that should simplify the detection of p30 but it could affect the appropriate localization of the protein. We used confocal microscopy to confirm the exact localization of p30. As we can see from the figure below, the p30 protein produced by p30LV transduced THP-1 cells, correctly localizes in the nucleus and mainly in the nucleoli (Figure 2), as reported in the literature [162].

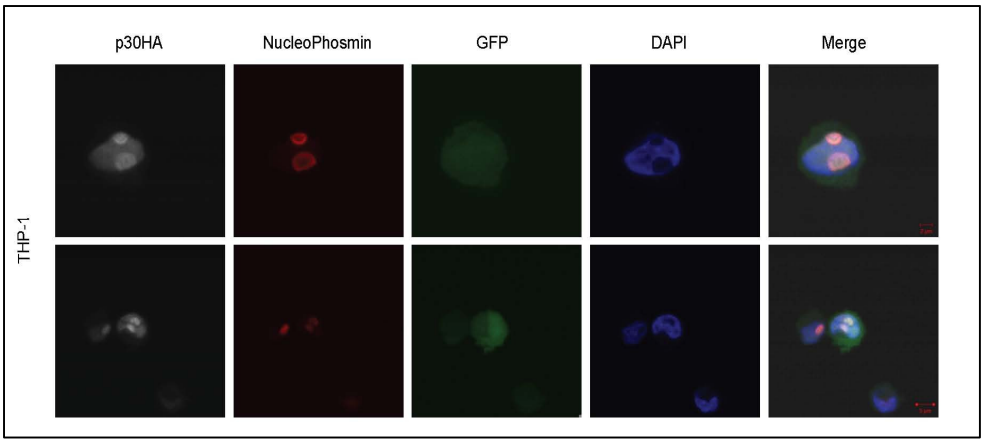


Figure 2. Confocal microscopy of p30LV transduced THP-1 cells. We performed different staining: with anti HA-antibody (p30HA), with a nucleolar marker (NucleoPhosmin), with a nuclear colorant (DAPI).

4.2 TLR4 expression in lentiviruses transduced THP-1 cells

It is known that HTLV-1-p30 protein interacts in the nucleus with PU.1 and is able to repress toll-like receptor 4 (TLR4) expression on the cell surface [264]. At first we verified if in our experimental conditions p30 affected TLR-4 expression. In order to do that, we transduced THP-1 cells with control lentiviruses (CTRL LV; gray line) or p30 lentivirus (p30 LV; red line).

TLR4 expression on the cell surface of transduced cells was analyzed by flow cytometry before and after 3 hour of stimulation with phorbol myristyl acetate (PMA). While untreated (-PMA) p30- or control-transduced THP-1 cells showed a similar level of TLR4 expression of the cell surface (left panel, Figure 3), treated cells (+PMA) showed an upregulation of TLR-4 expression in the control but not in the p30-transduced THP-1 cells (Fig. 3, middle panels). We then study on the same transduced and PMA treated cells the expression of CD14 surface marker, used as a control, by flow cytometry. As previously published [264], the CD14 surface marker expression, was not affected by p30 (Fig. 4, right panel) as the expression was the same in mock- transduced and p30 transduced THP-1 cells upon 3 hours of PMA stimulation.

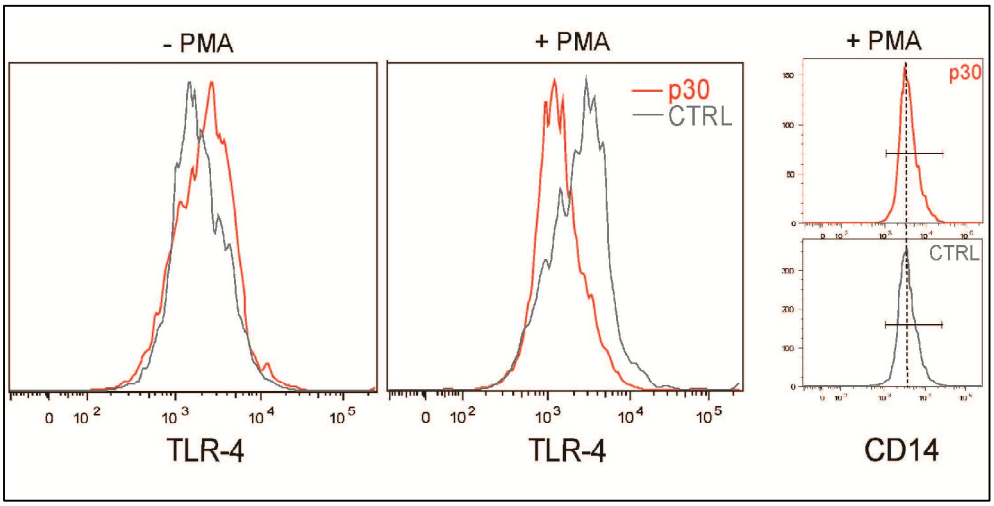


Figure 3. Flow cytometric analysis of THP-1 cells transduced with p30 (red line) or control (gray line) lentivirus. TLR4 expression on the cell surface was assessed following stimulation with PMA or not (left panel). The CD14 surface marker was used as a control (right panels).

To understand the mechanism that causes this downregulation of TLR4 expression, we performed a quantitative real-time PCR on TLR4 mRNA on

CTRL transduced and p30 transduced THP-1 cells. We confirmed that the mechanism underlying the effect of p30 was transcriptional (Figure 4). As we can see here, the expression of TLR4 mRNA was statistically significant decreased in presence of p30 protein upon PMA stimulation. In untreated cells (-PMA), we didn't observe any difference between THP-1 cells transduced with CTRL lentivirus or p30 lentivirus.

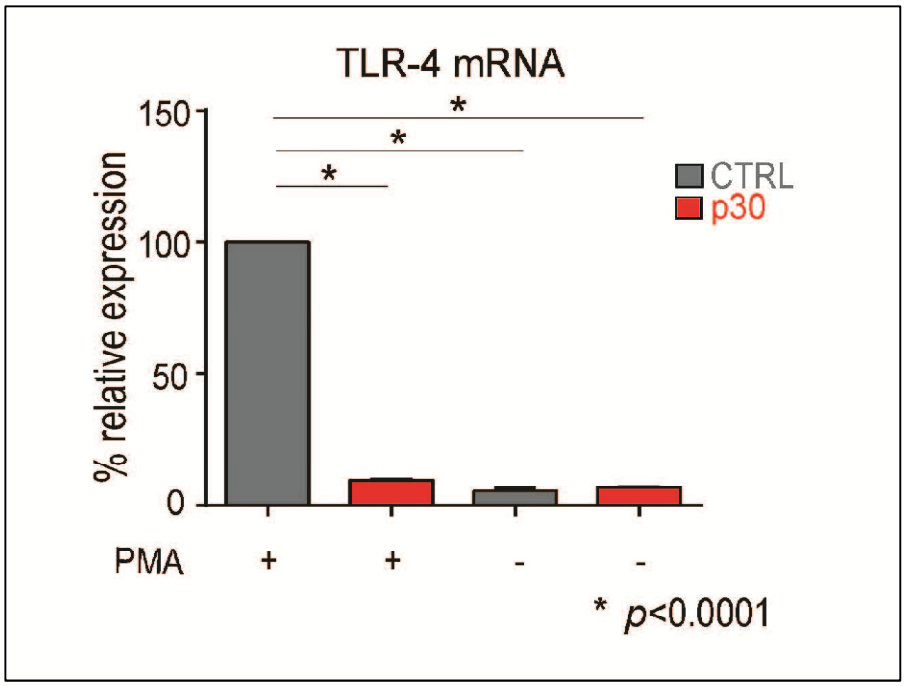


Figure 4: Real-time PCR was performed on the TLR4 mRNA in THP-1 cells transduced with p30- (in red) or control (in gray) lentivirus with or without PMA stimulation. The results are shown as a percentage of relative expression of the PMA stimulated mock-transduced cell. The statistically significant differences are marked with a star, which indicates a *p* value less than 0.0001.

4.3 Cytokines expression in lentiviruses transduced THP-1 cells upon LPS stimulation

Similarly, we transduced THP-1 cells with p30 lentivirus or with the control

lentivirus and after 18 hours of LPS stimulation, cells were collected and analyzed by flow cytometry for intracellular production of IL-12 and TNF-α (Figure 5). In both cases, the presence of p30 decreased the expression of such cytokines upon LPS stimulation when compared with the control.

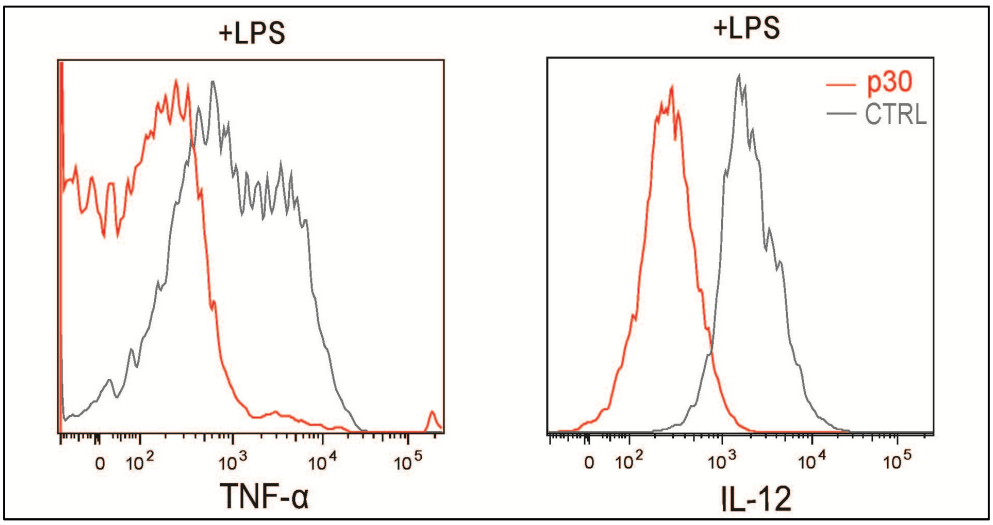


Figure 5: Flow cytometric analysis of THP-1 cells transduced with p30 (red line) or mock transduced (gray line). Twelve hours following LPS stimulation, cells were collected and analyzed for intracellular production of IL-12 and TNF-α.

These data are consistent with the finding of Datta and colleagues [264], which demonstrated that p30 decreases the release of *pro-inflammatory* cytokines, such as TNF-α, IL-8, and MCP-1 upon stimulation with the TLR4 specific agonist lipopolysaccharide (LPS) by decreasing the expression of TLR4 itself.

4.4 Expression of interferon stimulated genes (ISGs) in lentiviruses (p30 or CTRL) transduced THP-1 cells upon stimulation with TLR agonists

We next studied the effect of p30 on TLR3 and TLR7/8 because of their

relevance in virus immune-surveillance, since they recognize the double and the single strand RNA (dsRNA or ssRNA) respectively, which are characteristic of viral infections and particularly they are intermediates of HTLV-1 infection. As type 1 IFNs are the most potent innate antiviral factors able to stimulate the production of a wide variety of antiviral interferon stimulated genes (ISGs); and as both DNA and RNA viruses, as well as other pathogens have evolved a sophisticated and varied strategy to counteract these innate protective responses; we analayzed the expression of three ISGs after TLR stimulation with specific agonists.

In order to do that THP-1 cells were p30- or mock-transduced with the same lentiviruses used before, then stimulated with specific agonist of TLR3, such as polyI:C, or specific agonist of TLR7/8, such as Imiquimod. LPS stimulation on TLR4 was used as a control. We analyzed by real-time PCR the level of mRNA expression of interferon stimulated genes (ISGs) and selected the most common ISGs to measure the overall type I interferon response, such as myxovirus resistance A (MxA), APOBEC-3G (A3G) and 2'-5'-oligoadenylate synthetase (OAS). The results are shown in terms of percentage of relative expression of these ISGs genes, in the presence of p30 expression versus the mock-transduced control following 6 hours of TLR3 and -4 stimulation. Following the polyI:C stimulation, MxA and OAS expression was significantly inhibited by p30, and this difference was significant also when the expression of all genes together were analyzed ($p<0.01$) (Figure 6).

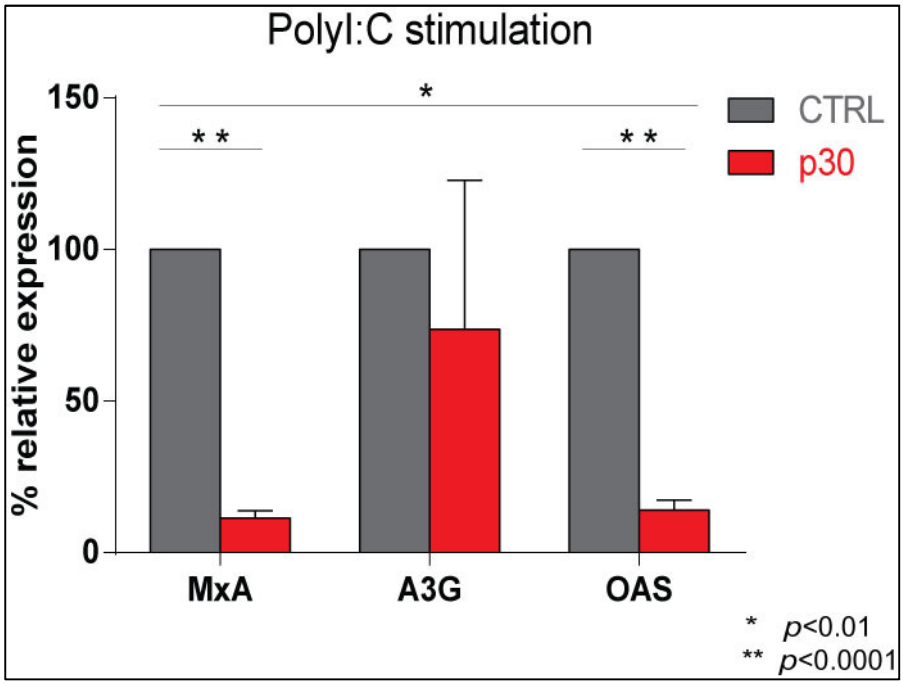


Figure 6: Real-time PCR quantitative analysis of type-1 interferon stimulated genes (ISGs). THP-1 cells were transduced with p30- (red bars) or control lentivirus (gray bars), kept in culture for 72 hours and stimulated for six hours with polyI:C. The results are presented as the percentage of inhibition of the expression of the MxA, A3G and OAS mRNAs versus the mock-transfected control. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

A similar effect was observed following LPS stimulation (Figure 7), as expected.

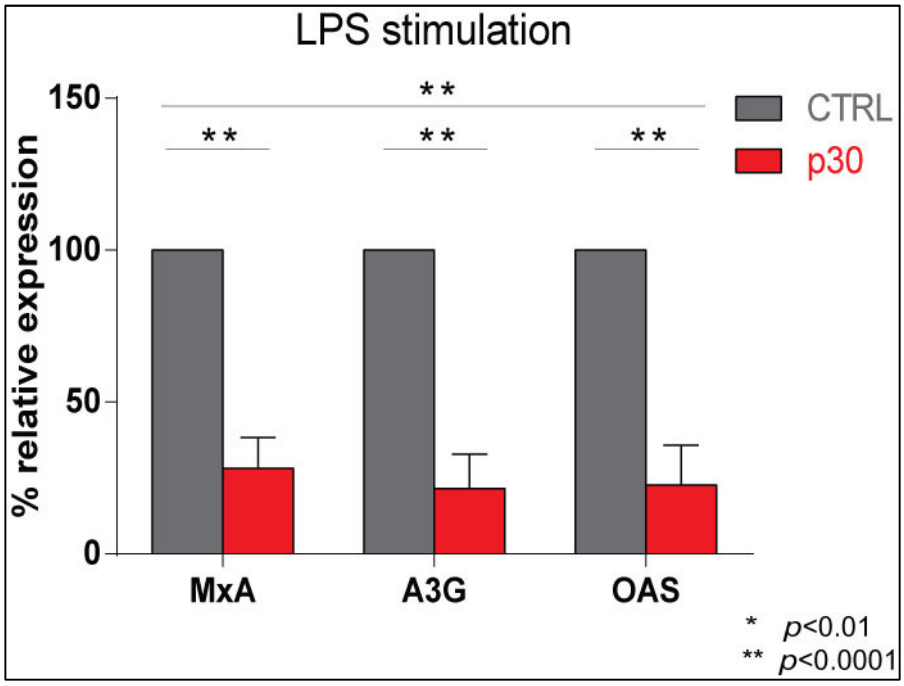


Figure 7: Real-time PCR quantitative analysis of type-1 interferon stimulated genes (ISGs). THP-1 cells were transduced with p30- (red bars) or control lentivirus (gray bars), kept in culture for 72 hours and stimulated for six hours with LPS The results are presented as the percentage of inhibition of the expression of the MxA A3G and OAS mRNAs versus the mock transfected control. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

Surprisingly, no difference was found following stimulation with Imiquimod (Figure 8).

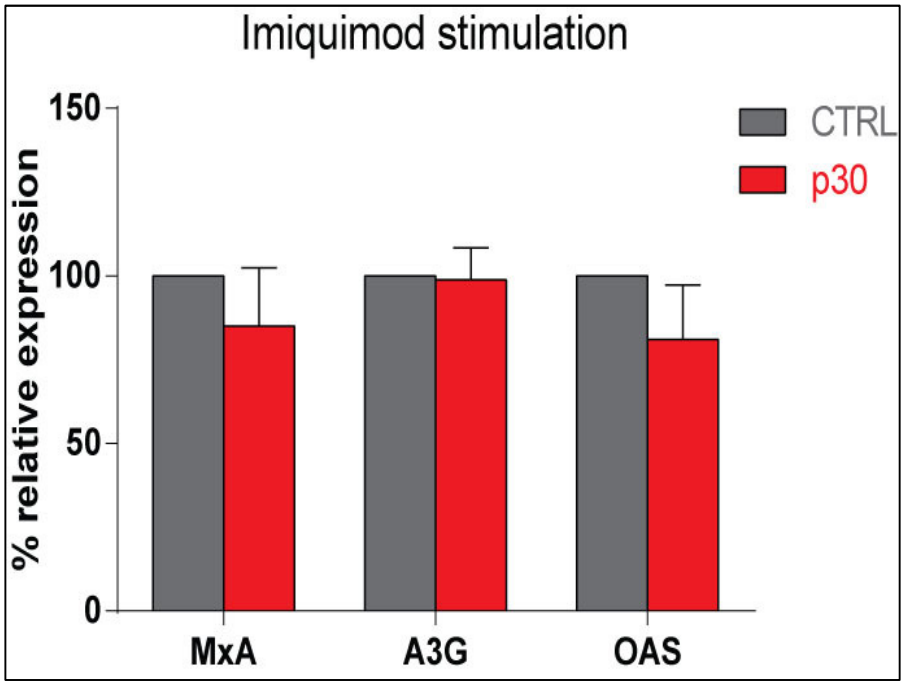


Figure 8: Real-time PCR quantitative analysis of type-1 interferon stimulated genes (ISGs). THP-1 cells were transduced with p30- (red bars) or control lentivirus (gray bars), kept in culture for 72 hours and stimulated for six hours with Imiquimod the results are presented as the percentage of inhibition of the expression of the MxA A3G and OAS mRNAs versus the mock transfected control.

4.4.1 Expression of proteins in lentiviruses transduced THP-1 cells upon stimulation with TLR agonists

To control if these differences observed in the expression of ISGs were caused by a different expression of quantity of proteins, we performed and immunoblot assay using antibody able to recognize p30 and tubulin as a control. The lack of p30- mediated inhibition of Imiquimod was not due to difference in the expression of p30, as demonstrated in Figure 9.

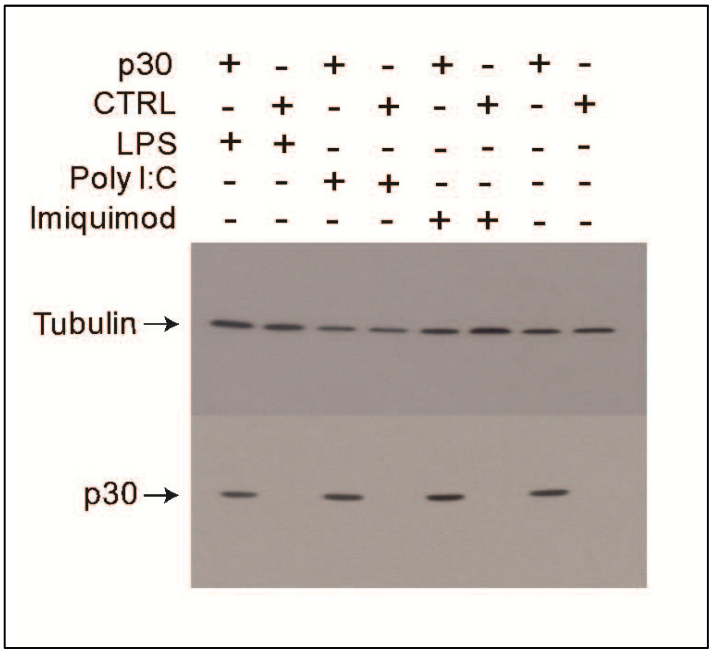
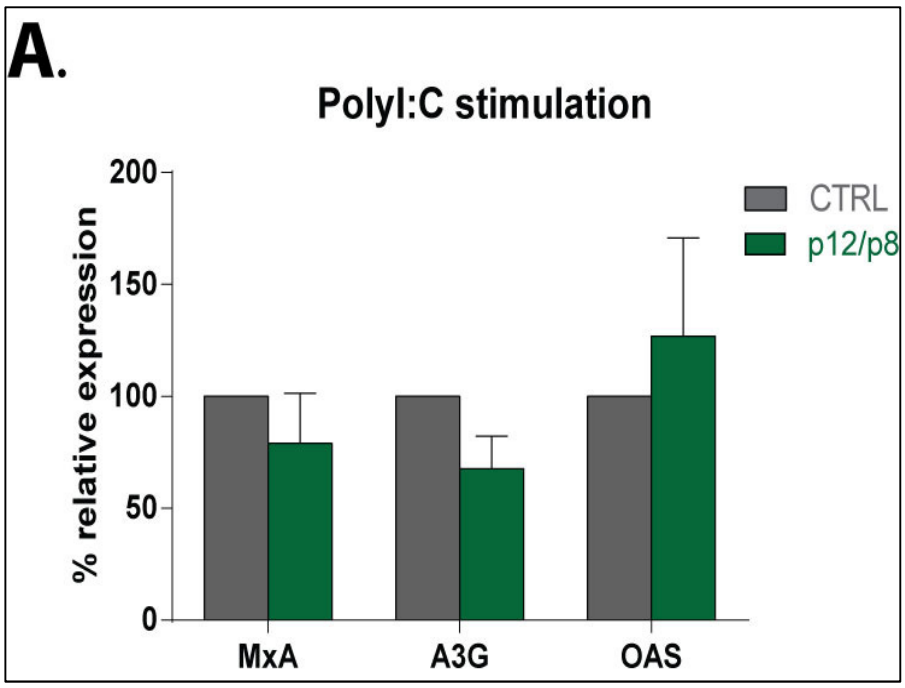
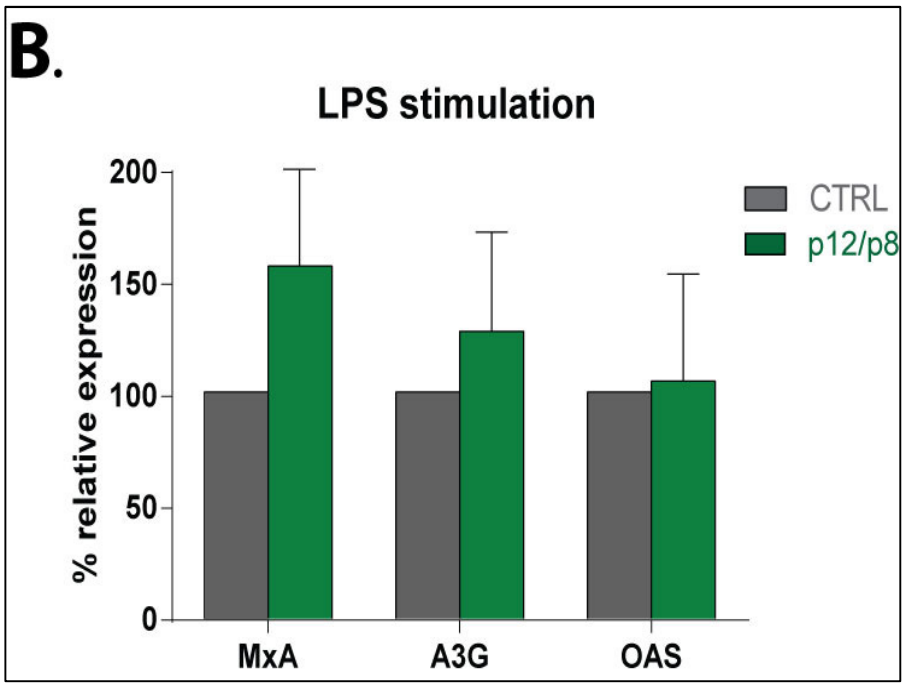


Figure 9: Immunoblot analysis of tubulin and p30 expression of the experiments presented in Figure 5; 6 and 7.

4.5 Expression of interferon stimulated genes (ISGs) in lentiviruses (p12/p8 or CTRL) transduced THP-1 cells upon stimulation with TLR agonists

Similar experiments were performed using cDNA endcoding p12/p8 since the presence of these proteins is essential for infectivity of monocytes as well [81]. So THP-1 cells were p12/p8- or mock-transduced, then stimulated with specific agonist of TLR3, such as polyI:C, or specific agonist of TLR7/8, such as Imiquimod. LPS stimulation on TLR4 was used as a control. We analyzed by real-time PCR the level of mRNA expression of interferon stimulated genes (ISGs) such as myxovirus resistance A (MxA), APOBEC-3G (A3G) and 2'-5'-oligoadenylate synthetase (OAS). In contrast to p30, p12/p8 did not have a significant effect on the ISGs upon specific TLR3 (Figure 10A), TLR4 (Figure 10A) and TLR7/8 (Figure 10C) stimulation.


A.
Polyl:C stimulation
% relative expression
200
150
100
50
0
CTRL
p12/p8
MxA
A3G
OAS


B.
LPS stimulation
% relative expression
200
150
100
50
0
CTRL
p12/p8
MxA
A3G
OAS

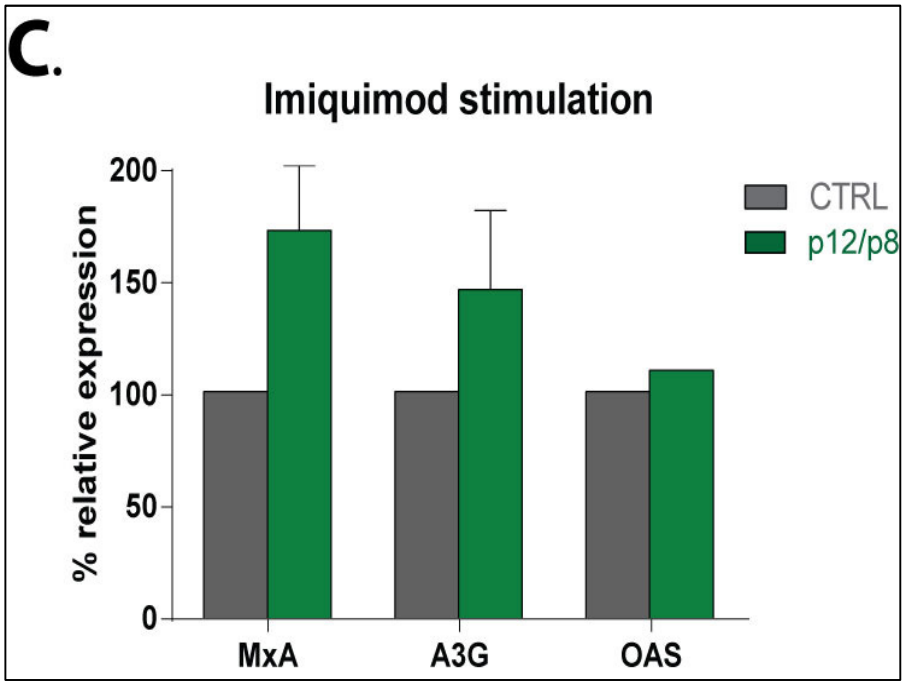


Figure 10. Real-time PCR quantitative analysis of type-1 interferon stimulated genes (ISGs). THP-1 cells were transduced with p12/p8- (green bars) or control lentivirus (gray bars), kept in culture for 72 hours and stimulated for six hours with polyI:C (A), LPS (B) or Imiquimod (C). The results are presented as the percentage of inhibition of the expression of the MxA A3G and OAS mRNAs versus the mock-transfected control.

4.6 Expression of interferon stimulated genes (ISGs) in lentiviruses transduced human primary cells

To validate the results obtained in the human tumor THP-1 cell line with p30, we performed the same experiments on *ex vivo* primary human monocytes and *in vitro* primary human Mo-mDCs.

4.6.1 Expression of ISGs in LV transduced primary human monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll gradient and primary monocytes were separated by adherence for 6 hours. Monocytes were transduced with p30 or CTRL lentivirus, and stimulated for

6 hours with polyI:C, LPS or Imiquimod. The expression of the three ISGs analyzed was significantly inhibited by the presence of p30 upon stimulation with polyI:C (Figure 11).

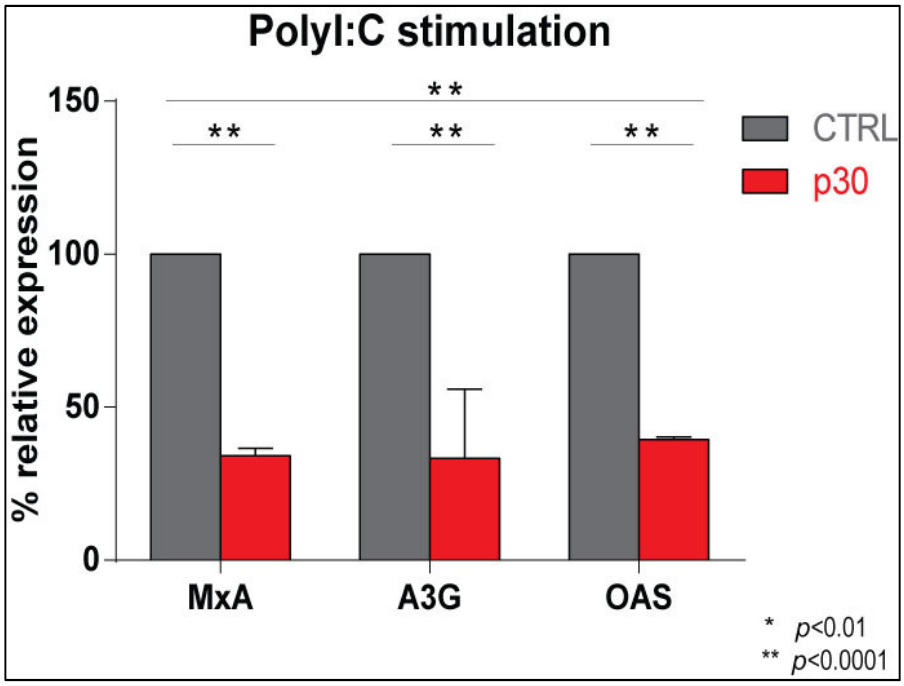


Figure 11. Real-time PCR quantitative analysis of the expression of (ISGs) on primary monocytes was performed. Primary monocytes were transduced with p30- (red bars) or control lentivirus (gray bars). 72 hours later the cells were stimulated with polyI:C. The results are shown as percentage of relative expression of ISGs mRNAs, such as MxA, A3G and OAS, following 6 hours of TLR stimulation. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

A similar effect was observed following LPS stimulation, as expected (Figure 12).

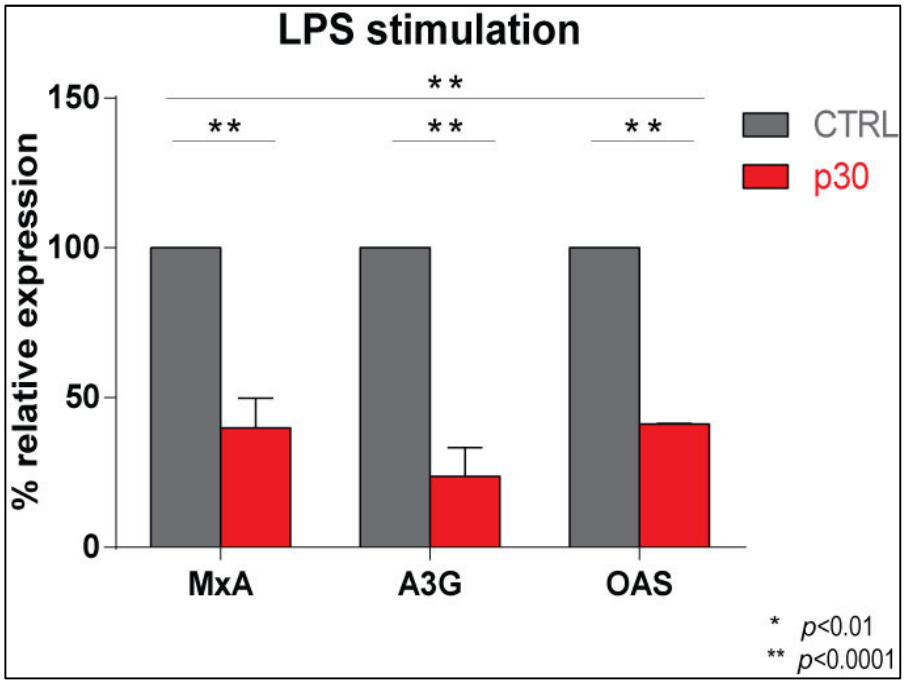


Figure 12. Real-time PCR quantitative analysis of the expression of (ISGs) on primary monocytes was performed. Primary monocytes were transduced with p30- (red bars) or control lentivirus (gray bars). 72 hours later the cells were stimulated with LPS. The results are shown as percentage of relative expression of ISGs mRNAs, such as MxA, A3G and OAS, following 6 hours of TLR stimulation. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

The expression of ISGs MxA, OAS and A3G was not significantly inhibited by the presence of p30 upon stimulation with Imiquimod (Figure 13).

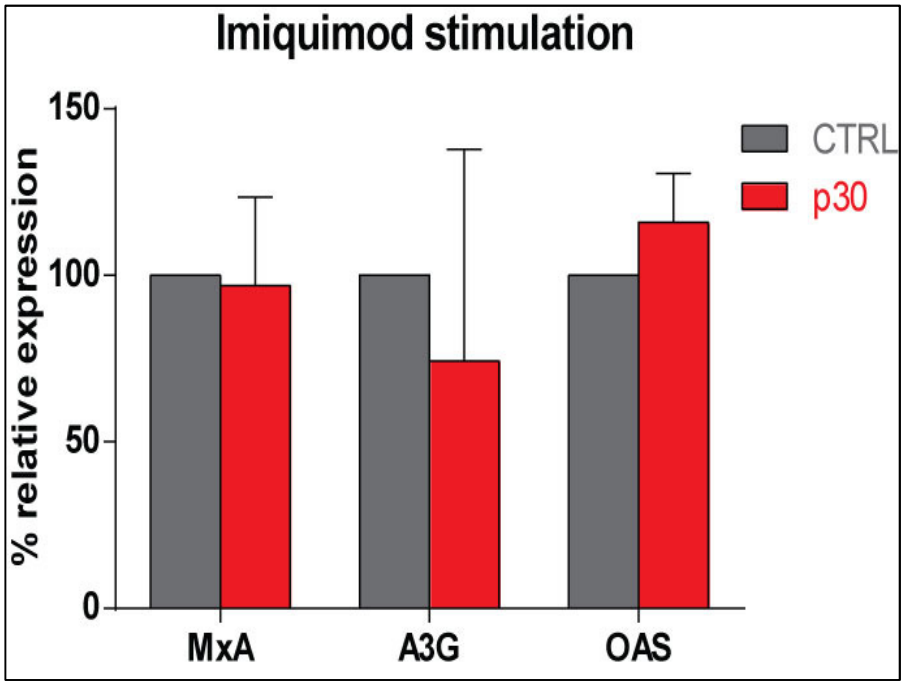


Figure 13. Real-time PCR quantitative analysis of the expression of (ISGs) on primary monocytes was performed. Primary monocytes were transduced with p30- (red bars) or control lentivirus (gray bars). 72 hours later the cells were stimulated with Imiquimod. The results are shown as percentage of relative expression of ISGs mRNAs, such as MxA, A3G and OAS, following 6 hours of TLR stimulation.

These data were consistent with our observations in THP-1 cells.

4.6.2 Expression of ISGs in LV transduced monocyte-derived dendritic cells (Mo-mDCs)

In the case of Mo-mDCs, after isolation of PBMCs by Ficoll gradient, primary monocytes were separated by elutriation to keep them inactivated and left “untouched”. After seven days of differentiation with IL4, GM-CSF, and TGFβ [81], the cell phenotype was verified by flow cytometry (data not shown) and cells were transduced with p30 or mock-transduced lentiviruses, and stimulated with polyI:C or LPS. Expression of p30 inhibited

significantly the mRNA expression of all the ISGs studied following stimulation with polyI:C as demonstrated in Figue 14.

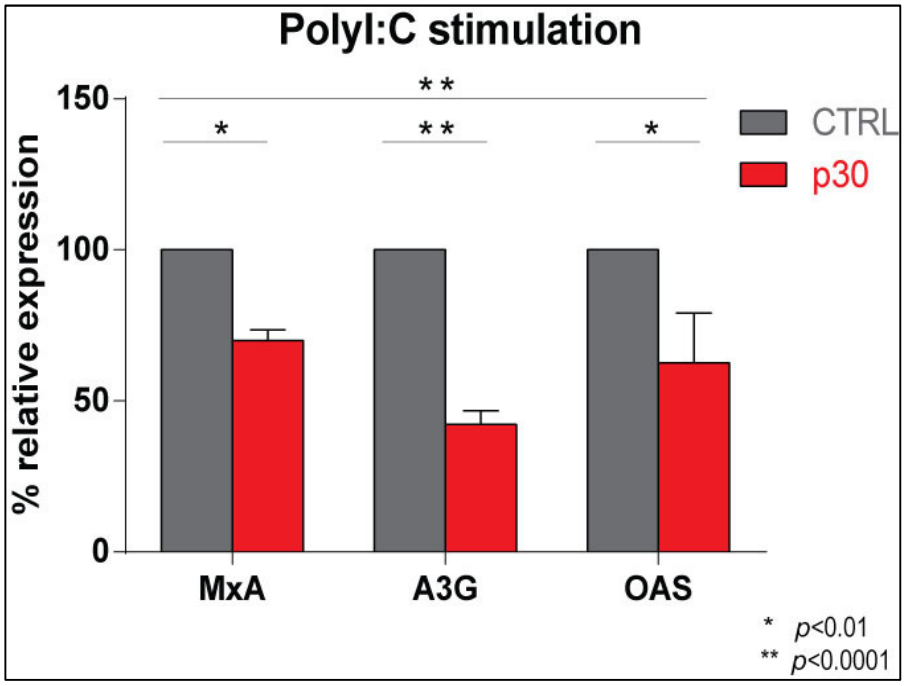


Figure 14. Real-time PCR quantitative analysis of the expression of (ISGs) on Mo-mDC was performed. Primary elutriated monocytes were differentiated into Mo-mDC and transduced with p30- (red bars) or control lentivirus (gray bars). 72 hours later the cells were stimulated with polyI:C. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

A similar effect was observed following LPS stimulation (Figure 15), as expected.

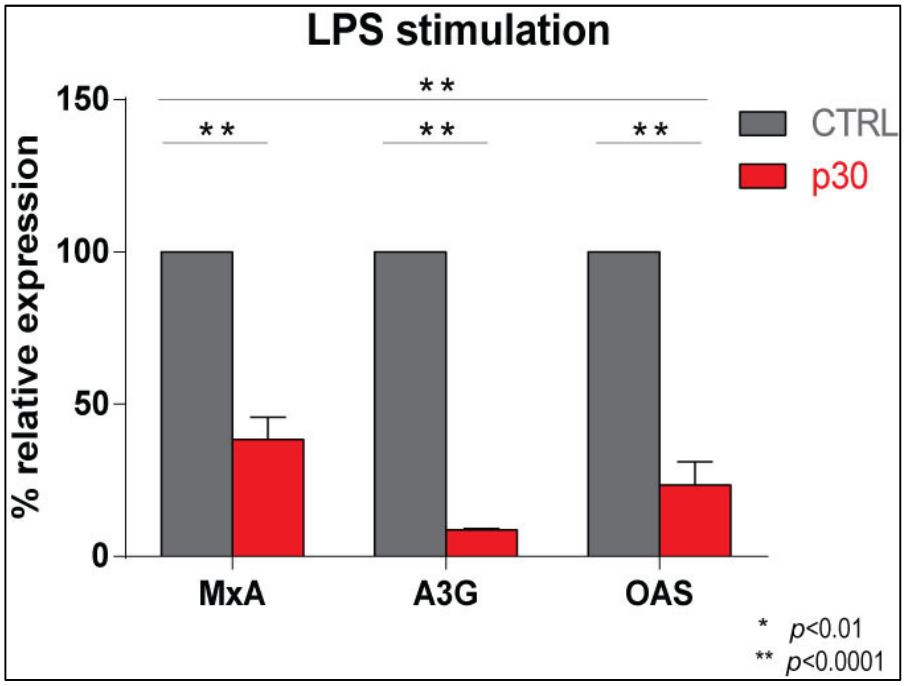


Figure 15. Real-time PCR quantitative analysis of the expression of (ISGs) on Mo-mDC was performed. Primary elutriated monocytes were differentiated into Mo-mDC and transduced with p30- (red bars) or control lentivirus (gray bars). 72 hours later the cells were stimulated with LPS. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.01 or 0.0001, respectively.

4.6.3 Expression of proteins in lentiviruses transduced Mo-mDCs upon stimulation with TLR agonists

To control if these differences observed in the expression of ISGs were caused by a different expression of quantity of proteins, we performed and immunoblot assay using antibody able to recognize p30 (anti-HA antibody) and tubulin as a control. The level of expression of p30 protein was comparable in all the conditions tested (Figure 16).

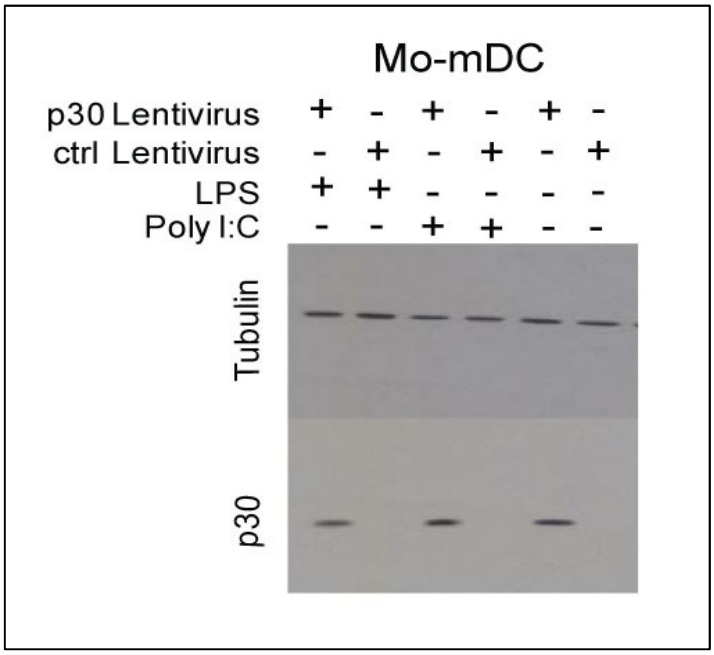


Figure 16: Immunoblot analysis of tubulin and p30 expression of the experiments presented in Figure 11; 12 and 13.

These data demonstrate that also in *ex vivo* primary monocytes and *in vitro* primary human dendritic cells, p30 expression inhibits TLR3 and TLR4 signaling and corroborate the THP-1 cell model.

4.7 Expression of virus in HTLV-1 infected THP-1 cells with different molecular clones (WT or p30-KO)

To test the contribution of p30 without using over expression systems, we infected THP-1 cells with the WT or the p30-KO HTLV-1 molecular clone [81] and tested the response of the cells to TLR3 and 4 stimulation (using TLR agonists PolyI:C and LPS respectively). The p30-KO HTLV-1, whereby the expression of p30 was ablated, produced a lower level of virus as measured by the p19 Gag levels released at peak into the supernatant when compared to WT (Figure 17, left panel) [81]. Accordingly, a lower level of viral DNA was found by quantitative real-time PCR in the cells

exposed to p30-KO than the WT with approximately 1 copy per cell versus 4 copies per cell, respectively (Figure 17, right panel).

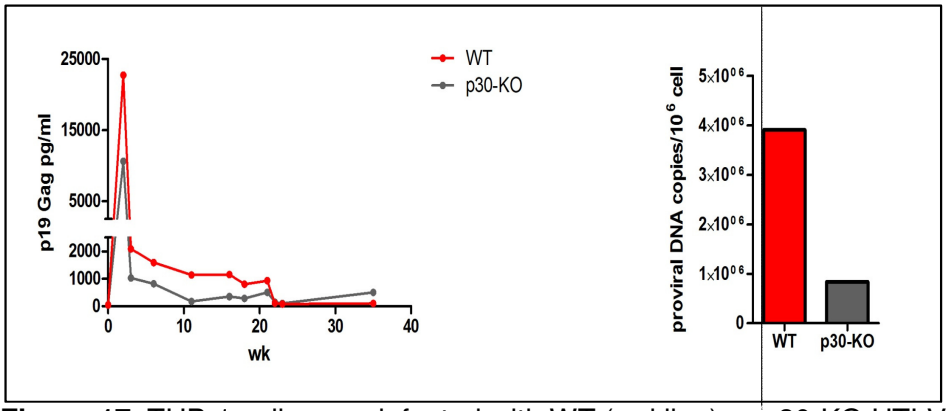


Figure 17. THP-1 cells were infected with WT (red line) or p30-KO HTLV-1 (gray line) virus. Productive infection was monitored by p19 Gag ELISA in the cell supernatant (left panel). Uninfected controls were negative for p19 Gag and are not represented in the graph. HTLV-1 proviral load was measured by real-time PCR performed on genomic DNA of THP-1 cells infected with WT (red bar) or p30-KO (gray bar) HTLV-1 molecular clones (right panel). The normalized value of the HTLV-1 proviral DNA load was calculated as HTLV-1 DNA copy number/albumin gene copy number and expressed as the number of HTLV-1 proviral DNA copies per 10^6 cells.

4.8 Phenotype of HTLV-1 infected THP-1 cells with different molecular clones (WT or p30-KO)

At week 18 post-infection we analyzed the phenotype of infected THP-1 cells by flow cytometry. Particularly we analyzed the expression of activation markers such as HLA-DR, CD80/83 and CD86 and of the homing marker CCR7. As we can see from the figure below, even in conditions of low levels of virus production the infected cells continued to express high levels of the CD80/83, CD86 immune activation markers and the CCR7 homing marker (Figure 18).

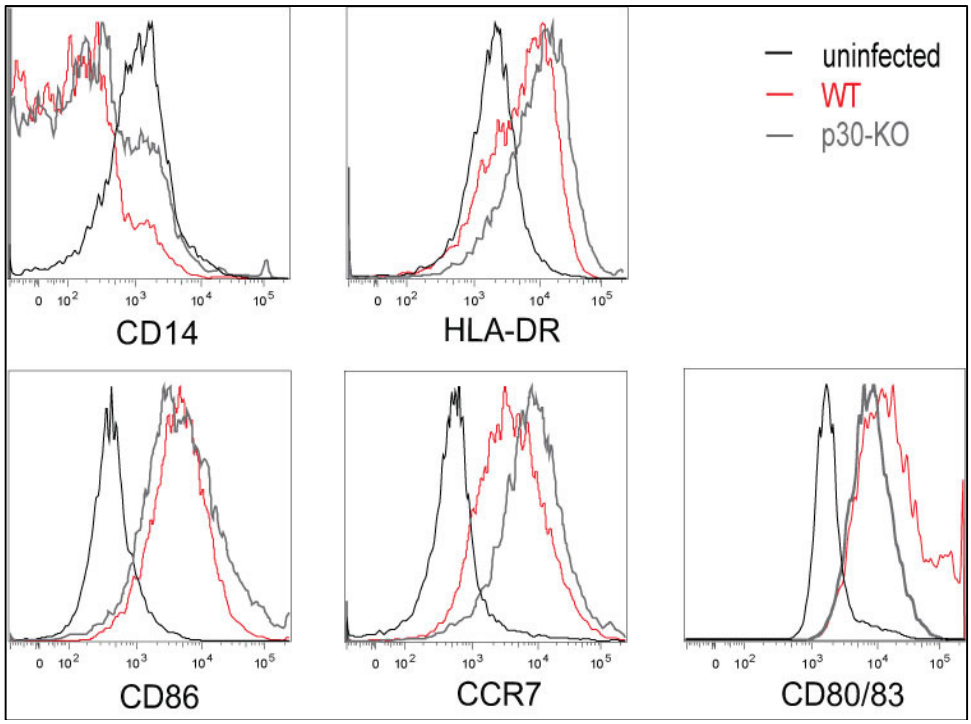


Figure 18. Several week post-infection the phenotypes of the THP-1 cells infected with WT or p30-KO HTLV-1 and uninfected were analyzed for activation markers, such as HLA-DR, CD80/83, CD86 and, the CCR7 homing marker.

4.9 Expression of interferon stimulated genes (ISGs) in HTLV-1 infected THP-1 cells

At week 19, when viral expression was equivalently low in both cell cultures (Figure 17), we stimulated the cells for 6 hours with polyI:C and LPS for TLR3 and TLR4, respectively. After stimulation, RNA was extracted and tested for ISGs expression. The results are shown as a percentage of relative expression of the ISGs in uninfected THP-1 cells versus the WT or p30-KO infected THP-1 cells. The response to TLR stimulation was significantly inhibited by infection with the WT virus but much less so in cells infected with the p30-KO virus upon polyI:C stimulation (Figure 19).

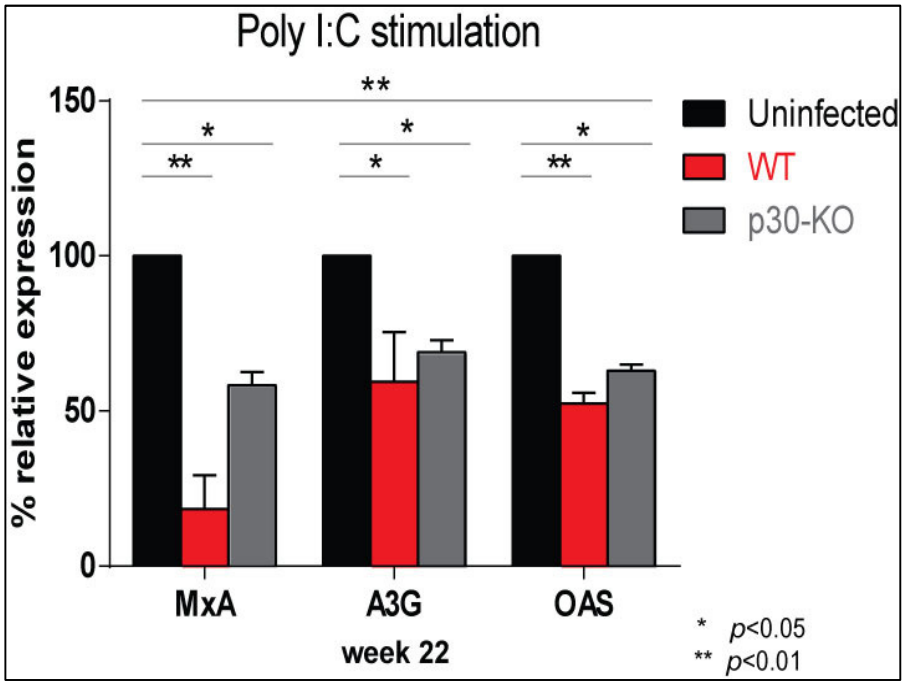


Figure 19. ISGs were measured at week 10 post infection, by real-time PCR on the RNA from the WT or p30-KO HTLV-1 chronically infected or uninfected THP-1 cells following stimulation with polyI:C for 6 hours. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.05 or 0.01, respectively.

A similar effect was observed following LPS stimulation (Figure 20), as expected.

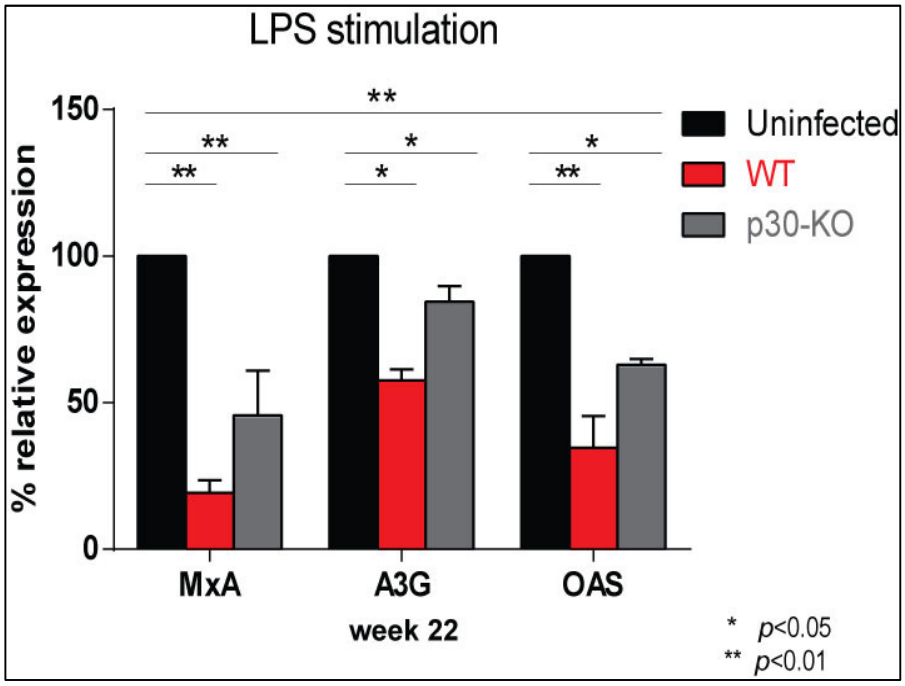


Figure 20. ISGs were measured at week 10 post infection, by real-time PCR on the RNA from the WT or p30-KO HTLV-1 chronically infected or uninfected THP-1 cells following stimulation with LPS for 6 hours. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.05 or 0.01, respectively.

4.10 Rescue of viral production and IFN responses in THP-1 cell infected with HTLV-1 molecular clone

4.10.1 Expression of p19 in lentiviruses transduced THP-1 cells previously infected with different HTLV-1 molecular clones

We next investigated whether we could rescue viral expression and IFN responses by overexpressing p30 in previously infected cells (week 10 post infection) (Figure 17). WT or p30-KO infected THP-1 cells were transduced with p30 or mock lentivirus. Un-transduced cells were used as control. After 2 days, the supernatants were collected and the p19 Gag was analyzed by ELISA. Interestingly, p30 expression restored virus production in cells

infected with the p30-KO virus (Figure 21).

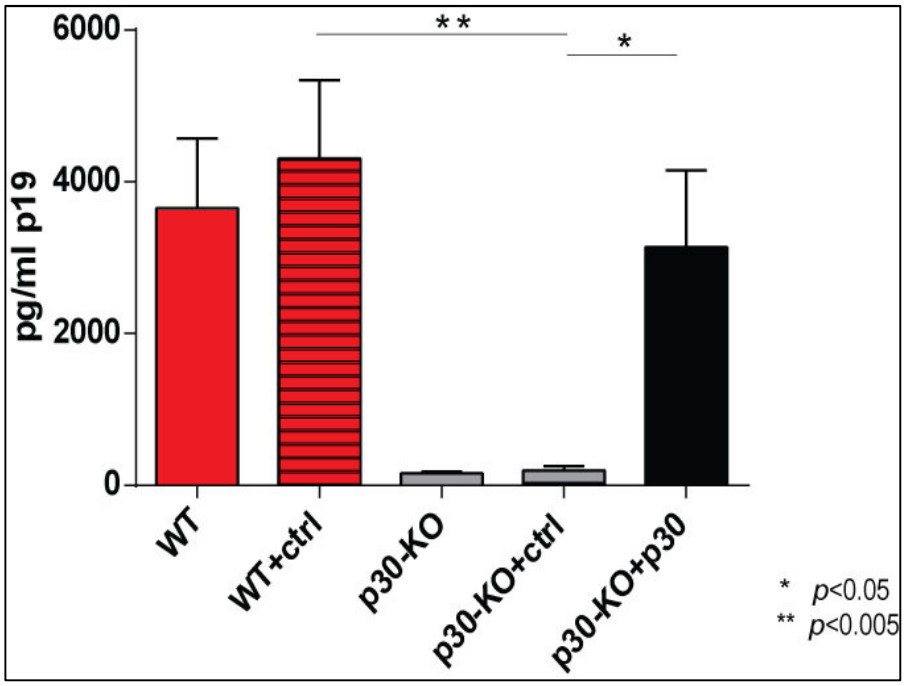


Figure 21. Level of p19 Gag in THP-1 cells chronically infected with WT or p30-KO HTLV-1 virus transduced with the lentivirus expressing p30 or the control lentivirus. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.05 or 0.005, respectively.

4.10.2 Expression of interferon stimulated genes (ISGs) in lentiviruses transduced THP-1 cells previously infected with different HTLV-1 molecular clones

Consistent with p30 reducing ISGs expression, RT-PCR analysis of cellular mRNA demonstrated that expression of MxA and A3G genes was higher in cells infected with p30-KO virus compared to cells infected with WT virus (Figure 22).

Transduction of p30-KO infected cells with lentivirus expressing p30 rescued only partially the MxA and A3G mRNA expression, suggesting that

other viral gene may participate in inhibition of ISG expression (Figure 22).

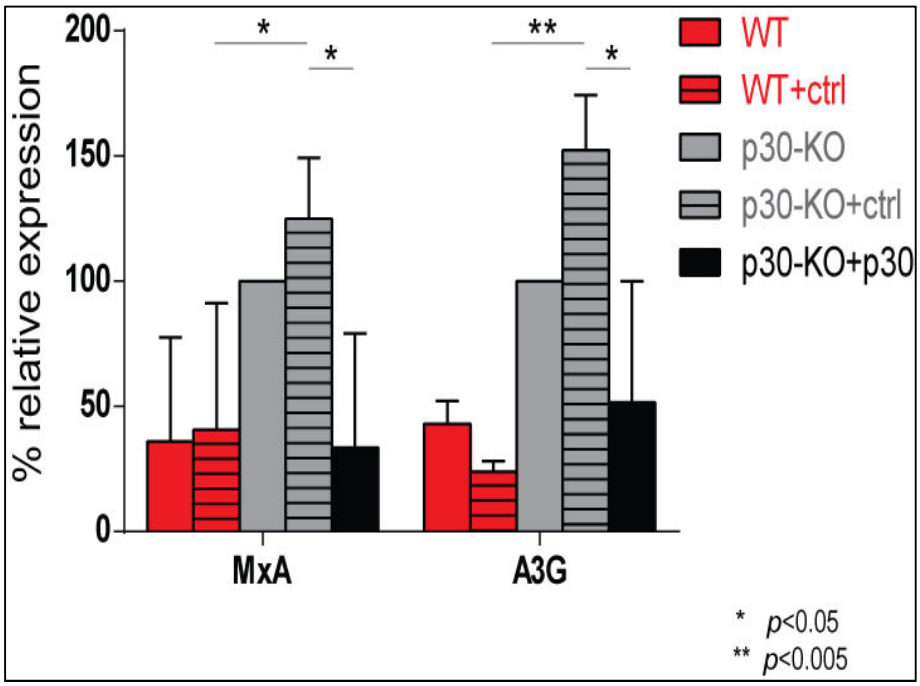


Figure 22. Real-time PCR for the MxA, A3G ISGs genes from the RNAs of the infected/transduced cells. The statistically significant differences are marked with one or two stars, which indicate a *p* value less than 0.05 or 0.005, respectively.

So we can conclude that p30-KO HTLV-1 virus production and ISGs inhibition is rescued by overexpression of p30.

4.10.3 Expression of proteins in lentiviruses transduced THP-1 cells previously infected with different HTLV-1 molecular clones

Proteins were extracted and tested by Western Blot, to demonstrate comparable expression of p30 and GFP (Figure 23).

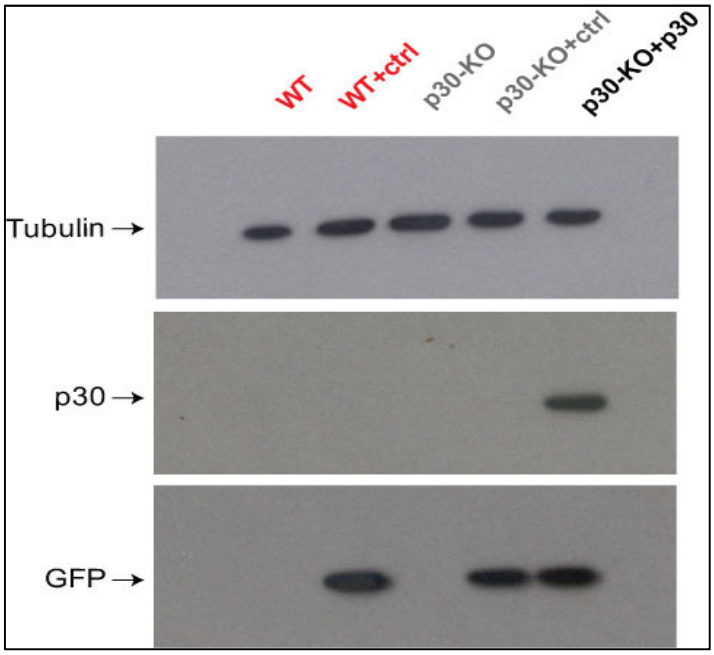


Figure 23. Western Blot analysis for tubulin, p30 or GFP on transduced cells.

4.11 Chromatin immuno-precipitation (ChIP)

To explore the mechanism by which p30 affects TLR3/4 function, we performed a chromatin immuno-precipitation assay. THP-1 cells were p30- or mock-transduced with lentivirus. Cells were stimulated for 1 hour with specific agonists polyI:C and LPS as a control, then cross-linked. The pull downs were performed with antibodies against PU.1, p30-HA, and total RNA Pol II, as well as with antibodies specific for the phosphorylated form of RNA Pol II CTD-Ser5 or RNA Pol II CTD-Ser2, which respectively identify the initiating or the elongating form of active RNA Pol II. Real-time PCR was performed on the ChIP enriched DNA to quantify the amount of PU.1 containing transcriptional complexes bound to the promoters of the IFNα1, IFNβ, TLR4 and the UbiquitinB genes. We observed that upon TLR4 stimulation with LPS and also TLR3 stimulation with polyI:C, lentiviral expression of p30 decreased the amount of PU.1 recruited to the IFNα1

promoter. We observed also a decrease on the same promoter of both initiating and elongating RNA Pol II, as assessed by Pol II-S5 and Pol II-S2 ChIP (Fig. 24).

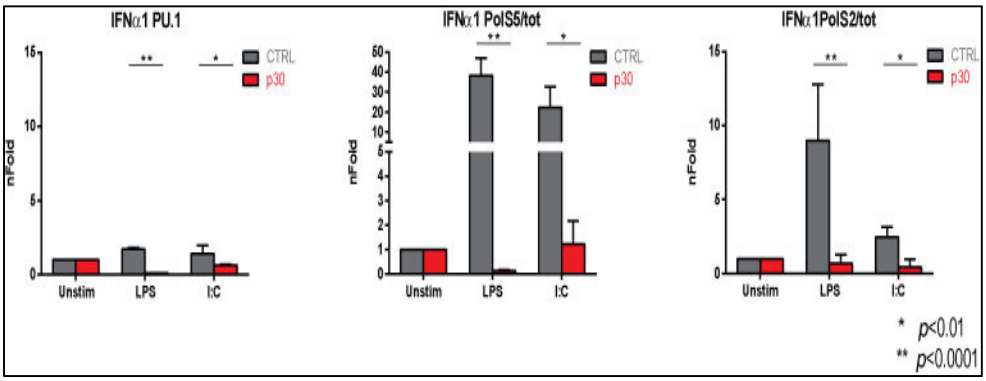


Figure 24. ChIP assay was performed on THP-1 cells transduced with p30 (red bars) or mock transduced (gray bars) lentivirus. Cells were stimulated for one hour with LPS or polyI:C (I:C) before the ChIP assay, or not. Depicted here, is the relative amount of DNA precipitated with the different antibodies normalized on the amount found in the input of the promoters for IFNα1 gene. The immunoprecipitations were performed with anti-HA, which detect the HA-tagged p30, anti-PU.1, anti-PolIItot, anti-PolIIS5 and anti-PolIIS2. The data are expressed in terms of nFold, where the unstimulated is 1 and represent the amount of promoter DNA for IFNα1 immunoprecipitated in cells stimulated in the presence or absence of p30. The statistically significant differences are marked with one or two stars, which indicate a *p* value minor than 0.01 or 0.0001, respectively.

Moreover, the presence of p30 resulted in a decreased binding of PU.1, Pol II-S5 and Pol II-S2 to the promoters of IFNβ and TLR4 upon LPS stimulation of TLR4 and polyI:C stimulation of TRL3 (Figure 25).

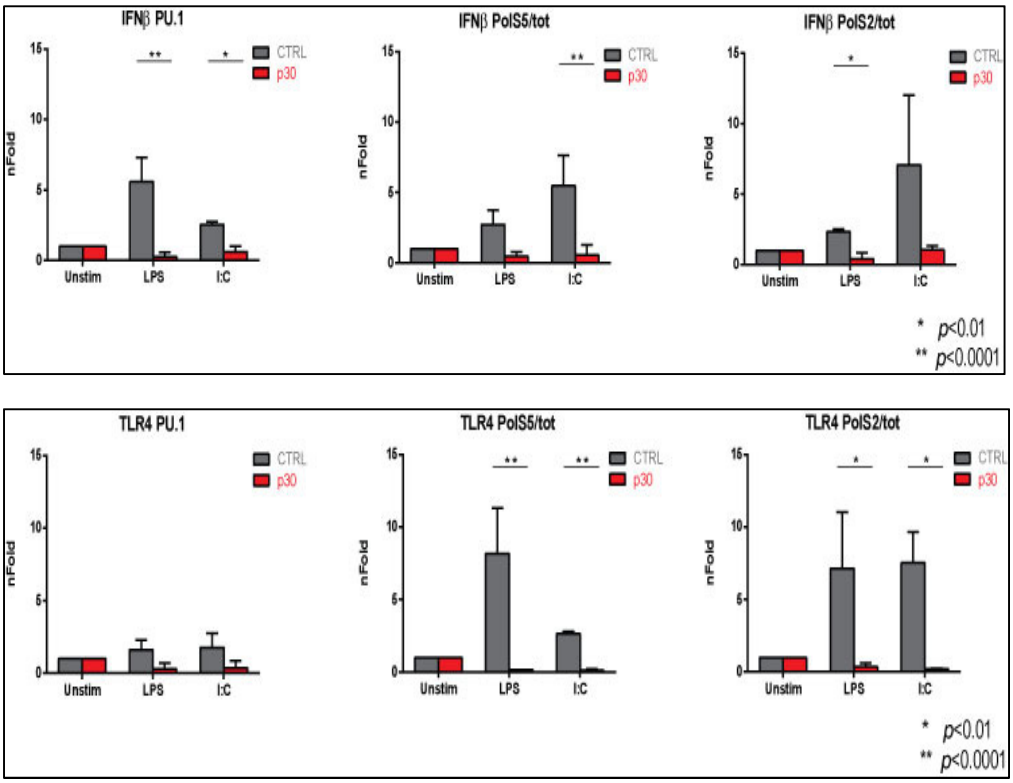


Figure 25. ChIP assay was performed on THP-1 cells transduced with p30 (red bars) or mock transduced (gray bars) lentivirus. Cells were stimulated for one hour with LPS or polyI:C (I:C) before the ChIP assay, or not. Depicted here, is the relative amount of DNA precipitated with the different antibodies normalized on the amount found in the input of the promoters for IFNβ and TLR4 genes. The immunoprecipitations were performed with anti-HA, which detect the HA-tagged p30, anti-PU.1, anti-PolIItot, anti-PolIIS5 and anti-PolIIS2. The data are expressed in terms of nFold, where the unstimulated is 1 and represent the amount of promoter DNA for IFNβ and TLR4 immunoprecipitated in cells stimulated in the presence or absence of p30. The statistically significant differences are marked with one or two stars, which indicate a *p* value minor than 0.01 or 0.0001, respectively.

The real time PCR performed on the UbiquitinB promoter, used as control,

showed no significant differences, as expected (Figure 26).

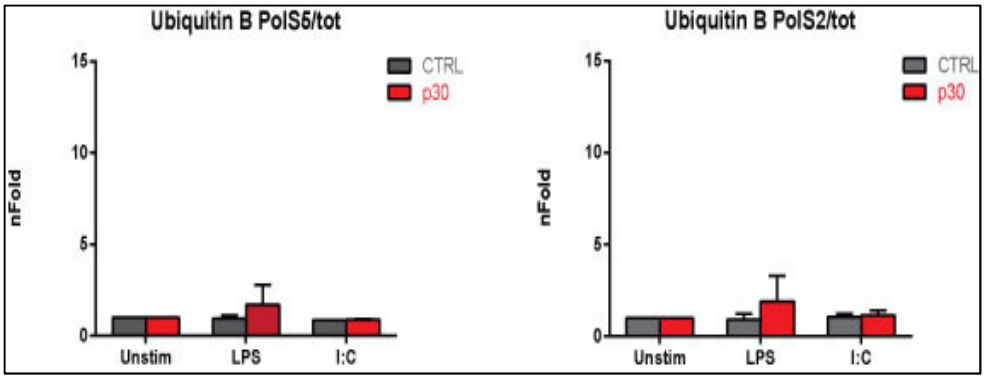


Figure 26. ChIP assay was performed on THP-1 cells transduced with p30 (red bars) or mock transduced (gray bars) lentiviral vector. Cells were stimulated for one hour with LPS or PolyI:C (I:C) before the ChIP assay, or not. Depicted here, is the relative amount of DNA precipitated on the amount found in the input of the promoters for UbiquitinB gene, as control. The immunoprecipitations were performed with anti-PolIIS5, anti-PolIIS2 and anti-Poltot antibody. Data are expressed in terms of nFold, where the unstimulated is 1 and represent the amount of promoter DNA for the UbiquitinB gene immunoprecipitated in cells in the presence or absence of p30. Depicted here the ratio of PolS5/Poltot (A) and PolS2/Poltot (B).

All these data are consistent with the reduced expression of IFN responsive genes following TLR3 and TLR4 stimulation being mediated by p30 at the transcriptional level, as was previously suggested for TLR4 [264].

Conclusions and future directions

5. CONCLUSIONS AND FUTURE DIRECTIONS

Interferons (IFNs) are key molecules that mediate antiviral innate immune response and are potent regulator of adaptive immune responses since they affect immune cell activation, cell growth, and apoptosis. Type I IFNs (α and β) are mainly produced by plasmacytoid dendritic cells but also by macrophages and fibroblasts [267]. The activation of IFN response leads to the upregulation of more than 300 genes (ISGs). These genes encode proteins that have antiviral and immunoregulatory activity and are able in some cases to suppress viral replication and clear virus [268]. Among them, myxovirus resistance GTPase protein A (MxA), 2', 5' oligoadenylate synthetase (OAS), apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G (A3G) exert a particularly important role in the immune intracellular response and are considered to be excellent markers for IFN action.

Viruses and often pathogens have evolved a wide variety of strategies to counteract and escape the IFN system [267]. The molecular mechanisms involved, span from the shutdown of the entire cell metabolism to specifically targeting IFN at different levels, such as to inhibit the type-1 IFN-production itself, to inactivate secreted IFN molecules, to block the signaling pathways or the functionality of IFN-induced protein. A variety of viruses such as Influenza virus, Sendai virus, Ebola, Herpes simplex virus [269], Human Papilloma virus [267], Adenovirus [269], Hepatitis C virus, have evolved a way to interfere with specific components of IFN induction pathway, mainly by blocking the IFN regulatory factors (IRFs). On the other hand, Dengue virus [269], Human Papilloma virus (26), Hepatitis C virus (8) and Paramixoviruses [269] target the JAK-STAT pathway. HIV interferes with the IFN-induced PKR and A3G through Tat and Vif, respectively (39, 44).

HTLV-1 has evolved its own strategy to counteract the IFN system.

Although the mechanism is not fully understood, Hishizawa *et al.* have shown that dendritic cells isolated from HTLV-1-infected individuals have an impaired ability to secrete type-1 IFN [251]. In addition, it has been reported that the cellular protein SOCS, which inhibits STAT1 phosphorylation, inhibits intracellular signal transduction downstream of the IFN receptor, IFNAR1/2, in both *ex vivo* $CD4^+$ cells from HTLV-1 infected individual and *in vitro* in HTLV-1 infected cells [232]. Moreover, in HTLV-1 infected cells *in vitro* the level of phosphorylation of Tyk2 and □17 STAT2, two key molecules in the activation cascade of the IFN pathway is decreased [265]. Tax also prevents the binding of CBP/p300 with STAT2 in a competitive manner. Despite all mentioned above, anti-IFN mechanisms evolved by HTLV-1, type-1 IFN maintains a potent antiviral function, and is used in the clinic as anti-HTLV-1 therapy [268] in ATL. While IFN-α treatment alone results in a modest and transient, although appreciable, beneficial effect on ATL patients, when combined with zidovudin (AZT) and arsenic trioxide it causes long lasting remission [251]. The mechanisms underlying this therapeutic effect, remains unclear. In fact, while some groups demonstrated *in vitro* that the treatment with IFN-α decreases HTLV-1 expression, Kannagi's group showed that the p19 Gag released in the supernatant is decreased, but not the expression of other viral genes [253]. However, the addition of AZT decreases viral transcription, p19 Gag and Tax production, leaving the p53 dependent apoptotic pathways unaffected and restoring the PKR antiviral activity [253].

Here, we demonstrate that HTLV-1 has evolved additional mechanisms to counteract the IFN system through the interaction of the viral regulatory protein p30 and the myeloid transcription factor PU.1.

In this study we found that HTLV-1 p30 decreases TLR4 expression in response to PMA stimulation in p30-LV transduced THP-1 cells. We confirmed that the mechanism underlying this effect of p30 was transcriptional. Moreover we showed that p30 decrease the expression of

intracellular cytokines TNF-α and IL-12 after LPS stimulation. These data are consistent with the finding of Datta and colleagues [264], which demonstrated that p30 decreases the release of *pro-inflammatory* cytokines, such as TNF-α, IL-8, and MCP1 upon stimulation with the TLR4 specific agonist lipopolysaccharide (LPS) by decreasing the expression of TLR4 itself.

Moreover, we demonstrated that p30 decreases TLR3 induction of interferon stimulated genes (ISGs) expression in THP-1 cells, but not of TLR7/8. Similar results were obtained in primary human monocytes and monocyte-derived dendritic cells (Mo-mDCs). Moreover the ablation of p30 from an HTLV-1 molecular clone results in decreased inhibition of TLR3 and TLR4 inducted ISGs expression in THP-1 cells that is partly restored by p30 overexpression. Finally our data demonstrated that p30-KO HTLV-1 virus production and ISGs inhibition is rescued by overexpression of p30.

Because p30 regulates the expression of other viral RNAs, it is still debatable whether p30 acts directly or other viral proteins are involved in decreasing ISGs. However, the ChIP assay we performed and the rescued inhibition of the ISGs upon p30 overexpression suggests a direct interaction between p30 and PU.1. This is consistent with the observations reported by Datta and colleagues [264]. To address the role of other viral proteins, detailed analysis of the viral expression pattern in the p30-KO mutant versus WT infected cells needs to be done.

We are currently investigating the expression level of viral transcripts and the differential expression of these in monocytes versus $CD4^+$ T-lymphocytes. Indeed, we found that after the initial peak of p19 Gag in the supernatant around day 10, the virus production drops to low level of expression in THP-1 (Figure 17). Preliminary data, not included in this thesis, shows that in THP-1 cells the total mRNA level of hbz is higher than tax/rex message during chronic infection. Together this suggests that in monocytes the regulation of viral transcription might follow a different

pattern. The effect of p30 is pivotal to allow infectivity and persistence, as demonstrated by *in vivo* experiments in macaques [81]. Our data demonstrates that in the presence of p30 the interferon response is abrogated during viral replication. Thus, strategies aimed at silencing the expression of p30 with siRNA in conjunction with other approaches may further curtail viral replication and possibly contribute to the eradication of infected cell through innate IFN mediated immune responses.

This work allowed us the possibility to write and publish a paper on Journal of Virology [270].

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