# MODULATION OF THE INNATE IMMUNE RESPONSE DURING HUMAN T-CELL LEUKEMIA VIRUS INFECTION: IMPLICATIONS FOR DEVELOPMENT OF AN ONCOLYTIC VIROTHERAPY FOR ATL

By

**Stéphanie Olière**

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements of the degree of Doctor of Philosophy

**Department of Microbiology and Immunology**

**McGill University, Montreal**

**August, 2010**

# *ABSTRACT*

Infection with human T cell Leukemia virus (HTLV-1) can cause Adult T-cell Leukemia (ATL) or the neurological disorder HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). Although the majority of HTLV-1–infected individuals remain asymptomatic carriers (AC) during their lifetime, 2-5% will develop either ATL or HAM/TSP. The factors that determine HTLV-1 pathogenesis remain elusive, and therefore represent a serious obstacle in the establishment of effective therapies for HTLV-1-associated diseases.

Using gene expression profiling of CD4+ T-lymphocytes isolated from HTLV-1-infected individuals, we identified candidate genes differentially regulated in HTLV-1-associated diseases. Of particular interest, SOCS1 was up-regulated in HAM/TSP and AC patients – but not in ATL. SOCS1 positively correlated with HTLV-1 mRNA in HAM/TSP patient samples. SOCS1-mediated degradation of IRF3 - inhibited antiviral signaling during HTLV-1 infection. Our study reveals a novel evasion mechanism utilized by HTLV-1 which leads to increased retroviral replication, without triggering an IRF3-dependent interferon response. Thus, targeting SOCS1 could represent a potential new approach to enhance the therapeutic potency of IFN-α/β treatment in HAM/TSP disease.

Although treatment of hematological malignancies has improved considerably, this has not benefited ATL patients, as they are completely refractory to conventional chemotherapeutic regimens. Oncolytic viruses, such as Vesicular stomatitis virus (VSV), have emerged as a potential treatment for cancer. Here we show that *in vitro* VSV infection induced significant oncolysis in highly proliferating primary ATL cells, but not in primary Chronic Lymphocytic Leukemia (CLL) cells which are arrested in the $G_0$ phase. As chronic activation and proliferation is characteristic of ATL cells, we examined the effect of T-cell activation on VSV permissiveness and lysis. Activation of primary CD4+ T-lymphocytes was sufficient to induce VSV replication and VSV-triggered cell death, suggesting that cellular signaling pathways - ERK, JNK or AKT - that promote VSV replication are engaged during T-cell activation. Similarly, mitogenic activation of

primary CLL promotes the exit from $G_0$ and entrance into the cell cycle, rendering them susceptible to VSV-mediated oncolysis. Moreover, a global increase in protein translation mediated by the activation of mTOR and eIF4E was crucial for VSV replication in primary lymphocytes. These findings provide novel molecular targets for ATL and CLL therapeutics.

# *RÉSUMÉ*

Le rétrovirus T-lymphotropique humain (HTLV-1) est l'agent étiologique de la leucémie à cellule T de l'adulte (ATL) - une leucémie agressive et fatale des lymphocytes T CD4+. HTLV-1 est également associé à une forme de myélopathie chronique appelée Paraparésie Spastique Tropicale ou atteinte neurologique connue sous le nom de HTLV-1 - Associated Myelopathy (HAM/TSP). Bien que la majorité des individus infectés avec HTLV-1 demeurent asymptomatiques (AC) au cours de leur vie, 2 à 5% développent soit une ATL soit une HAM/TSP. Les facteurs qui déterminent la pathogénèse de l'HTLV-1 restent inconnus, et représentent donc un sérieux obstacle à la mise en place de traitements efficaces contre les maladies associées au virus HTLV-1.

Les études sur l'expression des gènes des lymphocytes T CD4+ isolés de patients infectés par HTLV-1, ont permis l'identification de gènes d'intérêt exprimés de façon différentielle dans les maladies associées au virus HTLV-1. De façon intéressante, il a été mis en évidence que l'expression de SOCS1 est plus élevé chez les patients asymptomatiques ou HAM/TSP que chez les patients ATL qui expriment généralement très peu d'ARN viral. Chez les patients HAM, il existe une corrélation directe entre le niveau d'expression de SOCS1 et l'activité transcriptionelle provirale. Du point de vue fonctionnel, SOCS1 inhibe la réponse antivirale, entre autre via la dégradation du facteur de transcription IRF3. Cette étude a donc permis d'identifier un nouveau mécanisme utilisé par HTLV-1 afin d'inhiber la réponse antivirale et ainsi d'augmenter sa capacité de réplication.

Dans cette étude, nous démontrons également que l'infection *in vitro* par le VSV induit la lyse oncogénique des cellules ATL, à fort potentiel prolifératif, mais pas celle des cellules primaires de la leucémie lymphocytique chronique (CLL), qui sont quiescentes *in vitro*.

Puisque l'activation et la prolifération chronique est une caractéristique des cellules ATL, nous avons étudié l'effet de l'activation des cellules T sur leur permissivité au VSV ainsi que leur lyse induite par le virus. L'activation des cellules T CD4+ primaires de patients sains est suffisante pour permettre la réplication virale et la mort

cellulaire induite par le VSV. L'utilisation d'inhibiteurs de la signalisation cellulaire a montré que l'activation de ERK, JNK ou AKT est suffisante pour permettre la réplication de VSV dans les cellules T CD4+. De façon similaire, la stimulation mitogénique des cellules CLL de patients induisant leur entrée dans le cycle cellulaire, les rend susceptibles à l'oncolyse par le VSV. De plus, une augmentation globale de la traduction protéique induite par l'activation de mTOR et eIF4E est essentielle pour la réplication du VSV dans les lymphocytes primaires.

En conclusion, ces résultats permettent d'identifier de nouvelles voies thérapeutiques pour les leucémies de l'ATL et CLL.

# *PREFACE*

In accordance with the "Guidelines for Thesis Preparation", the candidate has chosen to present the results of her thesis in classical form. A general introduction is presented in Chapter I and appears in the following review article.

1- Nakhaei, P, Paz, S, Oliere, S, Tumilasci, V, Bell, JC and Hiscott, J. **Oncolytic virotherapy of cancer with vesicular stomatitis virus.** *Gene Ther Mol Biol*. Vol 9, 269-280, 2005.

The results are described in chapters III and appear in the following journal articles:

2- Olière, S, Hernandez, E, Lézin, A, Arguello, M, Douville, R, Nguyen, TL, Wilkinson, P, Olindo S, Panelatti, S, Kazanji, M, Rafick-Pierre Sekaly, Raymond Césaire and John Hiscott. **HTLV-1 evades type I interferon antiviral signaling by inducing the suppressor of cytokine signaling (SOCS1)**. *In revision to Plos Pathogen.*

3- Oliere S, Arguello M, Mesplede T, Tumilasci V, Nakhaei P, Stojdl D, Sonenberg N, Bell J, Hiscott J. **Vesicular stomatitis virus oncolysis of T lymphocytes requires cell cycle entry and translation initiation.** *J Virol*. 2008 Jun; 82(12):5735-49. This article has been selected as spotlight.

4- Oliere S, Cesaire R, Sharif -Askari E, Loignon M, Olindo S, Panelatti G, Kazanji M, Aloyz R, Panasci L, Bell JC, Hiscott J. **Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia.** *Oncogene*.2006 Jan 19; 25(3):349-58.

Specific contributions to the work described in chapter III (manuscript I) are in part derived from the aforementioned "Oncogene" publication listed above which was conducted as collaboration between the first three authors:
Raymond Césaire was responsible for providing patient samples, as well as clinical characterization of the patient cohort.
Sharif -Askari Ehssan help to design the experiments and write the paper.

The candidate was also involved in collaboration with other researchers in the laboratory, which resulted in the following publications:

1- Samuel, S, Fonseca Tumilasci V, Oliere, S, Nguyên, TL, Shamy, A, Bell, J and Hiscott, J. **VSV oncolysis in combination with the BCL-2 inhibitor obatoclax overcomes apoptosis resistance in chronic lymphocytic leukemia.** Accepted in Molecular Therapy (August 2010).

2- Tumilasci VF, Oliere S, Nguyên TL, Shamy A, Bell J, Hiscott J. **Targeting the apoptotic pathway with BCL-2 inhibitors sensitizes primary chronic lymphocytic leukemia cells to vesicular stomatitis virus-induced oncolysis.** *J Virol*. 2008 Sep; 82(17):8487-99. This article has been selected as spotlight.

3- Sharif-Askari E, Nakhaei P, Oliere S, Tumilasci V, Hernandez E, Wilkinson E, Lin R, Bell JC and Hiscott J. **Bax-dependent mitochondrial membrane permeabilization enhances IRF-mediated innate immune response during VSV infection.** *Virology.*2007 Aug 15;365(1):20-33.

4- Harris J, Oliere S, Sharma S, Sun Q, Hiscott J and Grandvaux N. Nuclear **Accumulation of cRel following C-Terminal phosphorylation by TBK1/IKK epsilon.** *Journal of Immunology*. 2006 Aug 15; 177(4):2527-35.

# *ACKNOWLEDGEMENTS*

I would like to express my gratitude to a number of people who helped shape the formation of my PhD degree. First, to my supervisor Dr. John Hiscott, for the support and scientific exposure he provided during my time at the Lady Davis Institute. I accredit him for instilling me with the skills required to manage independently in the world of science.

I had the opportunity to work with talented graduate students and postdoctoral fellow - especially Dr Meztli Arguello - to whom I am very grateful for all the support, advice and help throughout all those years. I enjoyed working in a very cooperative environment sharing expertise and knowledge. I had the opportunity to collaborate with many students and was rewarded by co-authorships on various papers dealing with different projects. Dr Rénée Douville, Dr Raphaëlle Romieu-Mourez, Dr Nathalie Grandvaux, Dr Thi Lien-Anh Nguyen, Dr Meztli Arguello, were kind enough to proofread different sections of my thesis and for that I am very thankful.

Especially, I need to express my gratitude and deep appreciation to members of the John Hiscott lab: Delphine alias “bouche-trou”, Vanessa, Suzanne, Simon, Peyman, Dr Lin, Eduardo, Tiejun, Sara, Marie-Line, Suzie, Laura, Thomas, Mehdi, Sun, Daniel as well as members of the Gatignol’s lab: Aïcha, Sylvane and Guerline. Their friendship, help and encouragement have consistently helped me keep perspective on what is important and what really matters in life. I am grateful to other students whose assistance and comments were an essence for a continuous progress. Not to forget someone, I will just thank them all.

I have a special acknowledgement to Renée Douville ☺ not only for here outstanding knowledge in the field of retroviruses and her expertise in laboratory tools, but mainly for her generosity in sharing her knowledge with whoever needs it. She taught me various

keys for success mainly perseverance, love for science, and professionalism. But mostly she thought me the meaning of the sentence “I can do it”.

☺Big thanks to not only my colleague but to my friend, an excellent scientist with a promising future, Mayra Solis without whom I would have never survived my PhD (Merçi, Grosse C!!!) ♪.

At last but not least, I would like to give my warmest thanks to my parents, sister, nephew and friends for their unlimited love, care and support and also for constantly asking me: “So, when are you finishing?” Well guess what guys – I am done!

I could not have done it without their presence around me.

# *DEDICATION*

I would like to dedicate this work to my parents, Solange and Georges; ma soeur Murielle (alias big beef!!!) and my nephew “mon Tito d’amour”. I hope I made them proud of me and compensated a small percentage for their sacrifices. I love you! ♥

♥ J’aimerai dédicacer ce travail à ma famille. J’espère que je vous ai rendu fière and que cette petite réussite pourra compenser pour tous vos sacrifices. Je vous aime!

To my partner Patrick for all his support and cheering me up. ♥

♥ Á mon conjoint Patrick pour son amour, support et ses encouragements.

# *TABLE OF CONTENTS*

# *LIST OF FIGURES AND TABLES*

## CHAPTER - I - INTRODUCTION

## CHAPTER - II - RESULTS

***Manuscript I***

***Manuscript II***



***Manuscript III***

## CHAPTER - IV - DISCUSSION

# *LIST OF ABBREVIATIONS*

| | |
|---|---|
| **AD** | Activation Domain |
| **Allo-HSCT** | Allogenic Hematopoietic Stem Cell Transplantation |
| **AP1** | Activator protein 1 |
| **APCs** | Antigen Presenting Cells |
| **ASK1** | Apoptosis Signal-regulating Kinase 1 |
| **ATF** | Activating Transcription Factor family |
| **ATLL** | Adult T-cell Leukemia/Lymphoma |
| **ATM** | Mutated in Ataxia Telangiectasia |
| **ATR** | Ataxia Telangiectasia and Rad-3-related |
| **BBB** | Blood Brain Barrier |
| **BER** | Base Excision Repair |
| **Btk** | Bruton's tyrosine kinase |
| **CA (p24)** | Capsid |
| **CAD** | Constitutive Activation Domain |
| **CARDs** | Caspase Recruitment Domains |
| **CDKs** | Cyclin Dependent Kinases |
| **CIS** | Cytokine-inducible Src-homology 2 (SH2) protein |
| **CKI** | CDK Inhibitory proteins |
| **CNS** | Central Nervous System |
| **CRE** | cAMP Responsive Element |
| **CREB** | Cyclic-AMP Response Element Binding |
| **CREM** | Cyclic AMP Response Element Modulator |
| **CRLs** | Cullin-Ring type E3 ligases |
| **CRM1** | Chromosome Region Maintenance interacting protein 1 |
| **CSF** | Cerebrospinal Fluid |
| **CTL** | Cytotoxic T-lymphocyte |
| **CYLD** | Cylindromatosis protein |

| | |
|---|---|
| **DBD** | DNA Binding Domain |
| **DCs** | Dendritic Cells |
| **DISC** | Death Inducible Signaling Complex |
| **DNA-PK** | DNA Dependent Protein Kinase complex |
| **DSBR** | Double Strand Break repair |
| **dsDNA** | Double Stranded DNA |
| **DUB** | Deubiquitinase |
| **DUBA** | Deubiquitinating enzyme A |
| **E2F** | Elongation Factor 2 |
| **ECMV** | Equine Cytomegalovirus |
| **EIA** | Enzyme Immuno Assay |
| **eIFs** | Eukaryotic Initiation Factors |
| **EMCV** | Encephalomyocaritis Virus |
| **Env** | Envelope |
| **ER** | Endoplasmic Reticulum |
| **FADD** | FAS Associated via Death Domain |
| **FAK** | Focal Adhesion Kinase |
| **GM-CSF** | Granulocyte Macrophage Colony Stimulating Factor |
| **gpG** | Glycoprotein G |
| **HAM** | HTLV-1-Associated Myelopathy |
| **HBZ** | HTLV-1 bZIP factor |
| **HCV** | Hepatitis C virus |
| **HDAC** | Histone Deacetylase |
| **HIF-1α** | Hypoxia Inducible Factor-1α |
| **HIV** | Human Immunodeficiency Virus |
| **HPV** | Human Papilloma Virus |
| **HSPGs** | Heparan Sulfate Proteoglycans |
| **HSV** | Herpes Simplex Virus |
| **HTLV-1** | Human T-Cell Leukemia Virus-I |
| **IκBs** | IkappaB |

| | |
|---|---|
| **IAD** | IRF Association Domain |
| **IAP** | Inhibitor of Apoptosis |
| **ID** | Inhibitory Domain |
| **IFNAR** | Interferon Alpha Receptor |
| **IFNGR** | Interferon Gamma Receptor |
| **IFN-α** | Interferon Alpha |
| **IKK** | IκB kinase complex |
| **IKKε** | Inducible IκB kinase |
| **IL-6** | Interleukin-6 |
| **IN** | Integrase |
| **IRAK** | Interleukin-1 Receptor (IL-1R)-Associated Kinase |
| **IRFs** | Interferon Regulatory Factors |
| **IRS** | Insulin Receptor Substrate |
| **ISGF3** | Interferon Stimulated Gene Factor 3 |
| **ISGs** | Interferon Stimulated Genes |
| **ISRE** | Interferon Stimulated Response Element |
| **IVIG** | Intravenous Gammaglobulin |
| **JAB** | JAK binding protein |
| **JAK1** | Janus Activated Kinase 1 |
| **JNK** | c-Jun N-terminal kinase |
| **Kb** | Kilobase |
| **KIR** | Kinase Inhibitory Region |
| **L** | Large polymerase protein |
| **LDH** | Lactate Dehydrogenase |
| **LPS** | Lipopolysaccharide |
| **LRR** | Leucine Reach Repeats |
| **LTR** | Long Terminal Repeat |
| **LZD** | Leucine Zipper Domain |
| **LZR** | Leucine Zipper-like Region |
| **MA (p19)** | Matrix layer |

| | |
|---|---|
| **MAD1** | Mitotic Arrest Defective protein 1 |
| **MAL** | MyD88 Adaptor-Like molecule |
| **MAPKs** | Mitogen-Activated Protein Kinases |
| **MAVS** | Mitochondrial Antiviral Signaling Adapter |
| **MCMV** | Murine Cytomegalovirus |
| **MDA-5** | Melanoma Differentiation-Associated gene 5 |
| **mDCs** | Myeloids DCs |
| **MEFs** | Mouse Embryonic Fibroblasts |
| **MH2** | Mad-homology 2 |
| **MHC** | Major Histocompability Complex |
| **MMR** | Mismatch Repair |
| **MMTV** | Mouse Mammary Tumor Virus |
| **mRNA** | Messenger RNA |
| **MV** | Measles Virus |
| **MyD88** | Myeloid Differentiation primary response gene 88 |
| **N** | Nucleoprotein |
| **NC (p15)** | Nucleocapsid protein |
| **NER** | Nucleotide Excision Repair |
| **NES** | Nuclear Export Sequence |
| **NF-κB** | Nuclear Factor Kappa B |
| **NHEJ** | Non Homologous End Joining |
| **NKs** | Natural Killer |
| **NLS** | Nuclear Localization Sequence |
| **NRP-1** | Neuropilin-1 |
| **OAS** | 2'-5'- oligoadenylate synthetase |
| **ORFs** | Open Reading Frames |
| **P** | Phosphoprotein |
| **P** | PDZ-binding domain |
| **P/CAF** | p300/CBP-associated factor |
| **PA** | Particle Agglutination |

| | |
|---|---|
| **PABP** | Poly(A) tail Binding Protein |
| **PAMPS** | Pathogen Associated Molecular Patterns |
| **PCNA** | Proliferating Cell Nuclear Antigen |
| **PCR** | Polymerase Chain Reaction |
| **pDCs** | Plasmacytoid DCs |
| **PI3K** | Phosphatidylinositol 3 Kinase |
| **PIAS** | Protein Inhibitors of Activated STATs |
| **Pin1** | Peptidyl-prolyl-isomerase |
| **PKR** | dsRNA-dependent Protein Kinase |
| **PKRA** | RNA Activated Protein kinase |
| **PLP2** | Papain-like protease domain |
| **PMA** | Phorbol Myristate Acetate |
| **poly(I:C)** | Polyinosinic-polycytidylic acid |
| **PR** | Protease |
| **PRRs** | Pattern Recognition Receptors |
| **PVL** | Proviral Load |
| **R** | Repeated |
| **Rb** | Retinoblastoma |
| **RBCK1** | RBCC Protein interacting with PKC1 |
| **RD** | Regulatory Domain |
| **Reovirus** | Respiratory Enteric Orphan virus |
| **RIG-I** | Retinoic acid Inducible Gene I |
| **RIP-1** | Receptor Interacting Protein 1 |
| **RLRs** | Retinoic acid-inducible gene I (RIG-I)-like receptors |
| **RSV** | Respiratory Syncytial Virus |
| **RT** | Reverse Transcriptase |
| **RxRE** | Rex-responsive elements |
| **S** | Serine |
| **SHPs** | SH2-containing protein tyrosine phosphatases |
| **SOCS** | Suppressor of Cytokine Signaling |

| | |
|---|---|
| **SRD** | Signal response domain |
| **SRF** | Serum Response Factor |
| **SSI** | STAT-induced STAT inhibitor |
| **ssRNA** | Single-Stranded RNA |
| **STAT** | Signal Transducer Activator of Transcription |
| **STLV** | Simian T-Lymphotropic Virus |
| **SU** | Surface Unit |
| **T** | Threonine |
| **TANK** | TRAF Family member Associated NF-κB activator |
| **TBK1** | (TANK)-Binding Kinase 1 |
| **Th** | T-helper cells |
| **TICAM2** | TIR-domain Containing Adaptor Molecule 2 |
| **TIR** | Toll/ (IL-1R) homology |
| **TLRs** | Toll-Like Receptors |
| **TM** | Transmembrane subunit |
| **TNF-α** | Tumor Necrosis Factor Alpha |
| **TRAF** | TNF-Receptor-Associated Factor |
| **TRAIL** | TNF-Related Apoptosis Inducing Ligand |
| **TRAM** | TRIF-Related Adaptor Molecule |
| **TRE** | Tax Responsive Element |
| **TRIF** | TIR-containing adaptor Inducing interferon β |
| **TRIM25** | Tripartite Motif 25 |
| **TSP** | Tropical Spastic Paraparesis |
| **TYK2** | Tyrosine kinase 2 |
| **U3** | Unique 3' |
| **U5** | Unique 5' |
| **VAD** | Virus-Activated Domain |
| **VEGF** | Vascular Endothelial Growth Factor |
| **VHL** | Von-Hippel Lidau |
| **VPA** | Valproic acid |

| | |
|---|---|
| **VRE** | Virus Responsive Element |
| **VSV** | Vesicular Stomatitis Virus |
| **VV** | Vaccinia Virus |
| **WB** | Western Blot |
| **WNV** | West Nile Virus |
| **XBP1** | X-box-binding protein 1 |
| **ZN** | Zinc-finger domain |

*"Few things are impossible to diligence and skill. Great works are performed not by strength, but perseverance."*

**Samuel Johnson (1709 – 1784)**

# ~ CHAPTER I ~

## *INTRODUCTION*

# 1. Human T-Cell Leukemia Virus-I (HTLV-1)

HTLV-1 is the first identified human retrovirus and was discovered by Dr. Robert Gallo (National Institute of Health, U.S.A) in 1980 [1]. It was isolated from the peripheral blood cells of a patient with a lymphoproliferative disease initially diagnosed as cutaneous T-cell lymphoma in the leukemic phase. Integrated HTLV-1 proviral sequences were detected in the malignant T-cells, but not in normal cells of the affected individual. This condition was later referred as Adult T-cell leukemia/Lymphoma (ATLL), a severe T-cell lymphoproliferation originally described by Dr. Takatsuki in 1977 in Japan [2]. Five years later, epidemiological studies linked HTLV-1 infection with a slowly progressive neurologic disorder termed HTLV-1 associated Myelopathy (HAM) in Japan [3] and Tropical Spastic Paraparesis (TSP) in Martinique (French West Indies) [4]. The two syndromes were later demonstrated to be identical and therefore, a single name - HAM/TSP - was adopted [5-6]. The list of other autoimmune and reactive disorders associated with HTLV-1 infection has been extended over the years to include HTLV-1-associated arthropathy, HTLV-1-associated uveitis, infective dermatitis, cutaneous T-cell lymphoma and several other less characterized diseases [7-9].

## 1.1. HTLV-1 gene expression

### *1.1.1. Structure*

HTLV-1 is a type C virus belonging to the family of Retroviridae and classified into the genus of Deltaretrovirus that includes HTLV-2, HTLV-3, HTLV-4, bovine leukemia virus (BLV) and simian T lymphotropic viruses (STLV). HTLV-1 is a round-shaped virus of approximately 100-140 nm diameter and contains two identical positive single-stranded RNA genomes associated with the nucleocapsid protein (p15/NC) (**Figure 1**) [10]. The mature virion is surrounded by an envelope, which is constituted of a lipid bilayer from the host cell membrane and viral glycoproteins spikes (gp46 and

gp21). The inner envelope contains the matrix layer (p19/MA), which is important for the organization of viral components at the inner cell membrane. The icosahedral capsid (p24/CA) protects: the viral RNA genome, the $tRNA^{Pro}$ of host cell (that serves as a primer for the initiation of reverse transcription), the functional protease (PR), the reverse transcriptase (RT), and the integrase (IN) which are all organized together with the nucleocapsid (NC) into a ribonucleoprotein complex [11].

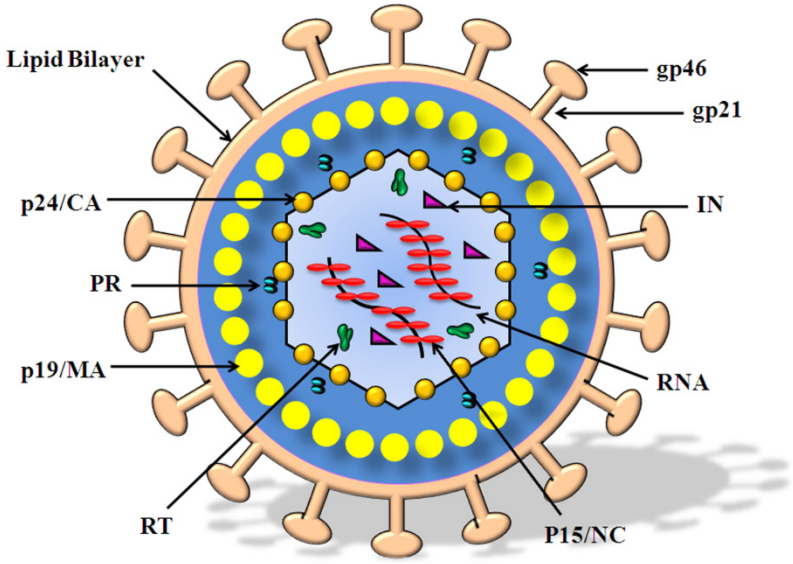



**Figure 1. HTLV-1 virus structure.** Schematic cross-section through a mature HTLV-1 virion depicting its structure and composition. HTLV-1 virion contains two identical single-stranded RNAs associated with nucleocapsid protein (p15/NC). The capsid (p24/CA) protein surrounds the RNAs and NC complex as well as the integrase (IN), reverse transcriptase (RT), tRNA proline ($tRNA^{pro}$) and the protease (PR). The whole structure is covered by the envelope (env), which is constituted of lipid bilayer of cellular origin and of viral glycoproteins spikes (gp46 and gp21).

### *1.1.2. HTLV-1 life cycle*

Although HTLV-1 can infect a large panel of human and non-human cells *in vitro*, studies in *in vivo* models demonstrate a preferential tropism for CD4+ and CD8+ T-

lymphocytes (**Figure 2**) [12-16]. The life cycle of HTLV-1 is very similar to that of other retroviruses and can be organized in two steps (**Figure 3**). First, HTLV-1 viral particles attach to the uninfected cells through the interaction with the viral surface glycoprotein gp46 and a host cell surface receptor complex containing the ubiquitous glucose-transporter GLUT-1, heparan sulfate proteoglycans (HSPGs), and neuropilin-1 (NRP-1). This leads to the fusion of the envelope of the virion and the membrane of the target cell [17-19]. Immediately after penetration into the cell, the virus is uncoated and the viral core is released into the cytoplasm. HTLV-1 RNA is reverse transcribed by the viral RT and double-stranded DNA (dsDNA) is generated using the $tRNA^{pro}$ as a primer. The synthesis of the first strand DNA starts when still packaged in the viral core. Then, the parental RNA is removed by RNAse-H activity allowing the complementary DNA strand to be produced. The linear viral dsDNA migrates into the nucleus and randomly integrates into the host genome through the enzymatic activity of the viral integrase [20]. The integrated HTLV-1 genome is referred to as a provirus and becomes a stable part of the target cell's genome. All these processes are realized in the absence of *de novo* viral gene expression owing to the viral structural proteins and the enzymatic proteins packaged in the infecting virion.

During the second step of HTLV-1 life cycle, the integrated provirus takes advantage of the host cellular machinery by using the host RNA polymerase II to initiate the transcription of primary genomic viral RNA. Viral transcription also results in the generation and accumulation of viral mRNAs that are processed, spliced, transported into the cytoplasm and translated into distinct viral proteins (**Figure 4**). Next, the viral core is assembled and packaged along with the genomic RNA at the plasma membrane. The encapsidation process requires a sequence called the psi element (ψ) [21]. This element is spliced out of all the other viral transcripts in order to ensure that the unspliced genomic RNA is the only one to be packaged. Finally, the progeny virus buds and is released from the cytoplasmic membrane. Recently, Pais-Correia *et al.* reported that HTLV-1-infected T-lymphocytes transiently store viral particles as carbohydrate-rich extracellular structures that are held together and attached to the cell surface by virally-induced

extracellular matrix components, including collagen and agrin, and cellular linker proteins, such as tetherin and galectin-3. Extracellular viral structures adhere to other cells upon cell-to-cell contact, allowing virus spread and new infection of target cells [22]. This newly immature virion requires the activity of the viral protease to cleave the structural proteins and maturate.

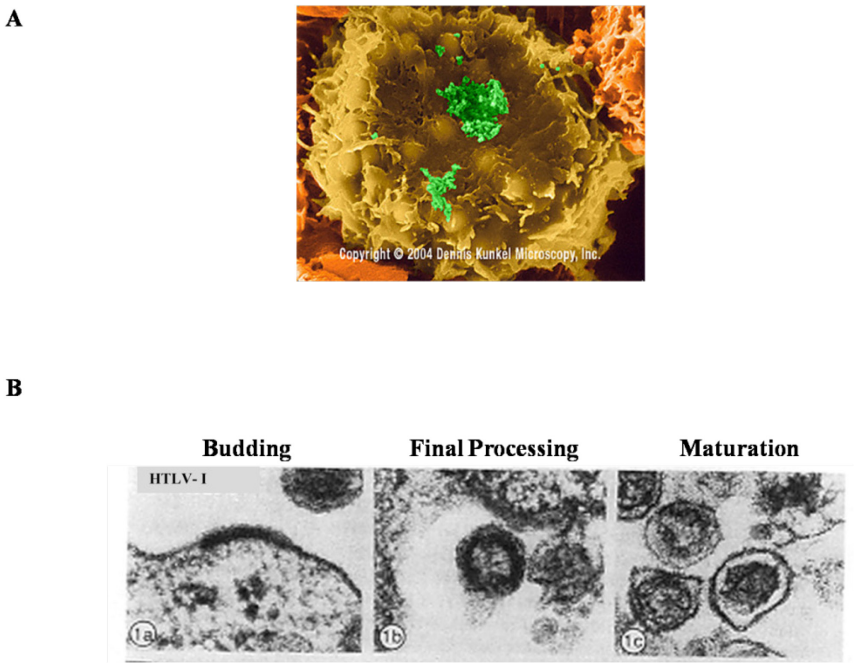



**Figure 2. HTLV-1 virion as seen by electron microscopy. (A)** HTLV-1 virions (green) attacking a CD4+ T-cell (brown) (Image Denis Kunkel microscopy). **(B)** HTLV-1 budding and maturing from an infected CD4+ T-lymphocyte (Cann Fields Virology, 1996).

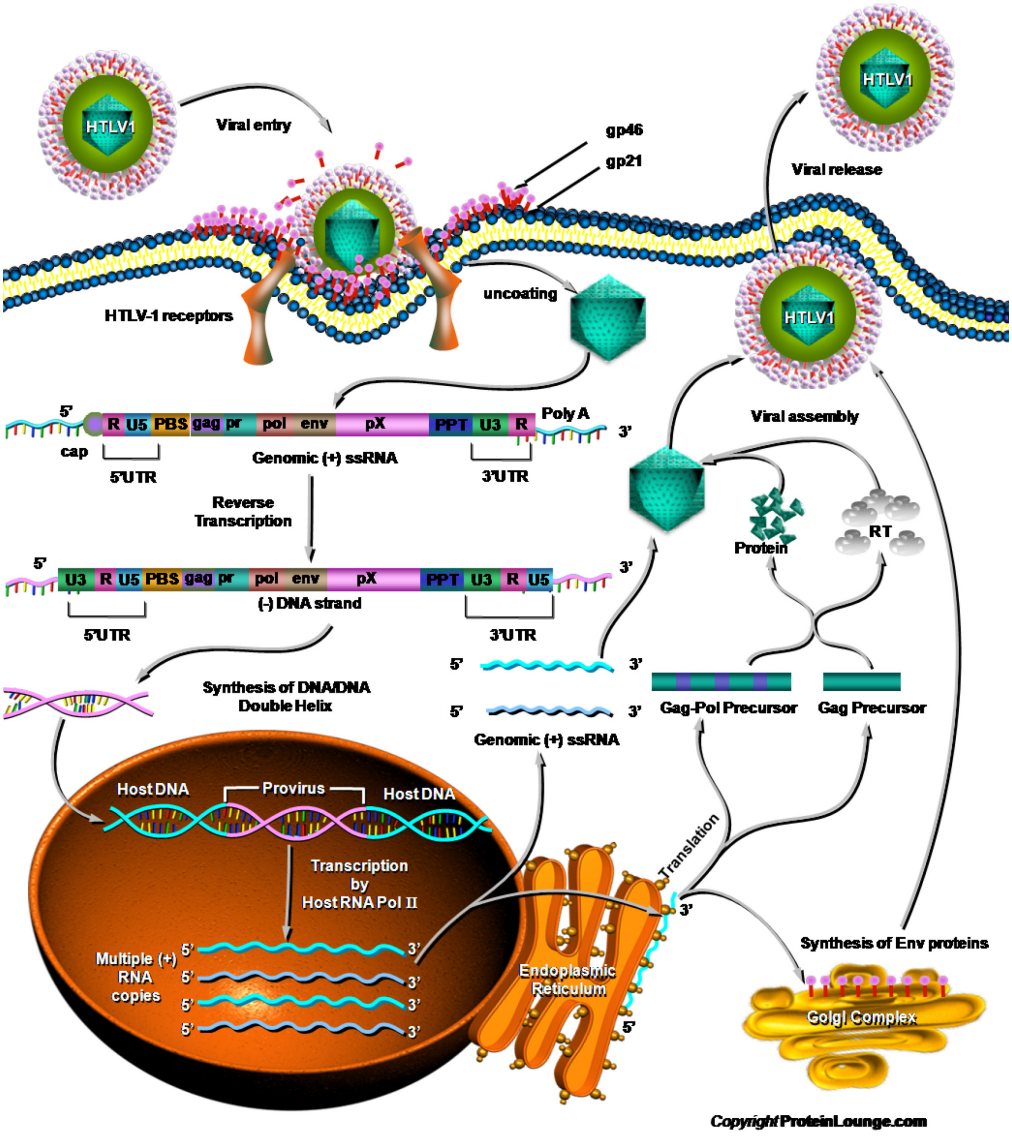



**Figure 3. HTLV-1 life cycle.** The HTLV-1 life cycle is described by the following events: receptor binding, fusion with cell membrane, uncoating of viral core, nuclear transport of viral core, reverse transcription of viral RNA into DNA and integration of proviral DNA into cellular DNA, transcription and translation of viral proteins, assembly and budding of immature virions from the cellular membrane and finally maturation. UTR (untranslated region), U (unique), gag (group antigen), pr (protease), pol (polymerase), env (envelope), Reverse Transciptase (RT), gp (glycoprotein), PPT (Polypurine Tract), polyA (polyadenylated), cap (capped), PBS (Primer Binding Site).

### *1.1.3. Genomic Organization*

The 9-kilobase (kb) genome of HTLV-1 is a positive single-stranded RNA capped at the 5' end and containing a ~200 nucleotides poly(A) tail at its 3' end. It contains elements common to all retroviruses such as *gag*, *pro*, *pol*, *env* (**Figure 4**). In addition to the structural proteins and retroviral enzymes, this complex retrovirus codes for a region at the 3' end of its genome, called pX. The elements of this specific region (Tax, Rex, p12I, p13II, p30II and HBZ) play important roles in the deregulation of cellular functions. The proviral genome is flanked at each end by a long terminal repeat (LTR), a hallmark of retroviral genomic structure which is essential to viral reverse transcription, integration and transcription. Each LTR contains a U3 (unique 3'), R (repeated) and U5 (unique 5') region. The U3 region of HTLV-1 is important in regulating virus transcription. It is composed of the TATA box required for viral gene expression, a sequence that promotes termination, polyadenylation of the messenger RNA (mRNA). The U3 region also contains several transcription factor-binding sites - the Tax responsive elements (TRE) - essential for Tax-mediated transactivation of the LTR. The R region of the LTR contains several Rex-responsive elements (RxRE) necessary for Rex-mediated nuclear export of viral transcripts. The specific functions of Tax and Rex will be discussed further in the following sections. Together the R and U5 regions of LTR provide the leader sequence for HTLV-1 mRNAs.

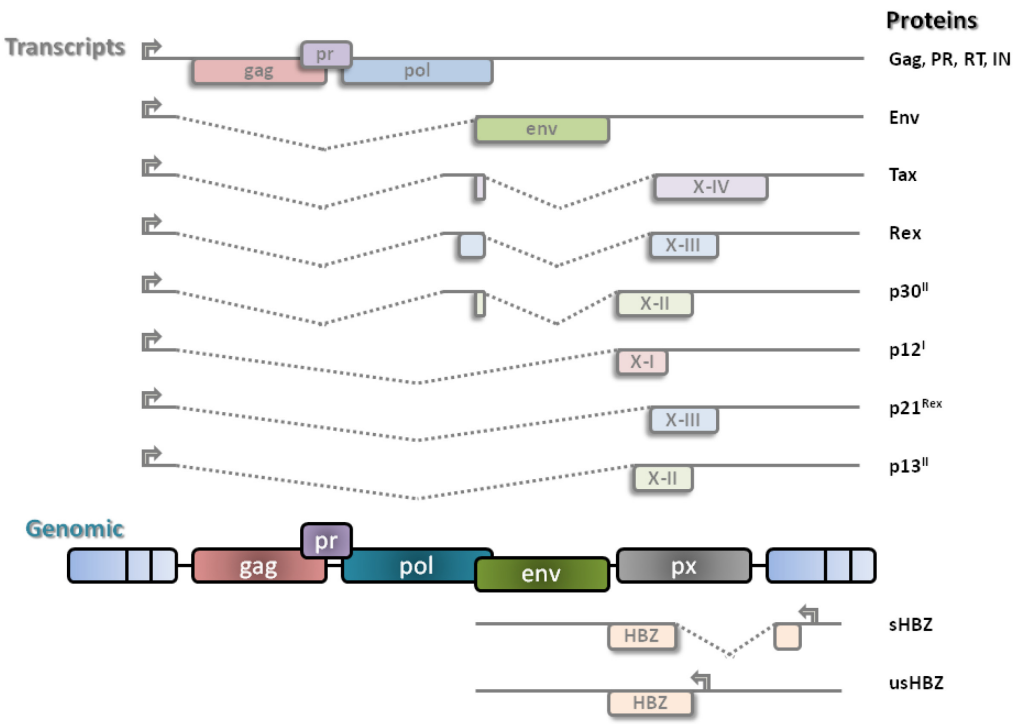



**Figure 4. HTLV-1 genome and mRNAs coding for viral proteins.** HTLV-1 encodes three classes of mRNA required for complete synthesis of viral structure and regulatory proteins: 1) unspliced mRNA coding for the capsid (p24/CA), nucleocapsid (p15/NC), matrix (p19/MA), reverse transcriptase (RT), integrase (IN) and protease (PR), as well as a mRNA coding for HBZ (usHBZ or unspliced HBZ); 2) single-spliced mRNAs coding for the envelope, $p12^{I}$, $p21^{rex}$, $p13^{II}$ and HBZ (sHBZ); and 3) double-spliced mRNAs coding for Tax, Rex and $p30^{II}$ . The region pX of the genome - located between the *env* gene and the′ 3 -LTR - contains four partially overlapping reading frames (ORFs) encoding accessory proteins ($p12^{I}$, $p13^{II}$, $p30^{II}$), the post-transcriptional regulator Rex (ORF X-III) and the Tax transactivator (ORF X-IV). In addition, HBZ is encoded from the 3′ LTR and pX on the complementary strand of the genome. Adapted from [23].

### *1.1.4. Structural proteins*

HTLV-1 makes maximal use of its genome by employing ribosomal frame shifting and alternative splicing mechanisms to generate several viral proteins from the same coding region. Ribosomal frame shifting is essential to produce structural, enzymatic and precursor proteins. The main function of the *gag* gene is to promote the assembly and release of virus particles. It is translated from an unspliced full-length mRNA as a single precursor polyprotein p55 (Gag). Gag precursor is targeted to the inner plasma membrane via a biochemical process called myristylation at the N-terminal end of the protein. During viral maturation, the gag precursor gets proteolytically cleaved by the viral protease into three products: 19 kD (matrix), 24 kD (capsid) and 15 kD (nucleocapsid) proteins. The HTLV-1 protease (PR) is encoded by an open reading frame that overlaps the *gag-pol* genes and is made by a frame shift that occurs during the synthesis of Gag. The protease is synthesized in an immature form that undergoes self-cleavage to generate the active form. The protease is required for proper maturation of HTLV-1 since it is responsible for the processing of the precursor Gag and Pol polypeptides. A second ribosomal frameshift event is required to produce the polymerase. The *pol* gene encodes several enzymes necessary for the synthesis of the viral DNA (RT), the degradation of the parental RNA (RNase H), and the integration of the generated dsDNA into the genome of the host cell (IN). The *env* gene product of HTLV-1 is encoded from a single spliced mRNA. This protein is synthesized as a glycosylated polyprotein of 61 kD, subsequently cleaved by cellular protease to generate the surface unit (SU/gp46) and the transmembrane unit (TM/gp21). The SU protein binds to the host cell receptors, while the fusion of cellular and viral membranes is caused by the TM protein.

### *1.1.5. Regulatory proteins*

The pX region of the HTLV-1 genome can be divided in four open reading frames (ORFs): X-I, X-II, X-III, X-IV and their expression is controlled by differential splicing of the single genomic RNA. Two regulatory proteins important for the life cycle - Tax

and Rex (ORF X-IV and X-III, respectively) - are encoded in the distal portion of the pX region and translated from double-spliced subgenomic mRNA. Recently, a novel ORF, encoding for the basic leucine-zipper factor HBZ (HTLV-1 bZIP factor) has been identified on the complementary strand of the pX region (**Figure 4**) [24].

#### *1.1.5.1. Rex, RNA processing and transport*

Rex is encoded by the X-III ORF and is a post-transcriptional regulator of viral gene expression. Rex (27 kD) is a RNA-binding protein that is important for transport of viral mRNAs. Rex facilitates the migration of unspliced RNA (*gag/pol/pro*) and singly-spliced RNA (*env*) from nucleus to cytoplasm [25-26]. Thus, Rex favours the expression of structural and enzymatic proteins as well as the production of viral particles. HTLV-1 Rex is necessary for viral spread and persistence *in vivo*, but is not required for cellular immortalization *in vitro* [27]. In contrast, HTLV-1 p30 protein, a nuclear protein, binds to the double spliced mRNA encoding Tax and Rex proteins and sequesters them in the nucleus. This leads to inhibition of viral gene expression, promoting viral latency and persistence *in vivo* [28-29]. Rex expression increases the stability of unspliced RNAs in T-cells [30].

Rex possesses multiple domains important for its function, including a nuclear localization sequence (NLS), nuclear export sequence (NES), and multimerization domain. Rex also contains a highly basic N-terminal RNA-binding domain located within amino acids (aa) 1-19 [31]. This domain functions as an NLS and is required for the transport of unspliced viral mRNAs to the cytoplasm [32-34]. Rex-mediated RNA transport occurs through the formation of an export complex with the chromosome region maintenance interacting protein 1 (CRM1)/(or exportin), a member of the karyopherin family of nuclear transport receptors [35-37]. The NES is required for the migration of Rex between the nucleus and cytoplasm. Rex function is mediated through direct interaction with a cis-acting target sequence called the Rex responsive element (RxRE) within the U3 and R regions of the 3' LTR [38]. RxRE forms a stable and complex secondary structure, consisting of four stem loops and a long stem structure [25, 39-40].

Because RxRE is present in all mRNAs (unspliced, single and double spliced mRNAs), it has been proposed that the stem-loop structure is also critical for the 3' processing of viral transcripts and appropriate polyadenylation of viral RNAs. The viral polyadenylation signal is separated from the 3' cleavage site by the RxRE sequence, a distance that does not allow processing of the 3' ends of viral RNAs. Formation of the correct RxRE secondary structure brings the two elements into close proximity with each other, thereby allowing correct polyadenylation [41-42].

The activity of Rex is affected through phosphorylation [43-44]. Treatment of HTLV-1 infected cells with protein kinase C inhibitor results in accumulation of unspliced mRNA and decreased gag protein synthesis [45]. A truncated 21 kd form of Rex ($p21^{Rex}$) has been detected in HTLV-1 infected cell lines and because $p21^{Rex}$ has a truncation in the N-terminal NLS domain, it inhibits the shuttling of the full-length form of Rex protein when over-expressed [46]. However, the function of $p21^{Rex}$ in physiologic HTLV-1 replication and pathogenesis is still unknown.

#### *1.1.5.2. Antisense encoded protein: HBZ*

Studies have recently demonstrated the involvement of another factor - HBZ - in the regulation of viral transcription [24, 47-48]. HBZ is located on the minus strand of the HTLV-1 genome and is encoded by an ORF located between the *env* and *Tax/Rex* genes (**Figure 4**) [49]. The *HBZ* gene is transcribed from a functional promoter positioned in the *3′* LTR [49-50]. Multiple HBZ mRNA transcripts have been identified in HTLV-1 positive cell lines [51]. These transcripts share about 95% aa sequence and differ only at their N-terminus. Spliced HBZ is the most abundant form of HBZ expressed in HTLV-1-infected T-cell lines and corresponds to the 206 aa long isoform produced from the alternative splice variant. Spliced HBZ is the only HBZ protein detectable by molecular approaches [50].

HBZ contains an N-terminal transcriptional activation domain (AD), a central domain involved in its nuclear localization and a C-terminal bZIP domain (LZD) (**Figure**

**5**) [52]. HBZ may play a role in HTLV-1 biology and ATL development by counteracting the effects of Tax-mediated inhibition and/or activation of transcription of cellular genes (reviewed in [53]. For example, HBZ inhibits the classical NF-κB pathway by interacting with p65, thereby preventing its DNA binding or by increasing the expression of PDLIM2 - the E3 ubiquitin ligase of p65 - leading to ubiquitin-mediated proteasomal degradation of p65 [54]. In addition, HBZ can also activate transcription mediated by JunD, a member of the AP-1 family. HBZ forms heterodimers with JunD through its bZIP domain, and the AD of HBZ is necessary for this activation [55]. HBZ/JunD dimers associate with the transcription factor Sp1 and activate transcription of the human telomerase catalytic subunit (hTERT) [56].

Recently, Arnold *et al.* investigated the role of HBZ on cellular immortalization *in vitro* and viral infectivity and persistence *in vivo* [47]. Deletion in the C-terminal leucine zipper region of HBZ from an HTLV-1 infectious proviral clone had no effect on the ability of the virus to replicate and immortalize lymphocytes in cell culture. However, rabbits inoculated with HBZ mutant cells, displayed a lower proviral load and an attenuated antibody response against viral proteins when compared to wild-type HTLV-1-infected rabbits [24, 47-48]. This suggests that HBZ plays an important role in the establishment of the chronic phase of HTLV-1 infection *in vivo*.

HBZ acts as a negative regulator of Tax-mediated viral transactivation and therefore acts as a repressor of HTLV-1 transcription. Specifically, HBZ forms heterodimers with several transcription factors such as CREB, CREB-2, CREM, and ATF-1. Thus, these transcription factors are no longer able to bind to TxRE, leading to the inability of Tax to trans-activate viral transcription [24, 48, 57]. HBZ also inhibits Tax-independent viral transcription by interacting with other transcription factors such as c-Jun and JunB, which are involved in basal HTLV-1 transcription [58-60]. These different mechanisms behind the negative regulation of viral transcription may help infected cells to escape the cytotoxic T-lymphocyte (CTL) response by inducing low levels of viral proteins in infected cells. Methylation analysis of LTRs from ATL cells

showed that the 5' LTR of the provirus is often hypermethylated or deleted whereas the 3' LTR, which contains the HBZ promoter, is hypomethylated and unaffected by epigenetic changes [61]. Recent studies have effectively shown that the *HBZ* gene is always expressed in ATL cells and up-regulates the cellular transcription factor E2F1 and subsequently expression of many cellular E2F1-responsive genes, including *PCNA*, *CDC2*, *CDC6* which are involved in cell proliferation [57, 62-63]. These studies provide further evidence that HBZ could be critical for the regulation of cell proliferation and involved in the onset or progression of ATL.

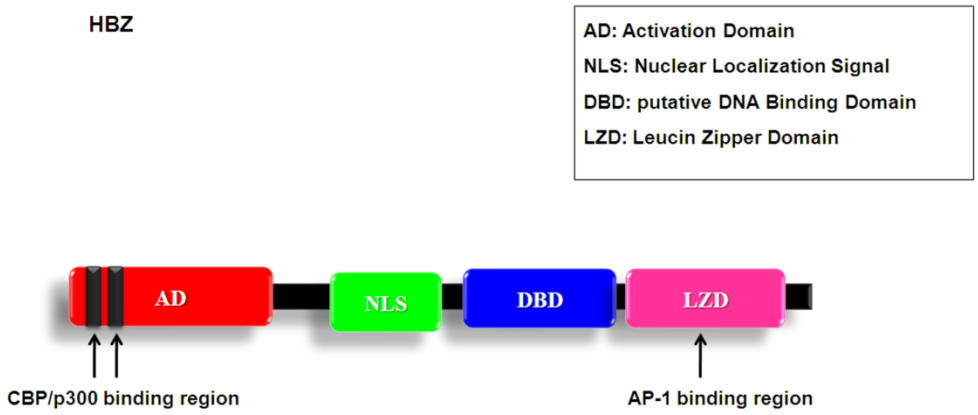



**Figure 5. Molecular organization of the HBZ protein.** HBZ is composed of an N-terminal activation domain, a nuclear localization signal, a DNA-binding domain, and a bZIP domain.

### *1.1.5.3. Tax transcriptional regulation*

Tax is a 40 kd nuclear protein encoded by the X-IV ORF and is known as a viral transcriptional activator since it can regulate viral gene transcription through its interaction with the 5' LTR of HTLV-1 genome. The predominant cellular pool of Tax is found in the nucleus but due to its pleiotropic functions, it shuttles between the nucleus and the cytoplasm [64]. Post-translational modifications such as sumoylation and ubiquitination influence the subcellular localization of Tax and its interaction with cellular proteins. The sumoylated form of Tax localizes in the nucleus while ubiquitinated form of Tax is found in the cytoplasm. Tax is also phosphorylated on several serine and threonine residues and this modification differentiates the "active" and "inactive" forms of Tax [65-66].

To exert its pleiotropic functions, Tax interacts with multiple transcription factors and signaling molecules through its functional domains. The domains responsible for the different functions of Tax include a nuclear localization signal (NLS) [67-68], a nuclear

export signal (NES) [64, 69], a CREB interaction domain, p300/CBP binding domain, NF-κB binding domain [70-72], a zinc-finger domain (ZN) [73-74], a dimerization domain [75-76], a PDZ-binding domain (P) [77], a KlX-interacting domain [78], two leucine zipper-like region (LZR) [71], and an activation specific domain [79] (**Figure 6**). Other motifs in Tax, such as the SH3 domain, the LIM domain, and the coiled-coil structure, have been identified and may be important for protein-protein interactions [25].

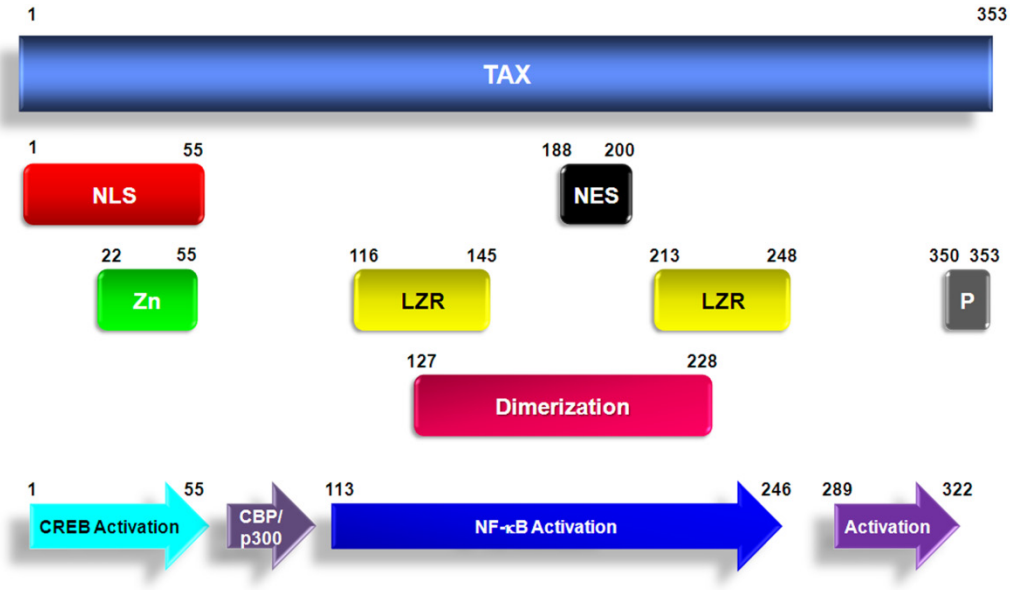



**Figure 6. Molecular organization of Tax protein.** NLS (nuclear localization sequence), NES (nuclear export sequence), LZR (leucine-zipper-like region), P (PDZ binding domain), Zn (zinc binding region). Adapted with permission from [74].

Tax is not a DNA-binding protein *per se,* but activates viral transcription through recruitment of cellular transcription factors to the viral LTR. The U3 region of the HTLV-1 LTR contains three highly conserved 21 base pair (bp) repeat elements called the Tax-responsive elements (TRE), which are important for Tax-mediated transcriptional activation [80-81]. Each TRE has three regions designated A, B, C containing 13 nucleotides of the 21-bp repeat. The B region of the TRE has a conserved 5-nucleotides core sequence TGACG(T/A)(C/G)(T/A) identical to a consensus of the cAMP responsive element (CRE) [82]. Tax brings the CRE-binding/activating transcription factors (CREB/ATF) - the cyclic AMP response element binding protein, CREB - to the HTLV-1 LTR and stabilizes the complex on the viral promoter through is N-terminus domain. In addition to CREB, a number of transcription factors belonging to the CREB/ATF family: the cyclic AMP response element modulator (CREM), the activating transcription factor family (ATF) and the X-box-binding protein 1 (XBP1) have been shown to bind both Tax and the 21-bp Tax-responsive element. These transcription factors also contain a leucine zipper (b-Zip) region important for homo- and heterodimerization. Tax enhances the dimerization of CREB and the binding of the Tax/CREB complex to the LTR serves as a high affinity binding site for the recruitment of transcriptional coactivators such as CREB binding protein (CBP)/p300 and p300/CBP-associated factor (P/CAF) [81, 83-84].These co- activators stabilize the complex on the promoter by inducing histone acetylation and chromatin remodeling [25, 85]. It has been reported that Tax can also bind directly the co activator CBP in the absence of CREB phosphorylation resulting in specific activation of the viral LTR [86]. Thus, Tax is able to recruit CREB/ATF factors in order to modulate the transcriptional activity of the LTR promoter.

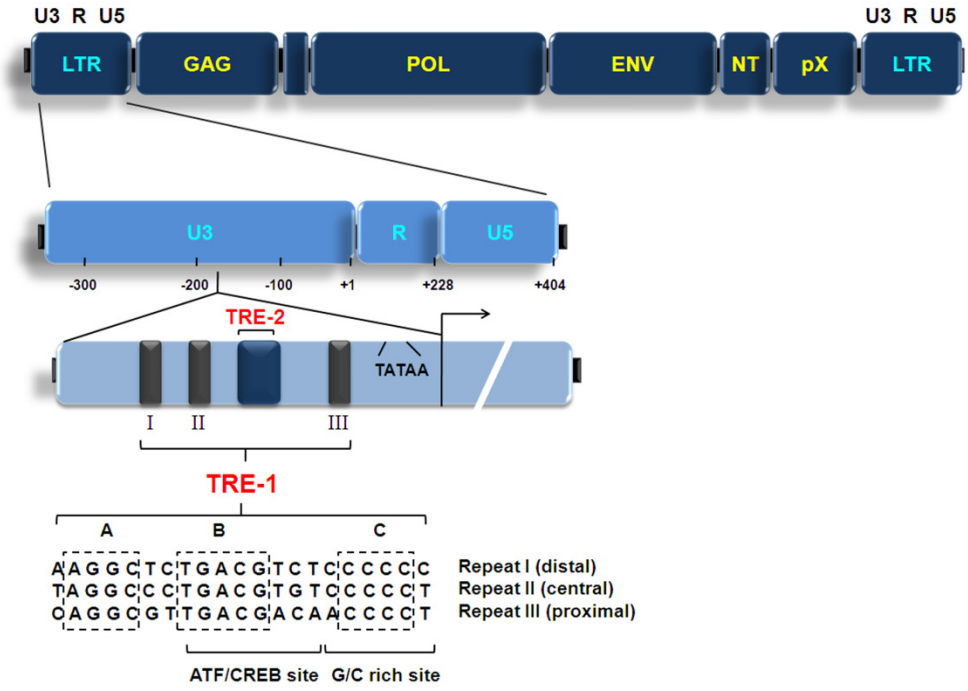



**Figure 7. HTLV-1 Long Terminal Repeats structure.** The viral LTRs are positioned at both ends of HTLV-1 genome. HTLV-1 transcription is controlled by the sequence within the U3 region of the 5'LTR. Three 21-bp Tax-responsive elements, which are collectively referred to as Tax-responsive element 1 (TRE-1) are found within the U3 region of the LTR. The TRE-1 contains several consensus binding sites for the host cellular transcription factors of the CREB/ATF family. Each 21-bp repeat of TRE-1 contains three completely conserved domains designated A, B and C from promoter distal end to proximal end. These three domains contain 13 nucleotides of the 21-bp repeat. A second Tax-responsive element 2 (TRE-2) is located between the promoter proximal repeat and the promoter central repeat and binds Myb (Myeloblastosis), Ets (E-twenty six), Sp1. Reproduced with permission from [81].

Although Tax regulates the transcription of HTLV-1 genes, another major function of this viral protein is to activate and repress the transcription of cellular genes through interaction with other transcription factors. In addition to CREB, the transcription factors interacting with Tax that are the most studied include: Serum Response Factor (SRF) and NF-kappaB (NF-κB) [59, 87]. These cellular factors containing CRE sites can be trans-activated by Tax in a similar fashion as within the viral LTR [88].

The NF-κB/Rel family is composed of five members, Rel A (p65), Rel B c-Rel, NF-κB1 (p50/p105) and NF-κB2 (p52/p100) organized in different homo and heterodimer NF-κB complexes. In resting cells, NF-κB complexes are sequestered in the cytoplasm by inhibitory proteins called IkappaB (IκB) such as p105, p100, IκBα, IκBβ and IκBγ. Activation of the NF-κB pathway by various stimuli (cytokines, virus infection, bacterial products and mitogens) leads to the phosphorylation of IκB inhibitors by the IκB kinase complex (IKK) followed by their subsequent ubiquitination and proteasomal degradation. Loss of IκBs allows NF-κB translocation into the nucleus where it simulates transcription of pro-inflammatory genes. The IKK complex is composed of three core subunits: the catalytic subunits IKKα and IKKβ and a regulatory subunit called the NF-κB essential modifier (NEMO or IKKγ). Most of the inducible NF-κB responses involve NF-κB p50-p65 heterodimers and is referred as the canonical pathway of NF-κB. IKKα mediated phosphorylation and processing of NF-κB2/p100 to p52/RelB dimers is a second NF-κB pathway and is called non-canonical pathway (reviewed in [89]).

HTLV-1-infected and Tax-expressing cells are characterized by a constitutively activated NF-κB pathway that induces the expression of several genes including interleukin-6 (IL-6), c-Myc and granulocyte-macrophage colony-stimulating factor (GM-CSF) [90-91]. Tax interacts directly with many components of the NF-κB pathway in

both the nucleus and cytoplasm (**Figure 8**) [91-92]. Several reports indicate that Tax stimulates NF-κB signaling in the cytoplasm by targeting the canonical and non-canonical NF-κB pathway through degradation of IκB or processing of p100, respectively [93-95]. Tax activates the canonical NF-κB pathway by directly interacting with the non-catalytic IKKγ/NEMO subunit of the IKK complex. The interaction between Tax and IKKγ activates IKKβ subunit and leads to phosphorylation and proteasomal degradation of IκBα and IκBβ. This results in the chronic induction of NF-κB that is observed in Tax-transformed cells. Knock-down of IKKγ in T-cell lines using anti-sense oligonucleotides abolishes Tax-mediated NF-κB activation [96]. Tax can also activate the non-canonical NF-κB pathway by interacting with IKKγ and p100. In fact, Tax binds to sequences within the Rel homology domain of p100. Tax-p100 interacts with the IKK complex through IKKγ [97] and activates IKKα resulting in the phosphorylation, ubiquitination, and processing of p100 to p52 subunit [98]. In the nucleus, Tax interacts with the NF-κB subunits p50, p65, c-Rel, NF-κB2 and stabilizes them on NF-κB responsive promoters by recruiting CBP/P300 [99-100]. Thus, Tax expression in HTLV-1-infected cells leads to the constitutive activation of the NF-κB pathway causing the deregulation of a myriad of cellular genes important for cell growth and signal transduction, such as cytokines and growth factors (IL-2, IL-6, IL-15, tumor necrosis factor alpha (TNF-α), GM-CSF), cytokine receptors (IL-2Rα, IL-15Rα), proto-oncogenes (c-Myc) and anti-apoptotic proteins (Bcl-xL).

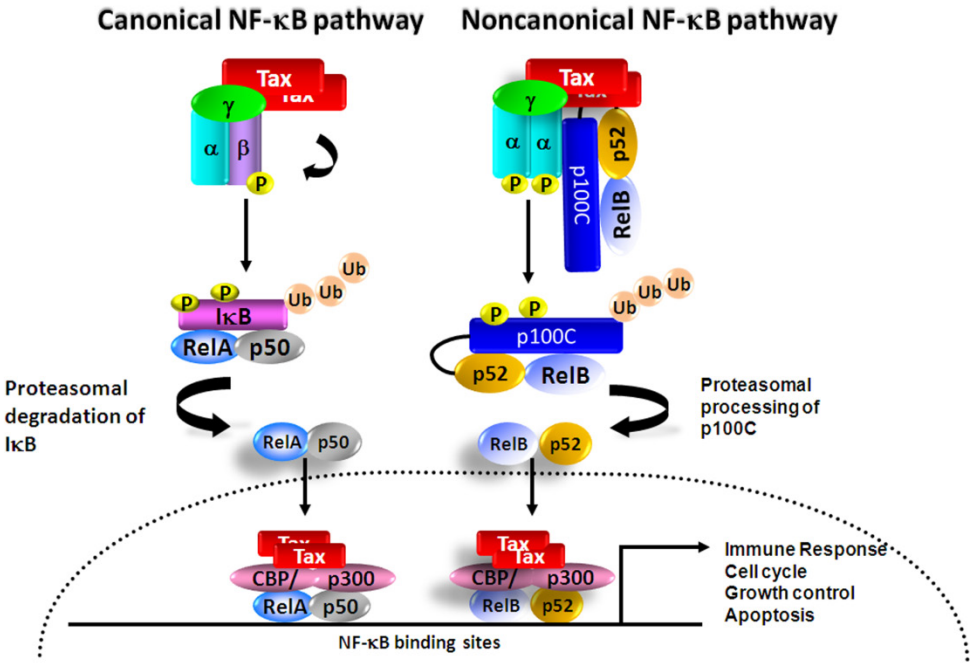



**Figure 8. HTLV-1 Tax interaction with the canonical and non-canonical NF-κB pathways.** Tax modulates NF-κB signaling pathway in both the nucleus and the cytoplasm. In the cytoplasm, Tax dimers associate with the non-catalytic IKK subunit NEMO, and facilitate Tax recruitment to the catalytic IKK subunits (α or β), resulting in the subsequent phosphorylation, followed by proteasomal degradation of IκB in the canonical pathway. Conversely, Tax can process the C-terminal inhibitory region (p100C) of p100 in the non-canonical pathways. In the nucleus, Tax binds to NF-κB subunits and recruits the transcriptional coactivators CBP/p300, leading to the synthesis of cytokines, genes involved in cell cycle regulation and apoptosis. Reproduced with permission from [91].

SRF is another cellular transcription factor targeted by Tax. SRF is a MADS box-binding protein and is classified as an immediate early gene, since its activation does not require *de novo* protein synthesis. Upon mitogenic stimulation the SRF is activated by phosphorylation. Tax takes advantage of this pathway by directly binding to SRF and the ternary complex factor (TCF) in a similar strategy to the one used for CREB activation. Thus, SRF-mediated transcriptional activation requires Tax interaction with CBP/P300 and P/CAF. Tax-mediated activation of SRF pathway results in the transcription of proto-oncogenes - c-Fos, c-Jun, JunB, JunD, and Fra-1 - which regulate expression of AP-1 responsive genes involved in evasion of apoptosis and cellular proliferation [101-102].

Tax can physically interact with many cellular proteins and thereby activate or repress their functions [25]. The downstream effects of Tax transcriptional activation of cellular genes and direct protein-protein interactions is deregulation of the cell cycle, accumulation of mutations due to interference with DNA repair, and evasion of apoptosis.

### 1.2. HTLV-1 induced transformation

One of the features of HTLV-1 is its ability to infect, immortalize and transform primary human CD4+/CD25+ T-cells *in vitro* and *in vivo*. As mentioned above, the incidence of ATL is low among HTLV-1 carriers, and those who develop the disease have a 20 to 30 year latency period from the time of infection to progression to ATL. This long latency period suggests that HTLV-1 infection of CD4+ T-cells initiates a multi-step oncogenic process. Tax appears to play a central role in both cellular and viral gene expression and is therefore essential for both viral replication and pathogenesis [103-104]. It has been reported that ectopic expression of Tax is sufficient to immortalize primary human T-lymphocytes [105-106]. Mice stably expressing Tax have been reported to

develop soft tissue tumors [107-108] or large granular lymphocytic leukemia [109]. The exact mechanism by which Tax contributes to the leukemic process remains unclear, but it has been proposed to involve several aspects of cellular deregulation leading to the accumulation of genetic mutations and uncontrolled lymphocyte growth.

### *1.2.1. Tax and cell cycle deregulation*

Cell proliferation is regulated by the cell cycle which can be divided into four phases: the $G_1$ phase (first gap phase), the DNA synthesis phase (S), the $G_2$ phase (second gap phase) and the Mitotic phase (M) for cell division. The $G_1$ phase is a preparatory phase during which cells produce the enzymes essential for DNA replication. In the S phase, chromosomes are duplicated, whereas in the $G_2$ phase cells prepare for the division which occurs during the M phase. Then, cells will either return to the $G_1$ phase or enter a quiescent state known as $G_0$ phase [110]. Cell cycle progression is tightly regulated by specific cyclins that are activated and phosphorylated by cyclin dependent kinases (CDKs). Each specific cyclin/CDK complex governs a specific phase of the cell cycle. Molecular checkpoints involving CDK inhibitory proteins (CKI) provide additional regulatory control by blocking cell cycle progression at specific phases. Phosphorylation of D- and E- type cyclins is required for cell cycle transition from G1 to S phase. The enzymatically active cyclin-D/CDK complex hyperphosphorylates retinoblastoma (Rb) protein which in turn releases the elongation factor E2F, facilitating cell cycle progression and cellular proliferation **(Figure 9)**. The A-type cyclins accumulate and functions during S phase, whereas the B-types cyclins are active during mitosis [111].

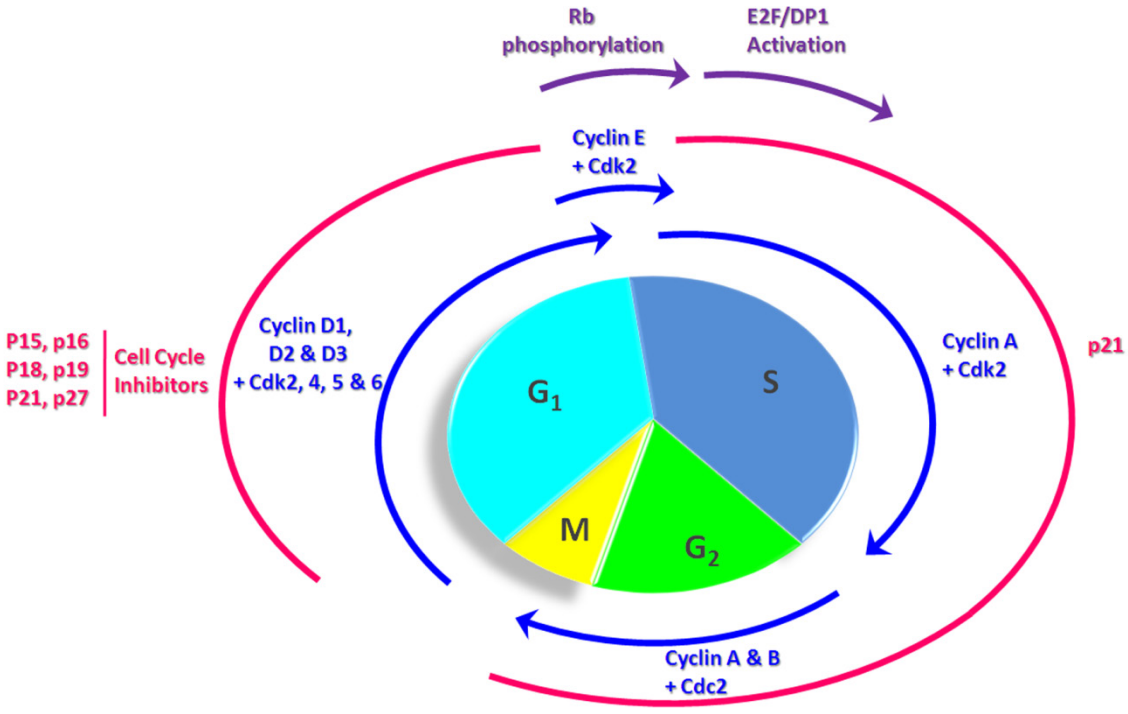



**Figure 9. Cell cycle control.** The cell cycle is composed of 5 distinct phases: $G_0$ (the quiescent phase), $G_1$ (the first gap phase), S (the DNA synthesis phase), $G_2$ (the second gap phase), M (the mitosis phase). Movement through the cell cycle is driven by the activities of cyclins and cyclin-dependent kinases (Cdks), which phosphorylates retinoblastoma (RB)-family "pocket proteins" and releases the transcription factor E2F, thereby suppressing their growth-inhibitory functions and allowing cell-cycle progression. Transition from $G_1$ phase to $G_0$ phase is facilitated by the D-types cyclins (D1, D2, D3), which form active complexes with Cdk4 or Cdk6. Transition from $G_1$ phase to S phase requires the participation of E-types cyclins in combination with Cdk2. Cyclin-D/Cdk4 and cyclin-D/Cdk6 complexes are inactive when associated with the Cdk-inhibitory proteins (CdkIs) - p21 or p27.

Tax binds to several regulatory proteins of the cell cycle resulting in accelerated progression past crucial checkpoints (**Figure 10**). During the $G_1$ phase of the cell cycle, Tax transcriptionally up-regulates the expression of several cyclins and Cdks including cyclin D2, cyclin E and Cdk2, Cdk4, Cdk6 [112-115]. Tax interacts directly with cyclins-D2, -D3 as well as Cdk4, stabilizes cyclin D2/Cdk4 complex and increases its kinase activity [116]. Hyperphosphorylation of Rb leads to the release of elongation factor 2 (E2F) which stimulates the transcription of a panel of cellular genes driving Tax-expressing cells into the S phase faster that non-Tax expressing cells. In addition, Tax transcriptionally represses the synthesis of CdkIs $p18^{INK4c}$ and $p19^{INK4d}$ and/or physically interacts with $p15^{INK4b}$ and $p16^{INK4a}$, in order to counteract their inhibitory activity on CDK4 and CDK6 [117-118]. By preventing the binding of $p15^{INK4b}$ and $p16^{INK4a}$ to Cdk4 and Cdk6, Tax shortens $G_1$ phase and accelerates cell cycle progression and cellular proliferation. Tax can also stimulate the transcription of E2F by directly altering the ratio of Rb-bound and Rb-unbound E2F [119-120]. Interestingly, Kehn *et al*. reported that Tax interacts and induces proteasomal degradation of the hypophosphorylated form of Rb, thereby promoting unregulated cell cycle progression [121].

During the $G_1$/S checkpoint and before DNA synthesis, cells must pause to repair DNA damage and therefore avoid transmission of errors to daughter cells. This checkpoint is controlled by the tumor suppressor protein p53 that acts as a downstream effector of the mutated in ataxia-telangiectasia (ATM)/ ataxiatelangiectasia and Rad-3-related (ATR) DNA damage recognition pathway [110]. Interestingly, 30% of ATL patients harbour a mutated p53 and even in absence of genetic mutations, p53 is not functionally active [122]. By inactivating p53, Tax can immortalize the HTLV-1-infected cells and destabilize their genome. Consequently, such cells can progress toward the ultimate leukemic state by a stepwise accumulation of oncogenic mutations and other types of chromosomal aberrations [123].

The half-life of p53 protein has been shown to increase rather than decreases in HTLV-1-transformed cells, regardless of p53 mutation. Further studies suggested that Tax is responsible for p53 stabilization and inactivation [124]. Since Tax does not bind directly or repress transcription of p53, the mechanism by which Tax inactivates p53 function is still unclear. One hypothesis is that Tax may inhibit p53-mediated trans-activation by interacting with CBP and preventing p53/CBP complex formation. Additionally, Tax disables p53-mediated $G_1$/S checkpoint by up-regulating the expression of the p53 target gene - $p21^{waf1}$ - in a p53-independent manner, thereby allowing cell cycle progression to occur even in the presence of DNA damage [125]. $p21^{waf1}$ has been characterized as an inhibitor of the G1/S progression through its direct association with cyclin E/cdk2 complex [126]. However, it also serves to stabilize cyclin D/cdk4/6cdk2 complex, increasing its kinase activity and promoting cell cycle progression. Therefore, the rapid progression of Tax-expressing cells in the $G_1$ phase and their inability to engage the $G_1$/S checkpoint can be explain by the up regulation of $p21^{waf1}$ and impaired p53 function.

During the S phase, Tax inhibits cyclin A expression and thus prevents cyclin A/cdk2-mediated phosphorylation of pre-replication complexes. The repression of cyclin A by Tax leads to redundant DNA replication thereby contributing to the aneuploidy often observed in ATL cells [127].

$G_2$/M checkpoint prevents cells from entering mitosis when cellular DNA is damaged. DNA damage activates the checkpoint kinases Chkl and Chk2 and stimulates their downstream targets including p53, Cdc25A and Cdc25A/C. Tax has been shown to deregulate the $G_2$/M checkpoint by interacting with both Chkl and Chk2 [128]. Several studies have shown that Tax *de novo* expression activates Chk2, resulting in an accumulation of cells in $G_2$/M phase [129-130]. By interacting with Chk1, Tax has been reported to inhibit Chk1-mediated phosphorylation of p53 [128]. Tax also binds to the mitotic arrest defective protein 1 - MAD1 - a component of mitotic checkpoint that prevents anaphase until chromosomal alignment is terminated. To facilitate cell division,

Tax impairs the function of MAD1 [131]. Tax can interact and activate the anaphase promoting complex, $APC^{cdc20p}$, an E3-ubiquitin ligase that mediates degradation of anaphase inhibitors and therefore responsible for the metaphase/anaphase transition. Activation of $APC^{cdc20p}$ by Tax leads to premature proteasomal degradation of the anaphase inhibitor - securin - as well as defective cytokinesis and improper chromatin separation resulting in aneuploidy [132].

### *1.2.2. Tax induced DNA mutations and genomic alterations*

Chromosomal abnormalities, deletions, translocations, rearrangements, duplications, micronuclei formation and aneuploidy are the hallmarks of Tax-expressing cells (**Figure 10**) [133-135]. As described above, Tax deregulates cell cycle checkpoints by amplifying the cell cycle speed beyond the time necessary for DNA repair. However, if DNA repair is not possible, other cellular mechanisms exist to prevent errors from being transmitted to daughter cells. Those mechanisms can be divided into four categories: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), and double-strand break repair (DSBR) pathways [136-137]. Tax represses BER activity which removes damaged bases from DNA by inhibiting transcription of the specific repair enzyme, DNA polymerase β (pol β) [138]. Tax also disrupts the NER pathway by inducing transcription of the proliferating cell nuclear antigen (PCNA) promoter. NER activity consists to repair bulky DNA adducts such as pyrimidine dimers. The PCNA protein is associated with DNA polymerase δ and ε enzymes involved in DNA replication and repair [134]. The robust expression of PCNA induced by Tax has been shown to favour synthesis of damaged DNA and thus, incorporation of unrepaired lesions into the genome [139]. Although little is known about the effect of Tax on DSB repair, it has been suggested that this mechanism plays an important role in the development of ATL. DSB are repaired via non-homologous end joining (NHEJ) mechanism. This error-prone process requires the help of several components including Ku70, Ku80, DNA-PK catalytic subunit to form the DNA dependent protein kinase complex (DNA-PK). DNA-PK is believed to recognize and regulate DSB. Microarray

analysis revealed that Tax-expressing cells have low levels of Ku80 mRNA compared to control cells [140], resulting in a weaker efficiency of DSB repair and a higher genomic mutation frequency.

### *1.2.3. Anti-apoptotic effects of Tax*

Cancer is a multistep processes that require several events for cellular transformation and ultimately, disease progression. Accumulating evidence indicates that the process of oncogenesis/transformation leading to the ATL condition reflects the outcome of an imbalanced cell death, survival, and proliferation. Thus, unbalanced activation of signal transduction pathways, inhibition of cell cycle checkpoints, and accumulation of genetic defects are generally associated with cell transformation and escape from apoptosis. Numerous studies have reported the influence of Tax on apoptotic pathways; however, the overall findings have been controversial since it was not clear whether Tax possess pro- and/or anti-apoptotic activities [141-145]. A possible explanation on the dual activity of Tax came from de la Fuente *et al*., whose microarray analysis on Tax-expressing cells demonstrated that induction of pro- and anti-apoptotic gene expression were linked to whether cells were at the $G_1$, S or $G_2$/M phase of the cell cycle [146]. This section will focus on anti-apoptotic activity mediated by Tax.

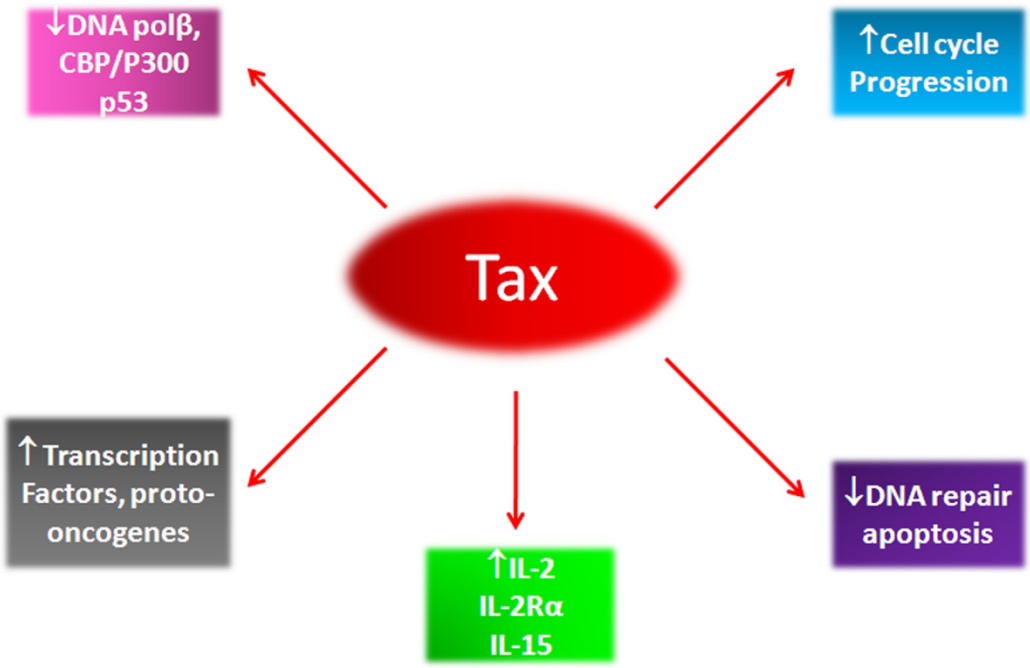



**Figure 10. Pleiotropic functions of Tax.** Summary of the diverse functions of Tax within an HTLV-1-infected T-cell, leading to uncontrolled cell proliferation.

Studies suggest that HTLV-1 uses Tax to control and take advantage of several pathways thereby facilitating cell survival. NF-κB is one of the major cell growth and survival pathways targeted by Tax. Although under tight regulation in normal T-cells, NF-κB is constitutively activated in HTLV-1-infected and Tax-expressing cells. Activation of NF-κB has been demonstrated to be important for Tax-induced interleukin-2 (IL-2) independent T-cell growth [115]. Tax-mediated constitutive activation of NF-κB in ATL cells leads to the synthesis of high levels of IL-2/ IL2 receptor α-chain (IL-2Rα) [72, 147] which in turn, stimulates an autocrine stimulatory loop driving the early stages of T-cell proliferation.

Thus, HTLV-1-infected T-cells undergo an initial phase of proliferation that is dependent on the IL-2/IL-2Rα-mediated autocrine proliferation. Over a period of decades *in vivo*, an IL-2 independent monoclonal population of leukemic T-cell emerges from leukemic clone(s) bearing multiple mutations in oncogenes and/or tumor suppressor genes (**Figure 10 and 11**). Proliferation of leukemic T-cells becomes IL-2 independent through the constitutive activation of critical components of the JAK/STAT pathway: JAK1, JAK3, STAT3 and STAT5 [148]. IL-15Rα is also upregulated by Tax in HTLV-1-infected cells and therefore it has been proposed that an IL-15 autocrine loop may also contribute to HTLV-1 pathogenesis [149-151].

In addition to growth factor synthesis, NF-κB activation by Tax leads to the transcriptional up-regulation of multiple anti-apoptotic proteins including Bcl2, c-FLIP, Bcl-$x_L$, surviving, A1, c-FLIP and inhibitor of apoptosis (IAP) family (**Figure 10**) [152-154]. Tax has also been shown to inhibit the expression of the pro-apoptotic gene Bax, leading to a decrease in apoptosis [155]. Since many ATL cells no longer express Tax but still harbour a constitutively activated NF-κB pathway, the link between Tax-mediated NF-κB activation and ATL outcome remains elusive. One explanation is that Tax-mediated NF-κB activation provides a critical proliferative or survival signal early in the cellular transformation event, but not for maintenance of the leukemic state. Viral HBZ has been proposed as critical maintenance factor sustaining the leukemic state observed in ATL condition (**Figure 11**). Indeed, Tax is a major target of cytotoxic T-cells (CTLs) and elicits a strong immune response that counterbalances infected T-cells propagation; in contrast, HBZ is a non-immunogenic protein and HBZ-specific CTLs are unable to efficiently eliminate HTLV-1-infected cells [156]. Therefore, Tax and HBZ cooperate to promote HTLV-1 infected T-cell proliferation in the early and late steps of infection as well as to limit of viral gene expression, allowing HTLV-1- infected cells to escape from the adaptive immune response [157].

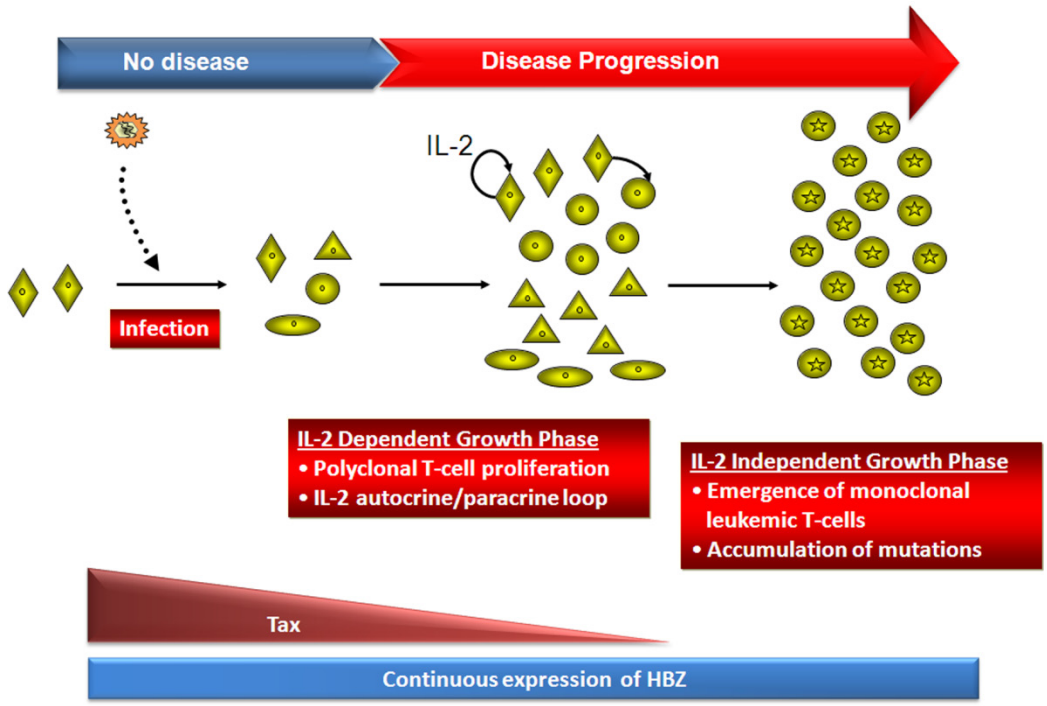



**Figure 11. Leukemic transformation of CD4+ T-cells by HTLV-1.** HTLV-1 induces T-cell proliferation and leukemogenesis. HTLV-1 infects CD4+ T-cells and initially causes at first a polyclonal/IL-2- dependent growth phase requiring the expression of the HTLV-1 regulatory proteins: Tax and HBZ. After decades of proliferation, a monoclonal T-cell population of leukemic T-cells emerges and leads to the ATL disease state. Tax expression is suppressed by several mechanisms (accumulation of non-sense mutations, deletion of the 5' LTR, DNA methylation of the provirus) suggesting that Tax is not necessary at late stage of transformation. HBZ encoded by the complementary strand of the HTLV-1 provirus down-regulates Tax-mediated viral transcription. HBZ is the only regulatory protein expressed in leukemic cells and is therefore believed to play a crucial role during the final steps of the leukemogneic process. HBZ is responsible for (1) the proliferation of CD4+ lymphocytes cells and (2) the viral persistence *in vivo*.

Phosphatidylinositol 3-kinase (PI3K) activation and its downstream target Akt is a second important pathway by which Tax represses apoptosis. PI3K pathway is an important mediator of cell survival and proliferation and is activated in response to cytokine and/or T-cell receptor signaling [158]. PI3K-Akt is thought to be involved in cellular transformation since it has been found to be constitutively activated in ATL patients and HTLV-1 transformed cells. Tax interacts directly with PI3K and subsequently activates the serine/threonine kinase - Akt - through site-specific phosphorylation. Akt activation acts on many downstream signaling pathways culminating in the activation of activator protein 1 (AP1), a transcription factor found to be highly expressed in invasive human cancers including ATL. AP1 ultimately leads to Bcl-$x_L$ expression, p53 repression, and cell survival [159]. This critical role for Tax-PI3K-Akt activation in survival and proliferation has been demonstrated by cell death of ATL cells treated with LY294002, a PI3K inhibitor [160-161].

## 1.3. Epidemiological aspects

### *1.3.1. Geographical distribution*

HTLV-1 is not a ubiquitous virus; only 15 to 20 million of individuals worldwide are estimated to be infected [162]. HTLV-1 prevalence is usually determined by serological screening of blood donors, pregnant women, and other selected population groups, such as HIV positive individuals and intravenous drug users. HTLV-1 is endemic in Japan, Africa, Central and South America, the Caribbean basin and few regions in the Middle East and Melanesia, but is also found in Northern First Nations People of Canada, Cosmopolitan areas of Europe, North America and Asia (**Figure 12**) [163-164]. In endemic zones, HTLV-1 seroprevalence rate in the population can be as high as 30% and varies depending on age and gender. It is predicted that after a latency period of 20-50 years, only 2% to 5% of infected individuals will develop an HTLV-1 associated disease, the remainder are asymptomatic carriers of the virus [165-167].

The geographic and ethnic clustering of individuals with HTLV-1-associated diseases may be explained by the natural history of HTLV-1 infection. Detection of HTLV-1 proviral sequences in a 1500-year-old Andrean mummy, indicates that HTLV-1 infection in humans has existed for a long period of time [168]. Anthropological evidence suggests that HTLV-1 and STLV came from the same ancestor virus that may have been transmitted to humans by contact with non-human primates [169]. It is believed that the interspecies transmission may have occurred through an accidental cut, a deep bite during hunting or by eating infected meat [170]. Interestingly, ATL-like manifestations have been observed in African green monkeys, gorillas and macaques infected with STLV-1 [171]. Further studies demonstrated that STLV-1 genome is highly similar to HTLV-1 genome (more than 90%) and that the sequence of gp21 of STLV-1 is 98% identical to that of HTLV-1 sub-type B [172-173]. The genome of HTLV-1 is very stable (less than 10 % diversity accumulated over a long period of evolution). This genetic stability may be due to the clonal expansion of HTLV-1 infected cells and the minimal use of the reverse transcriptase. This specificity makes the HTLV-1 genome an interesting tool for

studying the migration of infected human and simian populations and their inter- and intra-species contacts [174]. Although HTLV-1 was endemic in South America based on the HTLV-1 infected mummy discovery, the slave trade also contributed to the dissemination of the virus throughout the world [175-176]. Different strains of HTLV-1 and STLV-1 were detected in indigenous tribes from Melanesia, Africa and Australian aboriginals, [176-179].

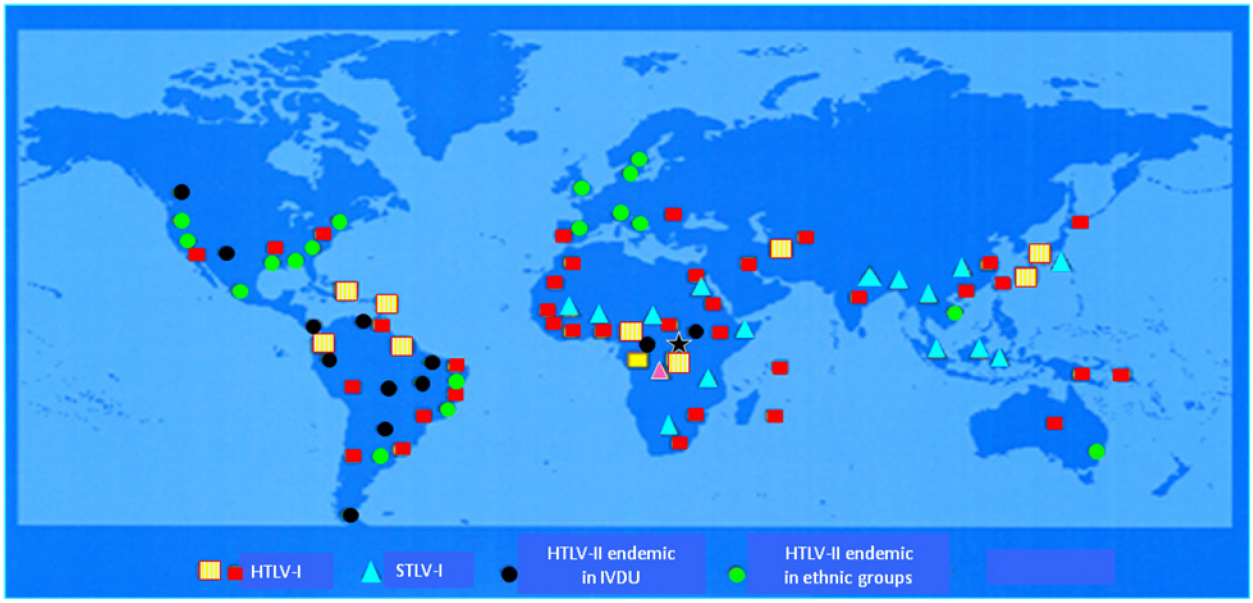



**Figure 12. HTLV-1, HTLV-2, and STLV-1 worldwide distribution.** HTLV-I infection is particularly prevalent in Japan, regions of South America, regions of Africa, and the Caribbean (yellow stripped squares). Presence of HTLV-1 in non-endemic regions is represented by smaller red square. HTLV-II is endemic in the Intravenous Drug Users population (IVDU) (black dot). Adapted from [180] (Copyright © 1999, by Cold Spring Harbor Laboratory Press).

### *1.3.2. Transmission of HTLV-1*

Unlike HIV-1, HTLV-1 is not an easily transmissible virus. Cell free viral particles are poorly infectious and HTLV-1 is thought to spread mostly by cell-to-cell contact between virally infected cells and target cells. *In vivo* studies revealed that cell free virions are not detected in the serum of HTLV-1 infected patients [181]. Recent evidence has suggested that HTLV-1 viral particles bud at the cell surface membrane and are transiently stored in adhesive extracellular structures rich in carbohydrates [22]. These structures are composed of virally induced extracellular matrix - collagen, agrin - and linker proteins - tetherin, galectin 3 - that are reminiscent of bacterial biofilms [182]. Extracellular viral aggregates rapidly stick to other cells upon cell contact, allowing the virus to spread and infection of target cells. *In vitro* infectivity by virions is less efficient than that achieved by cell-to-cell transmission [183]. Iga *et al.* reported that when HTLV-1 infected cells bind to target cells, "virological synapses" are formed and gag-viral genomic RNA complexes are transferred from the infected into the uninfected cells [184]. However, it is not clear whether this model reflects a major pathway of productive cell-mediated transmission of HTLV-1. Recently, Ruscetti et *al.* proposed that dendritic cells (DCs) could play a central role in HTLV-1 transmission, dissemination and persistence *in vivo*. Freshly isolated myeloids DCs (mDCs) and plasmacytoid DCs (pDCs) were efficiently and productively infected by HTLV-1 virions. Infected DCs rapidly and reproducibly transferred HTLV-1 to autologous primary CD4+ T-cells, resulting in productive infection of CD4+ T-cells [16].

Since the transmission of HTLV-1 is fairly difficult in human populations, the most efficient mode of transmission of this virus is by exposure to infected blood or whole cell blood products (e.g., during blood transfusion or through the use of contaminated needles). It is estimated that the sero-conversion rate in these specific cases is about 50 % [185]. Sharing of contaminated needles among intravenous drug users constitutes one of the most common routes of blood-to-blood transmission in metropolitan areas of the United States and Europe [166]. In endemic areas, transmission

of HTLV-1 from infected mother-to-child constitutes the predominant mode of infection. The probability of mother-to-child transmission is about 18 to 30% and the risk factors involved are: prolonged breast-feeding after 6 months of age (through infected milk-borne lymphocytes), and less commonly high HTLV-1 antibody titer, mother's proviral load and prolonged ruptured membranes during delivery [186]. Sexual transmission is relatively inefficient when compared to HIV but has the potential to introduce infection into previously unexposed groups. The risk of HTLV-1 transmission from male to female is about 60% while the risk from female to male is about 0.4% over a 10-year period. Transmission risk to females is believed to be higher if the partner has high antibody titer or antibody against Tax, while the risk of female-to-male transmission is associated with penile sores or ulcers [187].

## 1.4. Viral pathogenesis and clinical outcomes

### *1.4.1. Adult T cell Leukemia*

A small proportion of the individuals infected with HTLV-1 (1 in 1000-2000 seropositive per year) will develop an ATL phenotype in their lifetime with a 20-30 year latency period. Men are 40% more susceptible to suffer from ATL than women. It has been estimated that ~5% of individuals infected with HTLV-1 early in life - mainly through breastfeeding - develop ATL before the age of 20 years [81, 188].

ATL patients present clinical symptoms similar to those observed in non-Hodgkin's lymphoma, such as malaise, fever, lymphoadenopathy, hepatosplenomegaly, drowsiness, weight loss [189]. Typically widespread or localized skin involvement (in 40% of cases, there are large nodules, plaques, ulcers, and papular rash on the limbs, trunk, or face) and lytic bone lesions are reported in these patients. Immunosuppression causing bacterial and fungal infections has been also observed in ATL patients and contributes to a poor prognosis [8, 190].

ATL is an aggressive T-cell malignancy with a leukemic phase characterized by a high number of activated CD4+ T-cells circulating in the peripheral blood. The diagnosis of ATL is based on specific parameters including seropositivity to HTLV-1, marked leukocytosis and morphological analysis. ATL cells have a highly indented and convoluted multi-lobulated nucleus with markedly condensed chromatin and are named "flower cells" (**Figure 13**) [189, 191]. Hypercalcemia, elevated serum concentrations of lactate dehydrogenase (LDH) and soluble interleukin-2 (IL-2) receptor α chain (IL2Rα/CD25) are also clinical chemistry markers of an ATL condition. Immunophenotypically, ATL cells are defined by distinct mature helper T-cells surface marker: CD2+, CD7-, CD3+, CD4+, CD25+, CD45RA+, HLA-DR+, CD29-, CD45RO- and L-selectin+ [8]. The CD8+ marker is rarely detected on leukemic cells of ATL patients, although cases of ATL involving CD4/CD8 double-positive leukemic cells have been reported in the literature [192-193]. In contrast to ATL cell lines, ATL cells from patients express the Foxp3 marker, leading to the hypothesis that ATL cells may originate from regulatory T cells (Tregs) [194-196].

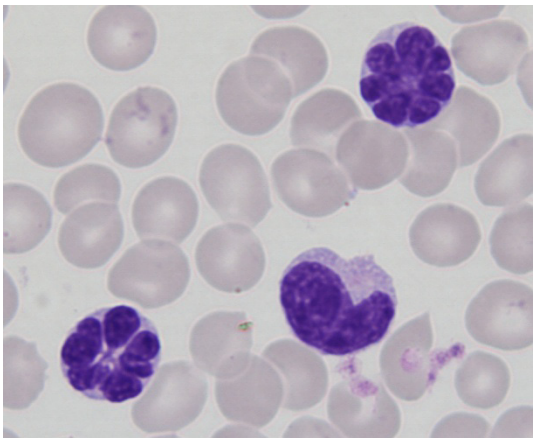


**Figure 13. Image of "flower cells".** Flower cells are atypical lymphoid cells with basophilic cytoplasm, multilobulated and convoluted nuclei, commonly seen in the peripheral blood of acute ATL. Reproduced with permission from [197] (Copyright © 2005 Matsuoka).

As defined by the Japanese Lymphoma Study Group, ATL is classified into four clinical subtypes: smoldering, chronic, lymphoma and acute, based on prognostic survival time as well as clinical and laboratory features described previously (**Figure 14**) [9, 189]. The smoldering type has the best prognosis amongst the four forms of the disease and is reported to have few ATL cells (less than 5%) in the peripheral blood over a long period of time. The chronic type is very similar to the smoldering form of ATL and is defined by a marginal increase of ATL cells, cough and skin lesions. Patients with the lymphoma form of ATL have almost no leukemic cells (less than 1%) detected in the peripheral blood but present with an enlargement of the lymph nodes. The acute phase has the highest incidence and is characterized by a rapid progression of the disease with a high number of ATL cells circulating in the peripheral blood, skin lesions, systemic lymphoadenopathy, hepatosplenomegaly, elevated level of serum LDH, calcium and a poor prognosis (less than 7 months) [9, 194, 198-199]. Thus, ATL is a fatal disease with median survival time from the onset of the disease ranging from 6.2 to 24.3 months [81].

The mechanisms by which leukemogenesis is initiated following HTLV-1 infection are complex. The viral transactivator Tax has been demonstrated to play a crucial role in the development of ATL through dysregulation of cell cycle and suppression of DNA repair pathways, leading to the gradual accumulation of oncogenic mutations over time and clinical onset of ATL.

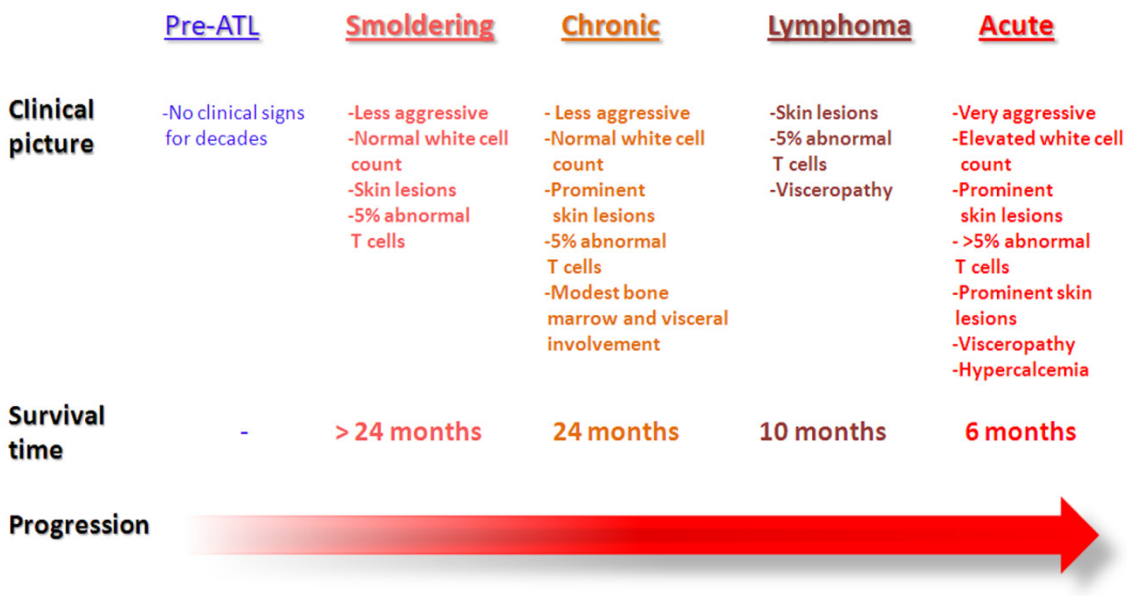



**Figure 14. Clinical features of Adult T-cell Leukemia.** ATL is classified into four clinical subtypes - smoldering, chronic, lymphoma and acute – based on the number of atypical CD4+ T-cells, tumor lesions and clinical course.

### *1.4.2. HTLV-1-associated myelopathies*

The prevalence of HAM/TSP amongst HTLV-1 carriers is estimated to be 1 for 1500 individuals [200]; women are more frequently affected than men with a ratio 3:1 and the disease progresses at a faster rate in women than in men [81]. The average age of occurrence of the disease is usually 35 to 45 years, although patients as young as 12 years of age have been reported to develop HAM/TSP [8]. In contrast to ATL, HAM/TSP has a relatively short latency period typically ranging from months to decades, but can be as fast as 18 weeks following blood transfusion with HTLV-1 contaminated blood.

HAM/TSP is a progressive neurological disorder similar to multiple sclerosis (or amyothrophic lateral sclerosis-like manifestations) with presenting symptoms such as paraparesis with spasticity in the lower extremities, hypereflexia, muscle weakness and sphincter disorders including dysfunction of the urinary bladder and intestines [201]. A cerebral syndrome with ataxia and intention tremor has also been observed occasionally [9]. It has been reported that patients can exhibit a predominance of sympathetic nervous system dysfunction [202].

Pathological manifestations of HAM/TSP are represented by a demyelinating disorder involving degradation of white matter within the lateral funiculi spinal cord, mainly concentrated in the thoracic and lumbar segments. Presence of HTLV-1-specific antibodies, infiltrating T-lymphocytes in the peripheral blood and cerebrospinal fluid (CSF) accompanied by the release of proinflammatory cytokines are features of the HAM/TSP condition [203-204]. It has been reported that the inflammatory response affects the integrity of the blood-brain barrier (BBB) and promotes the migration for further lymphocyte infiltration into the CNS [205-206]. Thus, HAM/TSP is believed to occur in two phases, initiated first as an inflammatory disorder, followed by a chronic long-term degenerative stage. $CD8^+$ cytotoxic T-lymphocyte (CTLs) cells specific for the HTLV-1 transactivator protein Tax, have been reported to be the primary proliferating

cell type in HAM/TSP [207]. Later stages of the disease are represented by the presence of extracellular Tax and expanding Tax-specific CTLs in the CSF [208-209].

The mechanisms associated with HTLV-1 infection and the subsequent development of HAM/TSP remains elusive. Three possible mechanisms have been proposed: 1) the direct damage mechanism (pathogenic immune response, infection of resident brain cells and excitotoxicity of neurons), 2) the bystander mechanism, and 3) the autoimmune mechanism of molecular mimicry (**Figure 15**) [210]. In the direct damage model, it has been proposed that the persistent activation of $CD8^+$ T-cells in the central nervous system (CNS) is due to the presence of replicating virus or viral proteins. In addition to CD4+ T-cells, HTLV-1 has been shown to infect the cell populations responsible for regulating the BBB such as astrocytes, endothelial cells and peripheral immune cells including monocytes and DCs. Oligodendrocytes and neurons are also potential targets for HTLV-1 [13, 211-212]. Infiltration of activated $CD8^+$ CTL cells specific for Tax leads to tissue destruction and release of inflammatory cytokines [213]. In the bystander damage model, proinflammatory cytokines/chemokines including TNF-α, IFN-γ, IL-1, IL-6, and GM-CSF are produced by the infiltrating lymphocytes and resident cell populations (astrocytes, microglia, etc.) infected with HTLV-1 [214]. High levels of these proinflammatory molecules cause dysfunction and death of resident cells in the CNS and ultimately contribute to the disruption of the BBB [215]. The BBB forms the interface between the blood and the CNS and is constituted of three cell types: astrocytes, pericytes and brain endothelial cells [202]. Recently, Afonso *et al.* demonstrated that human brain endothelial cells are productively infected *in vitro* consequently altering the BBB integrity and resulting in HTLV-1-infected cells migration through the endothelium [216]. Finally, in the autoimmune model, an immune response against the viral protein Tax is found to cross-react with the highly expressed neuronal antigen heterogeneous ribonuclear protein-A1 (hnRNP-A1) [210, 217]. Thus, infiltrating CD8+ CTL cells specific for Tax cause the incidental destruction of hnRNP-A1-presenting cells in the CNS.

Whatever the mechanism involved in HAM/TSP outcome, direct, bystander or autoimmune, an important hallmark of this disorder is the proviral load [218-219]. Indeed, Nagai *et al.* reported that peripheral mononuclear cells (PBMCs) from HAM/TSP patients harbour a HTLV-1 proviral load (PVL) 5 to 20-fold higher than asymptomatic carriers [218]. Similarly, Lezin *et al.* observed that HTLV-1 proviral load in CSF is higher in HAM/TSP patients than in asymptomatic carriers and conclude that HTLV-1 proviral load in PBMCs and CSF is directly correlated with the extent of CNS damage and may be used as an indicator to predict the outcome of HAM/TSP disease amongst HTLV-1 asymptomatic carriers [220].

Although the majority of HTLV-1-infected individuals remain healthy carriers during their lifetime, a small percentage of asymptomatic patients will develop either ATL or HAM/TSP, but never both. The factors contributing to HTLV-1 pathogenesis are poorly defined and under intense investigation. The genetic variation of HTLV-1 and the genetic background of the patients are believed to be important [221]. Furukawa *et al.* reported the existence of two Tax gene subgroups [222]. One subgroup of this gene was found with a high incidence in HAM/TSP patients but not ATL or asymptomatic patients, suggesting that the clade of HTLV-1 causing infection may determine clinical outcome. In addition, specific HLA alleles - HLA-A*26, HLA-B*4002, HLA-B*4006, HLA-B*4801 - have been shown to predispose to ATL condition [223]. Asymptomatics carriers with a HLA-A*02 and HLA-Cw*08 haplotype have a low proviral load and present less risk to develop HAM/TSP disease. In contrast, HLA-B*5401 was associated with a high proviral load and therefore a high susceptibility to HAM/TSP condition [224-225]. The initial route of viral infection has been also proposed as a factor contributing to the pathogenesis associated with HTLV-1 infection. For example, Osame *et al.* reported that exposure to HTLV-1 through the peripheral blood has been linked with a HAM/TSP outcome [226]. Kannagi *et al.* proposed that mucosal exposure to HTLV-1 favours the ATL condition [227].

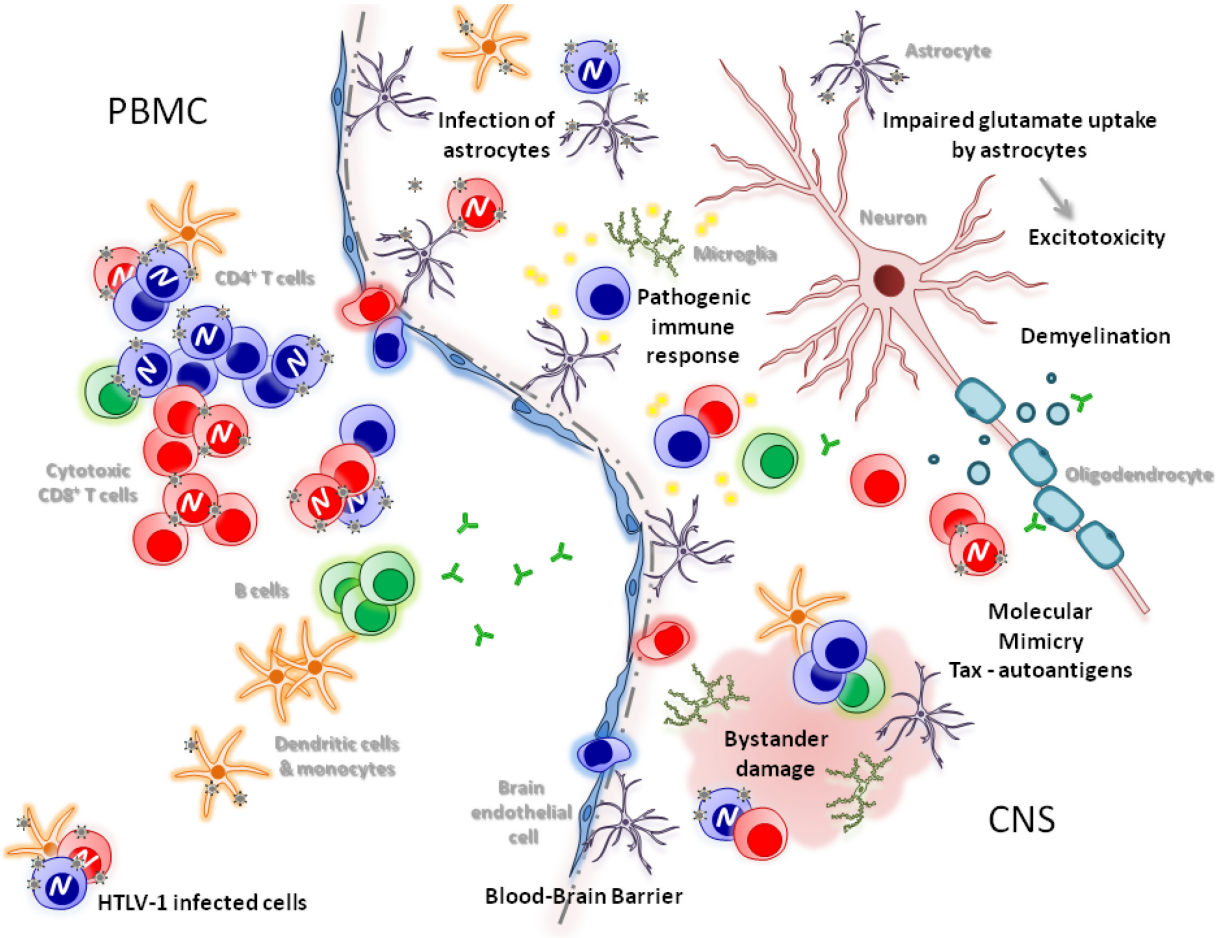



**Figure 15. HTLV-1-Associated Myelopathies pathogenesis.** Three possible mechanisms have been proposed: 1) the direct damage mechanism (pathogenic immune response, infection of resident brain cells and excitotoxicity of neurons), 2) the bystander mechanism, and 3) the autoimmune mechanism of molecular mimicry. Adapted from [228] (Copyright © 2010 Mineki Saito).

### *1.4.3. Other HTLV-1-associated diseases*

In addition to HAM/TSP and ATL, several extra-neural inflammatory disorders such as infective dermatitis, Sjogren's syndrome, thyroiditis, arthropathy, polymyositis, polyneuropathy, T-lymphocyte alveolitis and cutaneous T-cell lymphoma have been associated with HTLV-1 infection [8-9]. Seroepidemiological, clinical and virological data have demonstrated that HTLV-1 is closely linked with a certain type of uveitis. This pathology is a sight threatening intraocular inflammatory disorder [229]. Patients affected by this disease present with blurred vision, retinal vasculitis and exudates [230]. HTLV-1-associated arthropathy is a chronic arthritis of the joints. Pathological features of this condition are similar to those observed in rheumatoid arthritis and are characterized by a synovial proliferation and a positive rheumatoid factor [7, 231]. HTLV-1 associated with infective dermatitis is characterized by a severe exudative dermatitis of the scalp, ears, neck, axillae, and groin as well as a generalized fine papular rash [232]. It has been postulated that the presence of infective dermatitis in children may predispose them to subsequent development of ATL or HAM/TSP [233]. HTLV-1 has been also linked to Sjogren's syndrome - a chronic inflammatory disorder - characterized by lacrimal and salivary gland insufficiency resulting in xerostomia (dryness of mouth) [234-235]. The etiological role of HTLV-1 in these diseases remains a topic of controversy, since the direct evidence connecting these pathologies to HTLV-1 are not as well defined as in ATL and HAM/TSP.

### *1.4.4. HTLV-2*

In 1982, a closely related delta-retrovirus - HTLV-2 - was identified in a T-cell line established from a patient with atypical hairy cell leukemia [236]. HTLV-2 infection is generally rare and rather endemic in native Amerindian populations in the North [237], in Pygmy tribes in Central and West Africa [238-239] and amongst cohorts of intravenous drug users in the United States and Europe [240-241]. Although HTLV-2 tropism remains unclear, *in vivo* study showed that both CD4+ and CD8+ T-cells are susceptible to HTLV-2 infection with a greater proviral burden in CD8+ T-cells [242-

243]. Recently, the ability of HTLV-2 to immortalize *in vitro* human T-cells in an IL-2-dependent manner as effectively as HTLV-1 has been reported [85]. However, HTLV-2 infection has not been linked with the development of lymphoproliferative disorders but has been associated with sporadic cases of a myelopathy similar to HAM/TSP.

HTLV-1 and HTLV-2 share about 65% nucleotide sequence homology but differ in their clinical manifestations and epidemiological distribution [242]. Although the HTLV-1-encoded transactivator Tax (Tax-1) and the HTLV-2 Tax (Tax-2) have a 78 % homology at the amino acid level, it has been proposed that their specific Tax activity is in part responsible for these differences. Indeed, several reports showed that Tax-1 has a higher ability than Tax-2 to transactivate the viral LTR, thus, Tax-1 is more efficient than Tax-2 to induce viral and cellular gene expression. Moreover, Tax-1 enhances micronuclei formation, a marker for genetic instability while Tax-2 lacks this ability. Thus, Tax-1 is a great inducer of cellular DNA damage and has a stronger potential than Tax-2 to induce cell transformation [244-245].

Recently, two new HTLV-related viruses - HTLV-3 and HTLV-4 - have been identified in Central Africa [246]. Further investigation regarding these two viruses could provide useful insights in determining their prevalence, distribution and modes of transmission, as well as their possible association with human disease.

### *1.4.5. HTLV-1 and HIV-1*

Seroprevalence studies in developed countries such as USA and Europe have indicated that in the past few years, an increasing prevalence of Human immunodeficiency virus (HIV-1)/HTLV-1 co-infection amongst intravenous drug users [247-248]. HIV-1 and HTLV-1 are retroviruses that share several biological features, including route of transmission (sexual contact, blood transfusion, pregnancy and breast milk) and ability to infect CD4+ T-lymphocytes; however, the clinical and pathologic manifestations of these two viruses differ significantly. HIV-1 is highly cytopathic for CD4+ T-cells, while HTLV-1 is non-cytopathic and has the ability to cause clonal

proliferation and transformation of T-cells [249]. Although the real impact that HIV-1 and HTLV-1 co-infection exerts on clinical outcome is still controversial, the majority of studies suggest that HTLV-1 exacerbates the cytopathic effects of HIV-1, leading the co-infected individuals to a faster progression to Acquired Immuno Deficiency Syndrome (AIDS) and a shorter survival time [250-251].

In rare cases, HIV-1 infection has a stimulating effect on HTLV-1 replication. High HTLV-1 proviral load was detected in blood samples obtained from patients dually infected with HIV-1 and HTLV-1. Clinical studies of these co-infected patients suggests that HIV-1-enhances HTLV-1 replication and accelerates the development of HTLV-1 associated diseases such as HAM/TSP and peripheral neuropathy [252-254]. Although the mechanisms by which HIV-1 increases HTLV-1 gene transcription remain undetermined, it has been reported that HIV-1 trans-activating gene product - Tat - could potentially have inductive effects on HTLV-1 in co-infected micro-environments [255-257]. Further studies are required to evaluate the risk of recombinant HTLV-1/HIV-1viruses which may possess a broad cell tropism resulting in a faster dissemination or progression of disease.

### *1.4.6. Diagnosis and therapies*

HTLV-1 infection is life-long after it synthesizes its DNA copies by reverse transcription and integrates into the host's genome as a provirus. HTLV-1 infection can be determined by serological test, detection of genomic HTLV-1 or virus isolation. The diagnosis of HTLV-I infection is mainly based on detection of specific antibodies against HTLV-1, by enzyme immunoassay (EIA) or by a particle agglutination test (PA) that consists of gelatin particles coated with viral lysate [8, 191]. The first generation EIA was using HTLV-I whole viral lysate as the only antigen which frequently resulted on false-positive reactions [258-259]. More recently, highly sensitive assays based on recombinant proteins and/or synthetic HTLV-1 peptides alone or in combination with viral lysate have been developed to detect HTLV-1 and HTLV-2 infections [260-261]. These assays

contain recombinant and synthetic peptide antigens representing HTLV-1/HTLV-2 envelope and gag antigens [259]. Although this generation of assays has a higher specificity than the earlier tests, confirmatory testing is still recommended to eliminate false-positive reactions.

There are several serology-based confirmation tests, but the Western blot (WB) is most frequently used for this purpose. The HTLV WB kits use HTLV-1 viral lysate, to which recombinant envelope antigens have been added to improve the sensitivity [259, 262-263]. WB is also used as a confirmation assay to discriminate between HTLV-1 and HTLV-2. WB kits containing type-specific antigens such as recombinant proteins for the external glycoprotein of the HTLV-1 and HTLV-2 are commercially available. These kits enable not only confirmation but also differentiation between infections with the two viral strains.

Clonal integration of the HTLV-1 genome in infected T-cells can be detected by Southern blot analysis, Polymerase Chain Reaction (PCR) or real-time PCR [264]. PCR can provide the final diagnosis of infection. Generally, the most conserved region of the HTLV-1 genome - tax - is amplified to a detectable level [8, 265]. The advantage for using real-time PCR is that the HTLV-1 provirus load - the most frequently used marker for prognosis and progression of the disease - can be accurately quantified [219, 266-267].

Despite extensive progress in the understanding of the molecular immunology and virology of HTLV-1-associated diseases, there has been only minimal improvement in the treatment of ATL. Conventional high dose chemotherapies such as CHOP protocol (combination of cyclophoshamide, hydroxydoxorubicin, vincristine and prednisolone) have shown limited benefit in ATL and the median survival of patients with aggressive subtypes of ATL remains low: between 5 and 13 months [268].

Combination therapy with interferon alpha (IFN-α) and the antiretroviral nucleotide analog zidovudine has been shown to be effective against ATL [269]. The mechanism of action of this combination therapy is still unclear, but it was reported that IFN-α/zidovudine treatment in ATL patients decreased both HTLV-1 proviral load and vascular endothelial growth factor (VEGF) plasma levels, suggesting a potential anti-angiogenic effect of this therapy [270]. Clinical phase II study revealed a response rate of 92% for patients who received the therapy as an initial treatment [271]. However, the majority of the patients in remission relapsed when they were taken off the treatment; thus, IFN/zidovudine combination does not appear to be a cure for ATL. A combination of arsenic/IFN/zidovudine has been also developed to treat ATL patients [268, 272]. In ATL cell lines, arsenic trioxide inhibits constitutive activation of NF-κB and potentiates IFN-α apoptotic effects through proteasomal degradation of Tax. Clinical studies revealed that arsenic/interferon therapy exhibits some efficacy in refractory aggressive ATL patients. Treatment of ten chronic ATL patients with arsenic/interferon-alpha/zidovudine resulted in a 100% response rate (7 complete remissions, 2 complete remissions but with more than 5% circulating atypical lymphocytes, and 1 partial response) [272]. No relapse was observed during a follow-up of 8 months. However, long-term follow up is required to further clarify whether this combination therapy will translate into a cure or stabilization of the disease.

An alternative approach to the treatment of ATL is to target cell-surface markers overexpressed on the malignant cells with monoclonal antibodies. The first one to be tested was the anti-Tac monoclonal antibody directed against the alpha-chain of the interleukin-2 receptor (IL-2R-α/CD25), which is highly expressed on ATL cells but not on normal resting lymphocytes. Other molecules such as CD2, CD52, and recently CD30 and CCR4 have been targeted for pre-clinical studies of ATL treatment [273-277]. Antibodies directed against these molecules have shown to prolong the survival time in an immunodeficient mouse model of ATL.

NF-κB is constitutively activated in ATL cells and plays a major role in oncogenesis. Therefore, NF-κB constitutes an attractive molecular target for ATL treatment. Recently, two inhibitors of NF-κB activity - Bortezomib and histone deacetylase inhibitors - have been shown to exhibit an anti-ATL effect *in vitro* and *in vivo*. The proteasome inhibitor, Bortezomib, blocks the degradation of IκB, whereas the histone deacetylase inhibitors reduce the DNA binding of NF-κB [278-281]. However, further clinical studies are required to test the efficacy of these drugs in humans and their potential side effects as these pathways are essential for anti-viral defence against other pathogens.

Allogenic hematopoietic stem cell transplantation (allo-HSCT) is another approach that has been suggested to improve the ATL condition. Utsunomiya *et al.* reported that median leukemia-free survival time of ten ATL patients treated with allo-HSCT was 17.5 months and proviral load became undetectable in eight patients [268, 282]. Increasing levels of anti-Tax specific CTLs has been also observed in patients treated with allo-HSCT [283]. This suggests that enhanced CTL function might help to fight against HTLV-1 infected cells and contributes to induction of remission.

Various therapies, such as immunomodulatory or anti-viral drugs, have been developed to treat HAM/TSP patients [284]. However, patients treated with these therapies present only a transient clinical improvement. Treatments altering the long-term disability associated with HAM/TSP condition are still lacking.

Corticosteroids are one of the first agents to have been developed for HAM/TSP treatment but their effects have been elusive for many years. It has been suggested that corticosteroids have a higher efficacy early in the disease, in rapidly progressive disease and with a prolonged treatment. Now, it is proposed that corticosteroids confer only a transient clinical benefit. However, more data about the time of initiation, the dose, and the duration of the corticosteroid treatment are still needed [285].

Injection of plasmapheresis and intravenous gammaglobulin (IVIG) are sometimes used to modulate the humoral response of HAM/TSP patients since it has been suggested that anti-HTLV-1 antibodies could be neuropathic [286]. These therapies provoke diverse effects in the immune system such as inhibition of complement activation, modulation of proinflammatory cytokine production and signaling via Fc receptors. Clinical data demonstrated that treatment of HAM/TSP patients with IVIG and plasmapheresis resulted in a transient clinical improvement lasting only 2-4 weeks.

Type I interferons, known for their cytostatic and antiviral properties have been used for many years to treat HAM /TSP patients. IFN-α therapy produced minimal to moderate results and the efficacy of this treatment relies on the clinical state of the disease: degree of inflammation and tissue destruction [287-288]. HAM/TSP patients receiving IFN-β therapy for a long-time period present a significant decrease in the frequency of Tax-specific CD8+ T-cells along with a reduction in HTLV-1 Tax mRNA. However, HAM/TSP patients treated with IFN-β present only a slight improvement in motor function.

Antiretroviral compounds such as nucleosides analogues have been disappointing for the treatment of HAM/TSP disease [289]. Combination of zidovudine and lamivudine reduces proviral load *in vitro* but not *in vivo*; however, no significant clinical changes has been observed in HAM/TSP patients.

Some new therapies such as Hu MIK β1 and Valproic acid (VPA) are emerging. Hu MIK β1 is a monoclonal antibody that has been developed to block IL-15 signaling. Upregulation of IL-15 paracrine loop in HAM/TSP has been suggested to be responsible for the persistent expansion of HTLV-1 specific CD8+ T-cells. Lezin *et al.* reported that administration of the inhibitors of histone deacetylase (HDAC) - VPA - in HAM/TSP patients lead to increased viral expression and subsequent destruction of HTLV-1-

infected cells by the immune system [290]. Clinical trials are underway to test the safety and efficacy of these drugs.

## 2. Innate Immunity to RNA virus Infection

The immune system is constituted of several key components identified as innate (occurs early and throughout infection) and adaptive (activation at later times after primary infection and increases specificity to infection). In response to infection, numerous cytokines are produced and stimulate multiple signaling pathways which in turn induce antimicrobial states. These cytokines include members of the proinflammatory cytokine cascade: the interferons (IFNs), TNF-α and IL-12. During viral infections, some of the most prominent cytokines produced are the IFNs, named for their ability to induce genes that interfere with viral replication [291-293]. The IFN family is composed of transcriptionally activated and secreted proteins with multiple biological effects on the host. IFNs play a crucial role in the resistance to pathogens, and in the modulation of antiviral and immune responses. Beyond their antiviral activities, IFNs exert multiple immunoregulatory activities that control both innate and adaptive immunity. IFNs are classified into at least three distinct types: types I, II, and III based on the receptor complex used for signaling as well as sequence homology [294].

Type I IFNs are composed of various genes including 14-20 IFN-α genes depending on the animal species, one IFN-β gene [295], and the less extensively studied IFN-ω, -ε, and -κ, known as regulators of maternal recognition in pregnancy [296]. IFNs-α/β are rapidly released by most nucleated cells following the detection of invading pathogens. IFNs-α/β secretion elicits a cascade of events leading to the transcriptional regulation of hundreds of cellular genes known as interferon-stimulated genes (ISGs) encoding viral restriction factors such as RNase L, dsRNA-dependent protein kinase (PKR), and 2′–5′ oligoadenylate synthetase (OAS). These IFN-inducible proteins are believed to suppress viral replication by both cleaving viral RNA and suppressing the proliferation of virus-infected cells [297]. Importantly, type I IFN is essential for antibody production in B-cells and induction of cytotoxic T-cells (CTLs) and natural killer (NKs) cells. IFNs-α/β are widely administered in cancer therapy and viral-related diseases since

they are known to have strong antiviral, anti-proliferative and anti-angiogenic properties. The diverse and important actions of type I IFNs make them promising agents for a broad range of clinical diseases, including malignant tumors, immunodeficiency diseases, "cytokine storm" disease (hypercytokinemia), infectious diseases, as well as autoimmune diseases.

Type II IFN comprises only one family member, IFN-γ. This gene is structurally unrelated to type I IFNs and is typically secreted by specific cells of the immune system including CD4+ T-helper cells (Th), CD8+ cytotoxic T-cells, and NK cells and macrophages [298]. Although IFN-γ also displays antiviral activity, it is mostly considered as a powerful immunomodulatory cytokine.

Recently, a novel class of IFN-like molecules, namely IFN-λ1, -λ2, and -λ3 genes (also known as interleukin-29 (IL-29), IL-28A, and IL-28B, respectively), have been identified and classified as type III IFNs [294]. Type III IFN genes are induced by virally infected cells, and although structurally and functionally related to type I IFNs, they are considered as a distinct group and signal through a separate receptor complex. Recent evidence indicates type III IFNs mainly exert their antiviral activity *in vivo* via stimulation of the immune system rather than through initiation of an immediate antiviral state as seen with type I IFNs [299].

## 2.1. Early wave of IFN signaling

### *2.1.1. Viral detection by Pattern Recognition Receptors*

The innate immune system represents the first line of host defence against microbes (such as bacteria, viruses, fungi and parasites) and therefore plays a central role in the early recognition and subsequent establishment of an appropriate proinflammatory response against intruding pathogens. Pathogens are initially detected by cells expressing a variety of germ-line encoded pattern-recognition receptors (PRRs). Host PRRs

recognize evolutionary conserved signature molecules on microbes termed pathogen-associated molecular patterns (PAMPs). These PAMPs include, but are not limited to, lipopolysaccharide (LPS), dsRNA, ssRNA, flagellin, bacterial lipoprotein. PAMPs are generally an essential component for the survival of the pathogen as well as being distinguishable from "self" components. To date, three major classes of PRRs responsible for sensing the presence of invading pathogens have been identified: nucleotide-binding oligomerization domain (NOD)-like receptors, Toll-like receptors (TLRs), and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). Following engagement of PRRs by their cognate PAMPs, the host cell activates intracellular signaling pathways crucial for the establishment of an effective innate immune response. These events can result in the swift eradication of the incoming pathogen and induction of apoptosis of infected cells thus confering protection to surrounding cells. Moreover, this initial recognition of pathogens and innate response is essential for directing the ensuing adaptive immune response.

During viral infection, TLRs recognize specific PAMPs including ssRNA, dsRNA, RNA with 5'-triphosphate ends and viral proteins, present in the endosomes and/or at the cell surface, whereas RLRs detect PAMPs located in the cytoplasm of the cell. Sensing of these viral PAMPs by their cognate PRRs induces expression of type I IFNs, with evidence accumulating that cooperation between different pathways is needed to trigger a robust and regulated activation of the antiviral response (**Figure 16**). PRRs signal through various adaptor proteins leading to the activation of downstream kinases that are in turn responsible for the stimulation of latent transcription factors such as nuclear factor kappa B (NF-κB), interferon regulatory factors (IRFs) and ATF-2/c-Jun. Post-translational modifications mediate activation of NF-κB and IRFs - primarily phosphorylation events - ultimately resulting in the recruitment of these factors to the type I IFN promoters in a temporally and epigenetically coordinated manner. Secreted type I IFNs alert surrounding cells via binding to and subsequent activation of the type I IFN receptor (a heterodimer of IFNAR1 and IFNAR2) in an autocrine or paracrine manner leading to the activation of the JAK-STAT pathway and synthesis of hundreds of

IFN-stimulated genes (ISGs). These ISGs in turn antagonize many steps of the viral life cycle, shape the adaptive immune system and are responsible for global metabolic changes. Among them, IRF7 contributes to the positive-feedback amplification of the antiviral response, as well as anti-tumor immunity and antigen-presentation.

#### *2.1.1.1. The Endosomal Pathway - Recognition of RNA viruses via TLRs*

TLRs - a series of evolutionary-conserved receptors - are the best-characterized PRR family responsible for recognizing intruding pathogens present outside of the cell and in intracellular endosomes and lysosomes [300]. TLRs belong to the Toll/interleukin-1 receptor (IL-1R)/TLR superfamily of receptors. The original Toll receptor was characterized as a single-pass transmembrane protein, important for the establishment of a dorso-ventral pattern in *Drosophila melanogaster* [301]; its involvement in host defence against fungal and bacterial infections was later demonstrated [302]. Subsequently, several homologues of Toll were characterized in mammalian cells and named Toll-like receptors or TLRs (reviewed in [303]. To date, the TLR family comprises 12 and 13 members in human and mice, respectively, and the microbial components recognized by each TLR have mostly been identified [304]. Expression of TLRs is not static and can be induced in response to infection. Although these receptors are ubiquitous and can be expressed on non-immune cells, they are widely expressed in antigen-presenting cells (APCs) including macrophages and dendritic cells, which engulf and digest pathogens.

The Toll/interleukin-1 receptor superfamily is a family of proteins constituted of an extracellular domain containing multiple leucine rich repeats (LRRs), a transmembrane domain and a common cytoplasmic signaling domain which is designated the Toll/ (IL-1R) homology (TIR) domain. The LRRs are important for recognizing pathogen structures. The TIR domain contains three well conserved regions termed boxes 1, 2 and 3 which are essential for the interaction and recruitment of various adaptor molecules to activate downstream signaling pathways [305-306]. The sensing of viral nucleic acids is mediated by TLR3, human TLR7/mouse TLR8, and TLR9, which

specifically detect viral nucleic acid including dsRNA, ssRNA and unmethylated CpG DNA, respectively. Although TLR2 and TLR4 have been initially identified as sensors of bacterial components, they recognize also viral proteins. They are considered as immediate sensors of intruding pathogens as early as the viral entry stage of a virus infection. The envelope proteins from respiratory syncytial virus (RSV) and mouse mammary tumor virus (MMTV) are sensed by TLR4 and TLR2 [307]. TLR2 also recognizes the hemagglutinin protein of Measles virus. The detection of viral glycoproteins by TLR2 and TLR4 is generally associated with the synthesis of NF-κB-dependent proinflammatory cytokines such as TNF-α, IL-6 and IL-1 [308]. In addition, a study by Georgel *et al.* demonstrated that VSV-encoded glycoprotein G (gpG) stimulates IRF7, through both TLR4 and CD14, in mDCs and macrophages [309]. This study represented the first description of TLR-mediated recognition of a viral protein leading to IRF activation and type I IFN induction. Expression of TLRs is compartmentalized: TLR2 and TLR4 are present on the plasma membrane, whereas TLRs 3, 7, 8 and 9 localize within cytoplasmic vesicles such as endosomes and the endoplasmic reticulum (ER) [310]. The subcellular localization of nucleic acid-sensing TLRs is crucial for the discrimination of virus- versus self-derived components.

Upon stimulation with their cognate viral PAMPs, TLRs form active homodimers or heterodimers in order to induce different signaling cascades, and upregulate the transcription of distinct genes including proinflammatory cytokines and type I IFN genes. The difference in the signaling pathways activated by individual TLRs can be partly explained by the TIR domain-containing adaptors molecules recruited to TLRs. These TIR domain-containing adaptors molecules include myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like molecule (MAL), TIR-containing adaptor inducing interferon β/TIR-domain containing adaptor molecule 1 (TRIF/TICAM1), TIR-domain containing adaptor molecule 2/TRIF-related adaptor molecule (TICAM2/TRAM) [311-312]. TLR signaling is roughly divided into two distinct pathways: MyD88-dependent and TRIF-dependent pathways. TLR7 and TLR9 use MyD88 as the adaptor to relay downstream signaling, whereas, TLR3 does not

require MyD88 but rather uses TRIF for signaling. TLR4 is unique in the fact that it recruits all four adaptors, MyD88, MAL, TRIF, and TRAM to trigger two independent signaling cascades NF-κB and IRF3/7. MyD88 and TIRAP act in concert; in contrast, TRIF requires TRAM for interaction with TLR4.

TLR3 senses the synthetic analog of dsRNA, poly(I:C), often used as a mimic of viral infection, genomic RNA purified from dsRNA viruses (such as Reovirus) and dsRNA produced during the course of replication of ssRNA viruses such as RSV, EMCV and West Nile Virus (WNV) [313-314]. Stimulation of TLR3 with one of these RNA species, leads to the recruitment of TRIF, which subsequently associates with TNF-receptor-associated factor 3 and 6 (TRAF3 and TRAF6) and receptor-interacting protein 1 (RIP-1) [315-317]. TLR3 and the RLRs signaling pathways share TRAF3 to stimulate the synthesis of type I IFNs. On the other hand, TRAF6 and RIP1 activate NF-κB, resulting in the expression of proinflammatory cytokines. Mice deficient for TLR3 die earlier than wild-type mice following infection with murine cytomegalovirus (MCMV) and TLR3 deficiency in humans is associated with susceptibility to herpes simplex virus (HSV-1) infection. TLR3 recognizes dsRNA, in conventional DCs (cDCs), while TLR7 and TLR9 are highly expressed in pDCS, a cell type known to synthesize extremely large amounts of type I IFNs in response to virus infection.

TLR7 and TLR9 activate distinct signaling pathways in response to viral RNA or DNA in pDCs. TLR9 recognizes unmethylated CpG DNA motifs present in the genome of DNA viruses such as herpes simplex virus (HSV) and MCMV; these viruses will be not discussed in detail in this thesis since the focus is on the innate immune response to RNA viruses. In pDCs, TLR7 interacts with the adaptor MyD88, which in turn recruits IL-1R-associated kinase-1 (IRAK1), IRAK4 and IRF7. IRAK1 and IKKα have been identified as potential kinases mediating IRF7 phosphorylation and activation [318-320]. Once phosphorylated, IRF7 is released from the MyD88-containing complex and migrates into the nucleus to initiate the transcription of IFN-inducible genes. TRAF3 is

also involved in this signaling pathway. In addition of its role in the IFN pathway, the MyD88-dependent pathway is implicated in NF-κB activation leading to the expression of cytokines, including IL-12 and IL-6. In pDCs, autophagy is a mechanism essential for the expression of type I IFNs in response to Vesicular Stomatitis Virus (VSV) and HSV infection [321]. Mice with pDCs defective for autophagosome formation have impaired type I IFN production following VSV infection. It has been suggested that viral RNAs are captured by autophagosomes, which then fuse with lysosomes containing TLR7.

#### *2.1.1.2. The Cytosolic Pathway - Recognition of RNA viruses via RLRs*

Double stranded RNA (dsRNA) represents a molecular intermediate during the viral replication of many viruses within infected cells. Therefore, it is not surprising that the host has evolved many strategies to detect its presence. RLRs are cytoplasmic viral RNA sensors responsible for the production of type I IFNs. The RLRs family is composed of RIG-I (DDX58), melanoma differentiation-associated gene 5 (MDA-5), and LGP2 (Laboratory of Genetics and Physiology 2) [322-323]. RIG-I and MDA-5 contain two N-terminal caspase recruitment domains (CARDs) and a C-terminal regulatory domain (CTD), which are important for their activity. LGP2 shows 31 and 41% aa identities to the helicase domains of RIG-I and MDA5, respectively, but completely lacks the two N-terminal CARDs [324]. All three molecules have a central DEAD box helicase/ATPase domain. During virus infection, the expression of RLRs is strongly enhanced in response to type I IFN stimulation. Although RIG-I and MDA-5 share similar structures, they detect different viral RNAs and therefore their functions are not redundant. Much of the virus specificity between RIG-I and MDA-5 resides in the particular RNA structures or nucleotide composition recognized by each. Initially, RIG-I and MDA-5 were identified as sensors of polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of viral dsRNA. Kato *et al.* demonstrated a length-dependence for the activation of RIG-I and MDA-5 by poly(I:C). Long polymers of poly(I:C) activates MDA-5 whereas shortening the length of the poly(I:C) by RNase III treatment converts

poly(I:C) from an MDA5 ligand to a RIG-I ligand [325]. This suggests that RIG-I recognizes short viral dsRNA and MDA-5 long dsRNA.

Some viral RNAs are 5'-triphosphorylated and uncapped, whereas the 5' ends of host mRNAs are capped. RIG-I, but not MDA-5 recognizes dsRNA with a 5' triphosphate end although it also recognizes 3' monophosphate small RNAs [326]. Thus, RIG-I distinguishes viral RNAs from host RNAs based on differences in the 3' and 5' ends of RNAs. However, it has been reported that RIG-I can also detect short dsRNA (up to 1kb) without a 5' triphosphate end although its presence strongly increases type I IFN synthesis. Chemically synthesized dsRNAs bearing a 5' monophosphate end or those lacking 5' phosphate were able to potently activate RIG-I but produced lower amounts of IFNs compared to dsRNA with a 5' triphosphate end [327]. Depletion studies of RIG-I demonstrated its essential role in the production of type I IFNs in response to positive and negative RNA viruses such as paramyxoviruses, orthomyxoviruses, flaviviruses and rhabdoviruses. Although RIG-I was first described as a sensor for dsRNA, Pichlmair *et al.* reported that ssRNA with a 5'-triphosphate end can be also recognized by RIG-I [328]. However, recent contradictory reports indicates that the results obtained by Pichlmair *et al.*, were due to unintended hairpins produced during T7 *in vitro* transcription and thereby the RNA detected by RIG-I requires some double-stranded structure [329-330]. Therefore, the exact nature of the RNA recognized by RIG-I is still controversial and remains to be clarified. MDA-5 detects long dsRNA (more than 2kb) produced during theiler's virus, mango virus and Picornaviruses infection (EMCV).

Since LGP2 lacks a CARD domain, it is suggested to function as a negative regulator of RIG-I/MDA-5 signaling by sequestering dsRNA or inhibiting RIG-I conformational changes [331-333]. LGP2 contains a regulatory domain (RD), which has been demonstrated to interact with the RD of RIG-I, thereby inhibiting RIG-I self-association, possibly by disrupting homotypic CARD/helicase domain and/or C-terminus interactions. LGP2 controls the RIG-I signaling pathways at multiple steps, indirectly

through sequestration of RNA substrates and/or through disruption of the MAVS signaling complex. Crystollagraphy and *in vitro*/*in vivo* biochemical studies demonstrated that LGP2 CTD regulates RIG-I-dependent signaling by competiting with RIG-I for dsRNA binding independently of 5′-triphosphates, while a fully functional LGP2 is needed to augment MDA-5-dependent signaling [334]. Conversely, other studies showed that enzymatic activity of the helicase and RNA binding are not necessary for LGP2 mediated signaling inhibition, thus supporting an RNA-independent interference mechanism [335-336]. LGP2 knockout mice are resistant to VSV infection, since replicative RNA intermediates of VSV are detected by RIG-I rather than by MDA-5. Although LGP2 has been identified as a negative regulator of RIG-I/MDA-5 signaling, LGP2-deficient mice exhibited a defect in type I IFN production in response to infection by picornavirus such as ECMV indicating a positive role for LGP2 in antiviral signaling in response to picornavirus [337]. Further studies are needed to clarify the physiological role of LGP2 in the innate immune system.

### *2.1.2. Signaling downstream of RLR receptors*

Although the ligands and regulation mechanisms for the RLR members are different, RIG-I and MDA5 activate a common signaling pathway that induces type I IFN and creates the host's protective innate immune response against viral infection. Their helicase domain mediates recognition of viral RNA structures, triggering a conformational change and exposing their N-terminal CARD domains. This structural modification allows the two CARDs of RIG-I and MDA-5 to bind to the homotypic domain of the CARD-containing protein - the Mitochondrial Antiviral Signaling Adapter (MAVS) - also known as (IPS-1/VISA/Cardif).

Of note, dsRNA-mediated signaling is positively and negatively regulated by ubiquitination of RIG-I. First, the tripartite motif 25 (TRIM25) and Riplet (RNF135) act as E3 ubiquitin ligases that trigger K63-linked polyubiquitination of the CARDs of RIG-I [338]. This post-tranlational modification of RIG-I is required for conformational change,

efficient activation of RIG-I signaling pathway and thereby effective production of type I IFN. TRIM25-/- cells show impaired production of type I IFN after viral infection.

Several reports demonstrated the essential role of MAVS in RIG-I/MDA-5 signaling. Mice deficient for MAVS present impaired-proinflammatory cytokines and type I IFNs in response to all RNA viruses sensed by RIG-I and MDA-5 [339]. Additionally, ectopic expression of MAVS induces activation of both, IFN and NF-κB promoters [340]. MAVS contains an amino-terminal CARD domain, a proline-rich region (PRR) in the middle of the protein, and a C-terminal transmembrane domain (TM) that localized MAVS to the outer mitochondrial membrane - suggesting that beyond its role in metabolism and cell death, mitochondria may be essential for innate IFN responses. The NS3/4A protease of hepatitis C virus (HCV) cleaves the C-terminal end of MAVS – adjacent to the mitochondrial targeting region – suggesting that disruption of the mitochondrial association of MAVS is part of the innate immune evasion strategy used by HCV [341].

Interaction of RIG-I/MDA-5 with MAVS triggers dimerization/oligomerization of MAVS molecules and initiation of downstream signaling leading to activation of the classical IKK complex and the two atypical IKK-related kinases - TRAF family member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), the inducible IκB kinase (IKKε) [342] **(Figure 16)**. Activation of these upstream kinases leads to the phosphorylation of IRF3/IRF7 and NF-κB, resulting in the induction of the antiviral response. While MAVS binds to RIG-I and MDA-5 through its N-terminal CARD domain, its C-terminal effector-domain recruits signaling proteins such as TRADD, FADD (FAS associated via death domain), RIP1, TRAF6 and TRAF2, all involved in NF-κB activation pathway. On the other hand, the exact mechanisms by which MAVS activates TBK1 and IKKε remains to be clarified but it requires the participation of TRAF3. TRAF3 serves as a bridge to IRF3/IRF7 activation by interacting with both MAVS and either TBK1 or IKKε. Following recruitment of TRAF3 to the signaling complex, the RING-domain of TRAF3 – which is responsible for its E3 ubiquitin ligase

activity - preferentially assembles Lysine-63 polyubiquitin chains, thus forming a scaffold for the assembly of a signaling complex composed of TANK (TRAF family member-associated NF-κB activator), or NAP-1, NEMO (NF-κB essential modulator or IKKγ), IKKε and TBK1, leading to the activation of IRF3/IRF7.

NEMO, the regulatory subunit of the IKK complex, serves as a bridge to the NF-κB and IRF signaling pathways and promotes cross-talk between the two pathways during virus- and/or RIG-I-mediated signaling. Recently, our group demonstrated that NEMO is required for virus-induced activation of IRF3/IRF-7 and acts "upstream" of IKKs and TBK1 and "downstream" of MAVS and RIG-I [343-344]. NEMO was found to physically bind to TANK and mediates the recruitment of TBK1/IKKs to the RIG-I/MAVS complex leading to activation of IRF3 and IRF7. Activation of the NF-κB pathway necessitates the recruitment of TRAF2/6 to MAVS, followed by interaction and K63-linked ubiquitination of Receptor Interacting Protein 1 (RIP-1) and the binding to the regulatory subunit of the IKK complex, NEMO. Phosphorylation of the NF-κB inhibitor (IκBα) by IKKβ leads to its proteasomal degradation and the release of active NF-κB dimers.

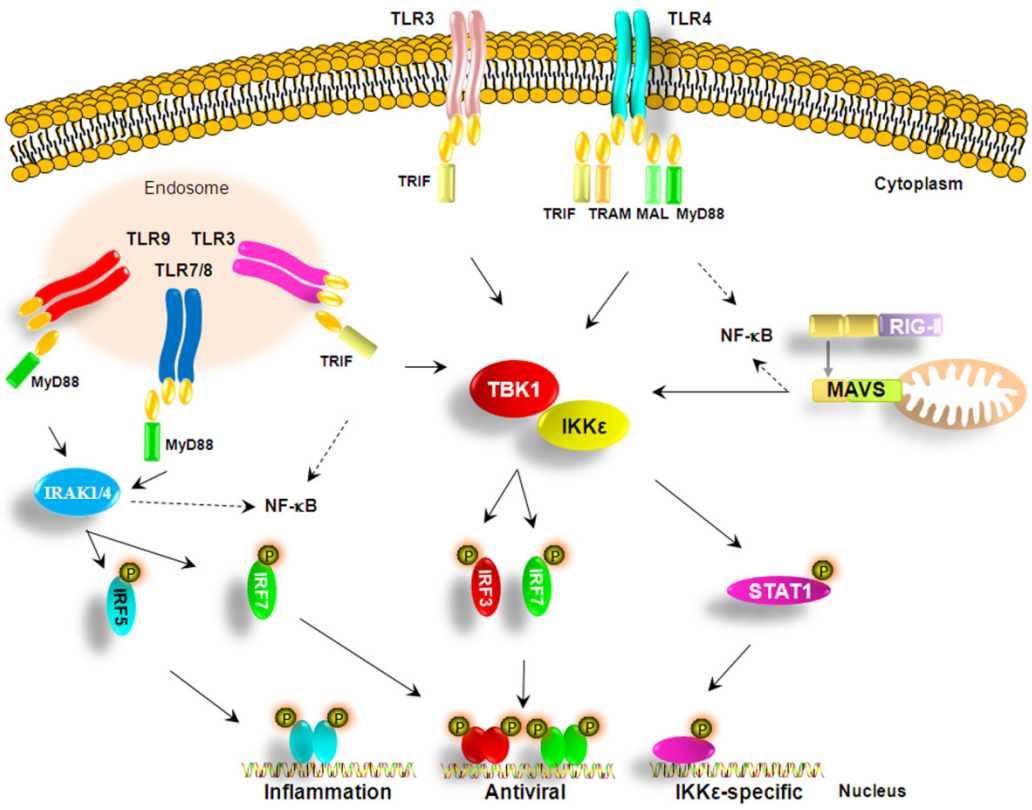



**Figure 16. TLR-dependent and -independent sensing of viral infection leads to TBK1/IKKε activation.** Simplified schematic representation of TLR-3, -4, -7/8, -9 and RIG-I signaling pathways, leading to TBK1 and IKKε activation. The following abbreviations are shown: TLR (toll-like receptor), RIG-I (retinoic acid inducible gene I), MAVS (mitochondrial antiviral-signaling protein), MyD88 (myeloid differentiation primary response gene 88), TRIF (TIR-containing adaptor inducing interferon β), TRAM (TRIF-related adaptor molecule), MAL (MyD88 adaptor-like molecule), IRF (interferon regulatory factor) and STAT1 (signal-transducer and activator of transcription 1). Reproduced with permission from [345].

### *2.1.3. Interferon regulatory factors*

Interferon Regulatory Factors (IRFs) fulfill a role as transcriptional mediators between viruses and IFN signaling pathways. IRF members play a critical role in the transcriptional regulation of type I IFN genes, ISGs, and other cytokines/chemokines. Beyond their role in the antiviral defense, IRFs are involved in apoptosis, immune regulation, cell cycle, and tumor suppression. The IRF family of transcription factors includes nine members: IRF1, IRF2, IRF3, IRF4 (PIP or ICSAT), IRF5, IRF6, IRF7, IRF8 (ICSBP), and IRF9 (ISGF3g) [346-347]. Each members of the IRF family is induced or expressed ubiquitously at different levels in both lymphoid and non-lymphoid cell lineages, with the exception of IRF4 and IRF8 which are restricted to hematopoietic cells. All IRFs are constituted of a well-conserved amino (N)-terminal DNA binding domain (DBD), characterized by five tryptophan repeat elements located within the first 150 amino acids of the protein and similar to the DBD of myb transcription factors [348]. Each IRF, with the exception of IRF1 and IRF2, possesses an unique C-terminal domain - the IRF association domain (IAD) - responsible for the interaction with other family members or transcription factors (such as PU.1), signal transducer, activator of transcription (STAT) and thereby formation of homo- and hetero-dimers [347]. Then, the dimers translocate into the nucleus and bind to specific consensus sequences through their DBD. Indeed, type I IFN induction is mainly due to the transcriptional activation of the sequence domain in the 5' end of IFN genes named the virus responsive element (VRE). The VRE consists of a complex enhancers constituted of at least four regulatory *cis* elements - positive regulatory domain I (PRDI), PRDII, PRDIII and PRDIV – interacting with different, cooperating transcription factors [349]. IRFs dimers bind to PRDI (GAAANNGAAANN) and (PRDIII AANNNGAA) also termed the IFN-stimulated regulatory element (ISRE) and IRF-binding element, respectively. NF-κB and ATF-2/c-Jun associate with PRDII and PRDIV, respectively, and cooperate with IRFs for the full induction of type IFN genes.

The unique function of a specific IRF stems from the ability of the IAD to bind to other members of the IRF family (and other factors), its intrinsic transactivation potential and cell type-specific expression of the IRFs. The sequence homology of the IAD domain amongst IRFs family members is low. Nevertheless, some structural similarities have been found between the IAD domain of IRFs and the Mad-homology 2 (MH2) regions of the Smad family of transcription factors, which mediate protein-protein interaction [350-351]. Indeed, the binding and cooperation of IRF7 with Smad3 for the activation of type I IFN gene expression has been reported previously [350, 352].

The majority of IRFs are implicated in distinct aspects of the antiviral response [353] while two members - IRF-4 and IRF-8 - are regulators of hematopoiesis [354-355]. Four members of the IRF family - IRF1, IRF3, IRF5 and IRF7 - have been characterized as positive regulators of type I IFN gene transcription in response to virus infection. IRF1 was the first IRF-member described as inducer of IFN-β genes induction, whereas IRF2 inhibits IFN expression [356-357]. Overexpression studies demonstrated that ectopic expression IRF1 and IRF5 induced type I IFN gene expression. However, the essential role of IRF1 in the regulation of type I IFN genes became controversial when Matsuyama *et al.* reported that IRF1-/- mouse embryonic fibroblasts (MEFs) cells were still able to produce IFN-β and IFN-α mRNAs in response to NDV infection [358]. Similar results were obtained in IRF5-/- cells, but demonstrated that inspite of its dispensable role in the regulation of type I IFN gene expression, IRF5 is a crucial inducer of proinflammatory cytokines such as IL-12 and TNF-α [359]. Altogether, these studies indicate that neither IRF1 nor IRF5 is essential for transcription of type I IFN genes in response to virus infection, and the exact role of IRF1 and IRF5 in type I IFN induction remains elusive. The subsequent search of others IRFs family members that would be involved in the transcriptional activation of IFN-α/β promoters led to the identification of IRF3 and IRF7.

Innate immune sensing of RNA viruses infection is mediated by host PRRs, including RIG-I and MDA-5 and several TLRs members such as TLR3 and TLR7. These PRRs function as detectors of dsRNA species that are synthesized by viruses during

replication, and trigger IFN gene induction. Activation of IFN gene transcription in virus infected cells is a central event in innate immunity and requires the assembly of three distinct families of transcription factors, including NF-κB, ATF2/c-Jun, and IRF3/IRF7. As the antiviral response relies on type I IFN signaling, IRF3 and IRF7 which participate in the cooperative production of IFNs-α/β, represent master regulators of the host recognition machinery that require virus specific activation.

#### *2.1.3.1. IRF3 master regulator of IFN signaling*

Interferon regulatory factor 3 (IRF3) gene encodes a 427 amino acid phosphoprotein of 55 kd constitutively expressed, in a latent form, in the cytoplasm of all cell types (reviewed in [360-362]. Transcriptional activity of IRF3 is controlled by viral PAMPs, such as dsRNA treatment or virus-induced extensive C-terminal phosphorylation at a serine/threonine cluster localized between amino acids 382 to 405. IRF3 phosphorylation leads to a cascade of events including conformational change allowing dimerization (either a homodimer or a heterodimer with IRF7), nuclear translocation, and association with the histone acetyltransferase co-activator CBP/p300 to form a holocomplex in the nucleus. The holocomplex then interacts with its target DNA sequence and modifies the local chromatin structure *via* the histone-acetyltransferase activity of the co-activator; thereby, efficient transcription of target genes including IFN-β and CCL5, CXCL10, NOXA, is initiated [363-366]. Viral PAMPs-mediated IRF3 phosphorylation ultimately leads to IRF3 ubiquitination and proteasomal degradation [363]. The importance of IRF3 in the antiviral response has been supported by studies showing that IRF-3-/- mice are more vulnerable to viral infection and their serum type IFN levels are significantly lower than IRF3 wild-type mice.

#### *2.1.3.2. Functional domains of IRF3*

IRF3 contains an intrinsic nuclear localization signal (NLS) and a nuclear export signal (NES). These two sequences are constitutively active and allow IRF3 to shuttle in and out of the nucleus. However, in absence of activating stimulus, the NES motif is dominant and the latent form of IRF3 is restricted to the cytoplasm, exhibiting little or no

DNA-binding properties [356] **(Figure 17)**. The three-dimensional crystal structures of the IRF-3 DNA binding domain and C-terminal domain have been independently and extensively reported [350-351, 367]. In these studies, they showed that IRF3 activity relies on the N- (aa 134-197) and C-terminal (aa 407-414) autoinhibitory domains within the C-terminal IRF association domain (IAD). In absence of activation, these two domains bind to each other to form a highly condensed hydrophobic core. This interaction hides the DNA Binding Domain (DBD), the NLS and several key residues within the C-terminal region of the IAD required for IRF3 dimerization, thereby preventing IRF3 nuclear accumulation, DNA binding and transactivation. Following exposition to PAMPS, IRF3 undergoes several post-translational modifications including hyperphosphorylation, which is responsible for its activity. Indeed, C-terminal phosphorylation events eliminate autoinhibitory interactions by adding charge repulsions within this region and thereby exposing the IAD active site and realigning the DBD. This event results in the formation of a transcriptionally active IRF3 protein, able to translocate into the nucleus, associate with CREB binding protein (CBP/p300) co-activator and participate in the transcriptional activation of the IFN-β and human IFN-α1 promoters [365, 368].

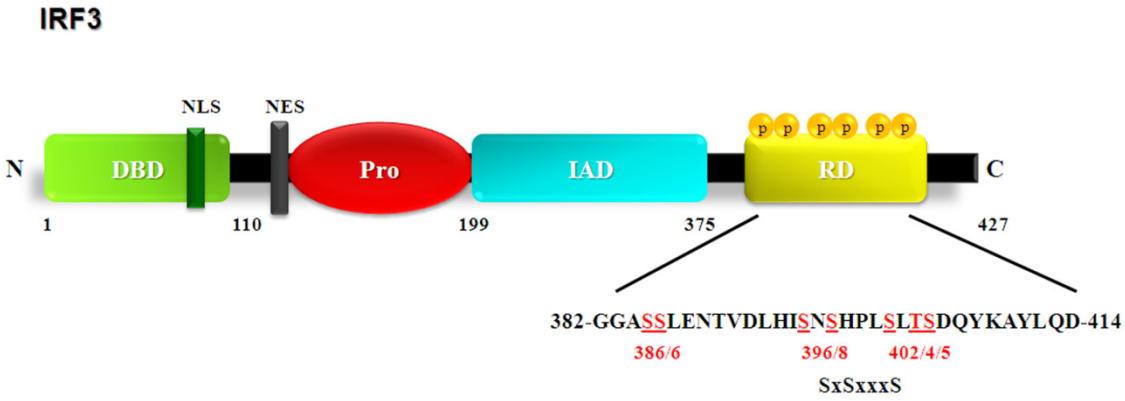



**Figure 17. Schematic representation of IRF3.** Schematic representing the principal domains of IRF3. Different domains are shown: the nuclear localization sequence (NLS), the nuclear export sequence (NES), the DNA-binding domain (DBD), the proline-rich region (Pro), the IRF-association domain (IAD), and the signal response domain (RD). The sequence of amino acids (aa) 382-414 are amplified below. The serine/threonine residues contributing to IRF3 activation are shown in red. TBK1/IKKε phosphorylation consensus site is also shown as SxSxxxS. Adapted with permission from [345].

### *2.1.3.3. Mechanisms of IRF3 activation*

#### 2.1.3.3.1. TBK1 and IKKε – trigger IRF3 phosphorylation

The expression patterns of TBK-1 and IKKε are distinct: TBK-1 expression is ubiquitous and constitutive in a large panel of cells, while IKKε is restricted to immune cells but is inducible in non-hematopoietic cells by stimulation such as TNF, phorbolmyristate acetate (PMA), LPS and virus infection [369-371]. It has been shown that TBK-1 and IKKε directly phosphorylate IRF3 and IRF7 at their C-terminal domain

*in vitro* and both kinases target identical serine residues [372-373]. Our group demonstrated by alignment of the primary sequence of the C-terminal domains of IRF3 and IRF7 that an extended SxSxxxS consensus motif is the target for TBK-1 and IKKε [374]. However, analysis of TBK-1-/- and IKKε-/- mice demonstrated that TBK-1 is the principal and essential kinase for the phosphorylation IRF3 and IRF7 and development of the antiviral response [375-376]. It has been thought that the role of IKKε in the development of the antiviral response was secondary and redundant with TBK-1. However, studies revealed that IKKε specifically induces a subset of key IFN-responsive genes during virus infection. It has been shown that mice lacking IKKε synthesized normal levels of IFN-β, but were hyper-susceptible to viral infection because of a defect in the IFN signaling pathway; indeed, a number of type I IFN-stimulated genes were not induced in the absence of IKKε because the interferon-stimulated gene factor 3 complex (ISGF3) was no longer able to interact with the promoters of these genes. TenOever *et al.* reported that IKKε -/- MEFs induced only 30% of the IFN-inducible ISGs including ADAR1, IFIT3 and IFI203, whereas other ISGs such as IRF7, PKRA (RNA-activated protein kinase) and STAT1, were normally synthesized. Phosphorylation of STAT1 by IKKε at Ser708 (Ser708), Ser744, and Ser747 is required for ISGF3 binding to IKKε-dependent promoters [320]. Phosphorylation of STAT1 by IKKε triggers the transcription of a subset of ISGs essential for a direct antiviral response. Following viral infection, TBK-1 and IKKε localize to different subcellular compartments. Confocal microscopy and studies revealed that VSV infection induces IKKε recruitment to the mitochondria while TBK-1 remaines cytoplasmic [377]. Overexpression of MAVS lacking the transmembrane domain prevented IKKε from localizing to the mitochondria and therefore suggests that sequestering of IKKε to the mitochondria is mediated by MAVS.

In the absence of infection, IRF-3 exists as two forms in the cytoplasm (designated I and II), However, four forms of IRF3 are induced following Sendai virus infection when separated by SDS-PAGE (Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis). Form II of IRF3 has been reported to be a N-terminal phosphorylation (aa 186 to 198) modification of form I. Treatment with stress inducers (sorbitol, DNA

damage inducers, TNF-α, PMA) induce activation of stress signaling pathways such as MAP kinase kinase kinase (MAPKKK) responsible for phosphorylation of IRF3 in its N-terminal domain. However, this phosphorylation is not sufficient to trigger a nuclear accumulation, a transcriptional activity or a degradation of IRF3. Following viral infection, a threonine residue situated at the amino acid 135 of IRF3 has been reported to be a substrate for the DNA dependent protein kinase (DNA-PK). Phosphorylation at T135 activates IRF3 and induces its sequestering in the nucleus and its stabilization.

#### 2.1.3.3.2. IRF3 phosphorylation

In response to a variety of stimulus including virus infection or dsRNA, forms I and II of IRF3 receive multiple phosphorylation modifications. Indeed, the C-terminal domain of IRF3 is phosphorylated on several serines (S) and threonines (T) residues localized on a region termed the signal response domain (RD) 381-VGGASSLENTVDLHISNSHPLSLTSDQYK-409. These phosphoacceptor sites can be divided in three clusters, all important for the optimal activity of IRF3: region I (Ser385/Ser386), region II (Ser396/Ser398) and region III (Ser402/Thr404/Ser405). It has been suggested that phosphorylation of IRF3 at Ser386 induces its dimerization. Regardless of several studies including mutagenesis phosphopeptide mapping and phosphospecific antibodies, the role of each phosphorylated Ser and Thr residue in the activation of IRF3 remains unclear. Fujita's group proposed that Ser385 and Ser386 are the key phosphorylation sites for IRF3 activation whereas a second phosphorylation site, spanning amino acids 396 to 405 play a secondary and non-essential role [378]. This report was supported by another study showing that mutation of Ser385 or Ser386 to Alanine (Ala) disrupts IRF3 activity. Phosphospecific antibody against Ser386 further demonstrated that virus-triggered phosphorylation of IRF3 is overexpressed at this site. In addition, substitution of Ser385/Ser386 with either Ala or Asp does not trigger a constitutively active form of IRF3 but rather inhibits virus-induced dimerization, association with CBP/p300, DNA binding and transcriptional activity. Our group demonstrated that the region between amino acids 395 and 407 is important for virus-mediated IRF3 activation. Substitution of the serine and threonine residues in this region

(395-ISNSHPLSLTSDQ- 407) (Ser396, Ser398, Ser402, Thr404 and Ser405) by alanine residues abrogated virus-induced activation of the protein, including C-terminal phosphorylation, dimerization and nuclear translocation [379]. However, mutation of these five serines or threonine to phosphomimetic Asp or Glu induced a constitutively active form of IRF3 known as IRF3-5D. IRF3-5D dimerizes, translocates to the nucleus, associates the CBP/p300, and activates the transcription of target genes even in the absence of viral infection. Mutagenesis studies and use of phospho-specific antibodies identified Ser-396 as a key residue that is phosphorylated *in vivo* upon virus infection. Studies from Panne's group gave some knowledge on the role of the different IRF3 phosphorylation sites [380]. This group proposed a two-step phosphorylation model based on an *in vitro* study showing that TBK-1 and not IKKε can directly activate full-length IRF3. Using purified proteins, they showed that TBK1 directly phosphorylates IRF3 *in vitro* and that the phosphorylation of residues found between amino acid 396 and 405 removes autoinhibition to allow interaction with CBP and facilitate Ser385/Ser386 phosphorylation. Ser386 is believed to be required for IRF3 dimerization. Two new phosphoacceptor sites, Ser339 and Thr390, have been identified and characterized independently. In combination with Ser396, Ser339 has been suggested to be responsible for inducing a hyperactive form of IRF3 giving the ultimate signal required for polyubiquitination and degradation [378]. Recently, Bergstroem *et al*. identified Thr390 as a novel *in vivo* phosphorylation site that modulates the phosphorylation status of TBK1-targeted Ser396 [381].

## 2.2. Late wave of IFN signaling

To swiftly eradicate incoming virus, the host induces the early expression of IFN-β through activation of IRF3, responsible for linking pathogen sensing to IFN-β production ("early phase"). The initial production of IFN-β relies in part on the C-terminal phosphorylation and dimerization of IRF3. Once activated, IRF3 translocates into the nucleus where it associates with the CREB binding protein (CBP/p300) co-activators to form a holocomplex. This complex contributes to the transcriptional activation of the human IFN-β and IFN-α1 promoters (mice IFN-α4). Since the amount of IFN secreted is not enough to protect neighbouring cells from the intruding virus, the newly secreted IFN-β/α1 act in an autocrine and/or paracrine-manner by binding to the type I IFN receptor and activating the classical JAK-STAT (signal transducer and activator of transcription-signaling pathway) to produce the other IFN-α subtypes, thus amplifying the antiviral response.

### *2.2.1. JAK/STAT signaling pathway*

Type I IFN receptor is composed of two distinct subunits: IFNAR1 and IFNAR2 associated with tyrosine kinase 2 (TYK2) and Janus activated kinase 1 (JAK1), respectively [382-384]. The members of the signal transducers and activators of transcription (STATs) family - STAT2 - has been shown to be associated with IFNAR2 before induction and is bound weakly with STAT1 [385]. Binding of type I IFNs to the transmembrane type I receptor leads to the multimerization of the IFNAR1 and IFNAR2 subunits (**Figure 18**). Because IFNAR itself has no intrinsic kinase activity, cytosolic, membrane-associated tyrosine kinases - TYK2 and JAK1 - mediate this signal transduction event. Ligand induces dimerization of the IFNAR receptor which provokes a conformational change bringing JAK1 and TYK2 into close proximity to each other, and results in the activation of both kinases. Indeed, TYK2 phosphorylates tyrosine 466 on the IFNAR1 which provides docking sites for STAT2. TYK2, in turn, phosphorylates

STAT2 on tyrosine 690, and STAT1 weakly associated with STAT2 is phosphorylated by JAK1 on tyrosine 701. Once phosphorylated, STAT1 and STAT2 are released from the receptor and form homodimers as well as heterodimers. The dimers translocate into the nucleus and the STAT1/STAT2 heterodimer interacts with a DNA binding protein - IRF9 (p48) - to form a heterotrimeric transcriptional activator known as the interferon-stimulated gene factor 3 (ISGF3). ISGF3 binds to the IFN-stimulated response element (ISRE), found in numerous IFN-reponsive genes, including IRF7, and initiates their transcription.

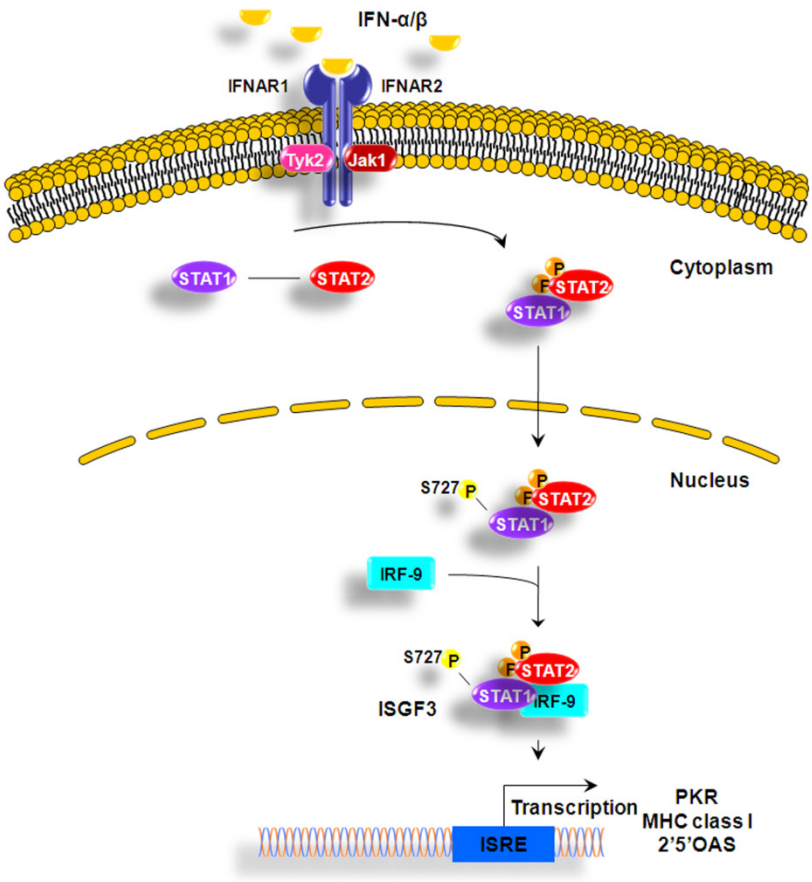



**Figure 18. JAK/STAT signaling pathway activated by type I IFN.** The biological activities of IFNα/β are initiated by binding to IFNAR, leading to the activation and phosphorylation of the receptor-associated tyrosine kinases TYK2 and JAK1, which in turn phosphorylate STAT1 and STAT2. Then, phosphorylated STAT1 and STAT2 interact with each other by recognizing their SH2 domains. STAT1-STAT2 heterodimers translocate into the nucleus where they bind to the DNA-binding protein IRF9 (p48) to form a complex called ISGF3. ISGF3 binds to a sequence motif (ISRE) in target promoters and stimulates transcription of genes such as serine/threonine protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS), myxovirus-resistance proteins (Mx), RNA-specific adenosine deaminase (ADAR), IRF7, MHC and many other ISGs. Adapted from [297].

### *2.2.2. IRF7 - Amplification loop*

Unlike IRF3, IRF7 is a potent activator of IFN gene transcription that can enhance the synthesis of all IFNα and IFNλ gene family members as well as IFNβ, resulting in the amplification of the IFN response [386-388]. IRF7 is expressed in low amounts in most cell types and can be strongly induced by type I IFN-mediated signaling. Constitutive IRF7 expression is restricted to B cells and dendritic cells. The turnover of IRF7 protein is fast, with a half-life of approximately 30 minutes, which represents a mechanism to guarantee a rapid shut-down of IFN induction [361, 389]. IRF7 is constituted of a DNA binding domain (DBD) in the N-terminus and multiple regulatory domains in the C-terminal region that control IRF7 activity, including a constitutive activation domain (CAD), a virus-activated domain (VAD), an inhibitory domain (ID) and a signal response domain (SRD) [390-391] **(Figure 19)**. IRF7 phosphorylation by TBK-1 relies upon viral infection, ensuring that IFN production is restricted to the infected cells. Similarly to IRF3, upon viral infection, IRF7 which is found in the cytosol, undergoes serine phosphorylation of its C-terminal domain, dimerizes and translocates into the nucleus. Biochemical data demonstrated that the C-terminal VAD between amino acids 471-487 is the target of extensive virus induced phosphorylation. Activation required phosphorylation at Serines 471/472 and 477/479 and substitution of these phosphoacceptor sites by alanine resulted in an IRF7 that was non-responsive to virus infection. Therefore, IRF7 was not longer able to translocate into the nucleus, bind to the DNA and transactivate the IFN-α4 promoter. On the other hand, substitution of the Ser477/479 with the phosphomimetic amino acid Asp leads to the generation of a constitutively active IRF7 [373]. It has been suggested that IRF7 is activated in a similar fashion than IRF3: the inactive IRF7 protein is folded on itself, the C-terminus ID hides the DBD and/or CAD. Upon activation, phosphorylation of key residues leads to electrostatic changes that force opening up of the structure, allowing dimerization and DNA binding.

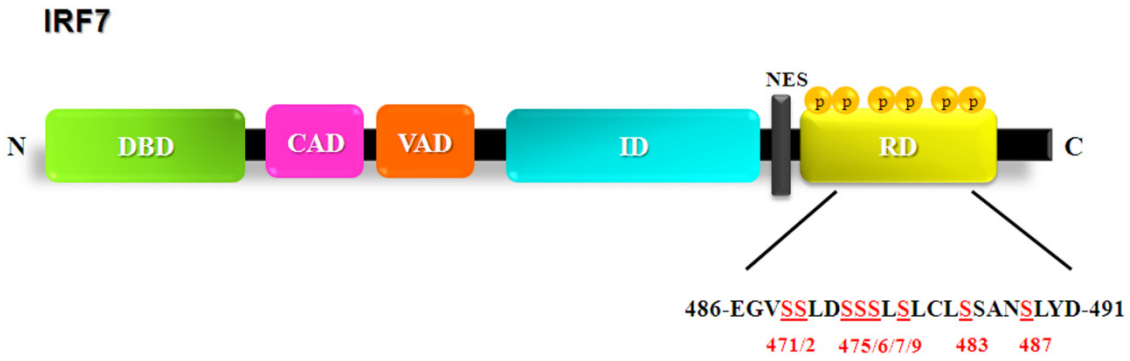



**Figure 19. Schematic representation of IRF7.** Schematic representing the principal domain of IRF7. Different domains are shown: the DNA-binding domain (DBD), the constitutive activation domain (CAD), the virus-activated domain (VAD), the inhibitory domain (ID), the nuclear export sequence (NES) and the signal response domain (RD). The sequence of amino acids 468-491 are detailed below. The serine residues contributing to IRF7 activation are shown in red. Adapted from [345].

Together IRF3 and IRF7 antagonize viral infection, as each plays a distinct role during the course of the antiviral response [350, 392-393]. IRF3 stimulates the transcription of the IFNβ genes whereas IRF7 activates the full complement of IFNα and IFNβ genes. Thus, IRF3 is required for the initial synthesis of the IFNβ gene whereas IRF7 is involved in the late phase of type I IFN gene expression. Suppression of both IRF3 and IRF7 in murine embryonic fibroblast cells (MEFs) fails to induce type I IFN mRNA in response to viral infection, an effect that could be reversed by ectopic expression of both proteins [394]. Honda *et al.* suggested that the contribution of IRF3 to type I IFN synthesis is minor in the absence of IRF7. IRF7-/- MEFs were deficient for

detectable type I IFN gene expression following ssRNA virus infection (VSV and EMCV) [386-387]. Therefore, a revised model of the type I IFN amplification loop has been suggested in which low levels of IRF7 would be essential for activating the initial phase of type IFN gene induction.

### *2.2.3. Biological effects of type IFNs*

Many viruses produce dsRNA during their life cycle, and both virus infection and exogenous dsRNA induces IFN synthesis. Type I IFNs are rapidly synthesized and exert diverse biological functions including prevention of viral invasion by blocking many steps of the virus life cycle. These cytokines also mediate an anti-viral state in surrounding cells by stimulating the transcription of more than 300 cellular genes - known as ISGs - involved in apoptosis, cell-cycle, innate and adaptive immune cell activation. The best-characterized ISGs encode proteins such as PKR, 2'-5'-oligoadenylate synthetase (OAS), RNase L and Mx that require dsRNA as a cofactor or substrate for their activity. Knock-out mice studies reveal that lost of one of these factors dramatically increased susceptibility to virus infection. PKR is constitutively expressed in an inactive form in the cytoplasm of most cell types and in response to dsRNA, actived PKR mediates translational control by phosphorylating the protein synthesis initiation factor eIF2α. Phosphorylation of eIF2α leads to a rapid inhibition of translation of most viral and cellular mRNAs, thereby preventing viral spread and full amplification of the virus-induced cellular stress response. PKR is also involved in cell death, a process that results from the combination of two simultaneous mechanisms: a) phosphorylation of the elongation initiation factor alpha (eIF2α), which blocks protein synthesis and b) activation of NF-κB, which induces transcription of both the death receptor Fas (CD95) and TNF-related apoptosis inducing ligand (TRAIL). IFN also induces OAS gene expression which, upon binding to viral double-stranded RNA, becomes activated and synthesizes short 2′-5′oligoadenylates (2-5A). The only well-established function of 2-5A is binding and activation of the ubiquitous enzyme RNASE L. Once activated, RNASE L dimerizes into an endoribonuclease that cleaves single-stranded regions of RNA. The

OAS-RNASE L pathway has been reported to inhibit the replication of a panel of RNA viruses including encephalomyocarditis virus, Coxsackie virus B4, West Nile virus and HCV. RNASE L has been reported to possess antitumor properties and to be involved in apoptosis since, in addition to viral RNAs, it degrades cellular mRNAs, rRNAs and thus damages the host cell machinery essential for viral replication. The ISG-encoded antiviral proteins Mx are large GTPases related to dynamin - a family of proteins involved in endocytosis and intracellular vesicle transport - that sequester viral ribonucleoproteins to specific subcellular compartments [395]. Mx inhibits replication of many RNA viruses such as influenza and measles viruses by interfering with intracellular trafficking and activity of viral polymerases. The p56-related proteins (IFIT gene product) including p56 and p54 constitute another ISG family which influences the translational machinery by blocking different subunits of the translation initiation factor eIF3, thus inhibiting viral and cellular protein synthesis.

Type I IFNs possess several immunoregulatory effects, and these can also enhance antiviral states within an infected individual. Type I IFNs act on other cytokines and directly on the growth, differentiation and function of immune cells such as B-cells, T-cells, mDCs and NK-cells to regulate innate and adaptive responses. The most studied cellular effects of type I IFNs are those shaping responses of NK-cells (innate cellular response), DCs (which participate in the innate response and control adaptive immunity) and T-cells (the main effectors of the adaptive arm of the immune system). In terms of immunoregulatory effects on innate immune cells, IFNs activate NK-cell cytotoxicity by upregulating levels of perforin rapidly after viral infection, as well as promoting the maturation, accumulation and activation of pDCs - major producer of type I IFNs during viral infection. Release of type I IFN by infected cells triggers DC maturation by upregulating the major histocompability complex (MHC) molecules - used to present antigen to T-cells and NK-cells - and the co-stimulatory molecules CD80, CD86, and CD40 - used to drive the activation of naïve and memory T-cells. Type I IFNs also induce IL-15 synthesis to promote NK-cells and memory CD8+ T-cells survival and proliferation early after infection. Type I IFNs play an important role in activating naïve

CD8+ T-cells, enhance IFN-γ production by CD4+ T-cell, the survival of activated CD4+ and CD8+ T-cells, as well as the development and proliferation of B-cells. Therefore, in addition to stimulating multiple direct defence mechanisms within infected cells, the immunoregulatory effects triggered by type I IFNs lead to independent effector mechanisms mediated by uninfected immune cells which block viral replication, and ultimately eradicate the virus from the host.

In addition to establishing an antiviral state, IFNs has been shown to possess an antiproliferative activity and thus protect the host from tumor formation (reviewed by [396]). In clinical settings, IFN-α/β is injected to patients suffering from malignancies such as melanoma, hairy cell leukemia, renal cell carcinoma and Kaposi's sarcoma. Type I IFN mediate its cytostatic properties by arresting and/or lengthening the cell cycle phases, and by interacting with key components of cell cycle regulation including Rb protein, E2F, cyclins D3, E, A, CDK6, CDK6 and cdc25. IFNs block the G1 to S phase transition by upregulating the CDK inhibitor p21. In addition to their ability to modulate cell cycle, IFNα/β are also involved in apoptosis by upregulating several pro-apoptotic molecules including TNF-related apoptosis inducing ligand or TRAIL/APO2L Fas/FasL, p53, Bax, Bak, Noxa, the death-inducible signaling complex (DISC) as well as activation of pro-caspases 8 and 3.

## 2.3. Negative regulation of IFN signaling

### *2.3.1. Limiting the IFN response through IRF3 ubiquitination*

Activation and shutdown of the antiviral response is tightly regulated by post-translational modification events. Cellular mechanisms that limit or shutdown type I IFN production downstream of PRRs are essential to protection against excess production of these cytokines, which can contribute to the development of chronic inflammatory disease, and systemic autoimmune diseases, which include systemic lupus erythematosus

(SLE), myositis, Sjögren syndrome (SS), systemic sclerosis (SSc) and rheumatoid arthritis (RA). These autoimmune diseases are characterized by an antigen-driven immune response against self proteins resulting in the destruction of host tissue [397].

Ubiquitination of IRF3 and IRF7 is an effective mechanism used by the cellular machinery (as well as viruses) to modulate type I IFN production and contributes to many other cellular biological events including cell cycle control, signal transduction, DNA repair and apoptosis. The ubiquitination process promotes covalent attachment of ubiquitin molecules to target proteins through the serially coordinated activity of three classes of enzymes: ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s) [398-399]. First, E1s interact with and activate an ubiquitin molecule, leading to the formation of a thioester-linked complex between the ubiquitin and the catalytic cysteine of E1. The charged E1 interacts with E2s, then, E1s transfer the activated ubiquitin to E2s and whereby a second thioester bond is formed between E1s and E2s. E2s bind to the E3-ubiquitin ligase that recognizes and recruits the target protein for ubiquitin ligation. The E3 ubiquitin ligase is important because it provides the specificity in the ubiquitin mechanism as it recruits both the E2s-ubiquitin complex and the substrate protein. Accumulation of additional ubiquitin molecules through lysine-glycine linkage leads to the formation of a polyubiquitin chain. Addition of monoubiquitin or polyubiquitin chains dictates diverse biological consequence for the substrate protein: lysine 48 (K48)-linked monoubiquitin or polyubiquitin chains, promote proteasomal-dependent degradation by the 26S complex, whereas Lysine 63 (K63)-linkage is generally associated with protein-protein interaction, protein cellular localization and thereby appears to promote activation of the protein of interest.

#### *2.3.1.1. Cellular protein-mediated IRF3 ubiquitination*

The existence of cellular proteins that target IRF3 for degradation has been demonstrated in a previous study in which the proteasomal inhibitor MG-132 blocks IRF3 degradation in response to viral infection. Several cellular components have been

proposed to contribute in the negative regulation of IRF3. These factors include the cytoplasmic peptidyl-prolyl-isomerase (Pin1), Ro52 (TRIM21) and RBCC protein interacting with PKC1 (RBCK1). Stimulation of HEK-293T cells with Poly(I:C) induced phosphorylation of IRF3 at Ser 339, which induces the recruitment of Pin1 resulting in polyubiquitination induced proteasomal degradation and ultimately leading to the subsequent shutdown of IFN-β production [400]. Moreover, mice deficient for Pin1 presented higher levels of IFN-β than wild-type mice following poly(I:C) injection, supporting the negative role of Pin1 in IFN-β production. Ro52 is another cellular protein targeting IRF3 for ubiquitination and degradation [401]. This inducible protein is present in patients with systemic lupus erythematosus and Sjogran syndrome and recognized as an autoantigen. Ro52 plays an important role in antiviral defense and has been identified as member of the TRIM/RBCC (RING (really interesting new gene) finger B-box coiled – coil) family of single-protein E3 ligase. Ro52 has a dual function in the antiviral response since it can positively or negatively regulate various IRFs. Ro52 ubiquitinates IRF8 through lysine K63-linkage and enhances inflammatory cytokine expression such as IL-12p40 in murine macrophages. In contrast, Higgs *et al.* reported that over-expression of Ro52 in HEK-293T cells increased ubiquitination and degradation of IRF3, an effect that was inhibited with the proteasome inhibitor MG-132. Furthermore, knock-down of Ro52 with shRNA (small-hairpin RNA) enhances IFN-β and CCL5 production in cells infected with Sendai virus [401]. Another E3 ubiquitin ligase - RBCC protein interacting with PKC1 (RBCK1) - has been shown to catalyze the ubiquitination and degradation of IRF3. RBCK1 has been previously identified as a negative regulator of TNF- and IL-1-induced NF-κB activation by targeting TAB2/3 for degradation [402]. Zhang *et al.* showed that overexpression of RBCK1 suppress Sendai virus-triggered type I IFNs synthesis, while knockdown of RBCK1 has the opposite effect [403].

#### *2.3.1.2. Virus-mediated IRF3 ubiquitination: a viral evasion mechanism*

To escape from host innate immune responses, viruses have evolved strategies to block or inhibit innate immune receptor-induced type I IFN production. Viral proteins can act as E3 ubiquitin ligases and target IRF3 for proteasomal degradation, thus inhibiting the antiviral response and providing a better cellular environment for viral replication. Pitha's group showed that HIV-1 accessory proteins VPR and VIF target IRF3 for ubiquitination and proteasomal degradation, thus limiting the antiviral response [404]. The infected cell protein (ICPO) from bovine herpes virus (BHV1) and herpes simplex virus 1 (HSV-1) has been reported to act as an E3 ubiquitin ligases by promoting proteasomal degradation of IRF3 and suppressing IFN-β promoter activity [405-406]. Other viral proteins such as Npro from classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV) associate with and induce degradation of IRF3 [407-408]. In contrast to ubiquitin-mediated degradation of IRF3, Zheng *et al.* reported that Papain-like protease domain (PLP2) of the nonstructural protein (nsp3) of mouse hepatitis virus A59 (MHV-A59), a member of coronaviruses family, interacts with IRF3 and provokes its de-ubiquitination, preventing its nuclear localization, thus inhibiting IFN-β production. They also showed that PLP2 which contains conserved deubiquitinase motifs (DUB) can de-ubiquitinates IRF3 modified by both K48- and K63-linked polyubiquitination chains [409].

### *2.3.2. Negative regulators of TLR and/or RLR dependent signaling*

Since IRF3 and IRF7 are considered are master regulators of type I IFN signaling, their activity may also be indirectly negatively regulated through other factors that act at the level of TLR-dependent and or RLRs-dependent signaling.

Recently, the deubiquitinating enzyme A (DUBA), an ovarian tumor (OTU) domain-containing deubiquitinating enzyme, has been identified as a novel regulator of interferon signaling. Kayagaki *et al.* reported that DUBA negatively regulates IFN signaling following RIG-I, MDA-5 or TLR3 stimulation [410]. Knock-down of DUBA increases the PRR-induced type I IFN response, whereas ectopic expression of DUBA has the opposite effect. DUBA physically associates with TRAF3, an adaptor protein

connecting TBK-1 and IKKε to upstream signaling molecules - stimulation of TLR3 with poly(I:C) increases this interaction. TRAF3 is an E3 ubiquitin ligase which undergoes K63-linked polyubiquitination, via its own RING finger domain [411]. By removing the K63-linked ubiquitin chains from TRAF3, DUBA disrupts the interaction between TRAF3 and the downstream kinases - IKKε/TBK-1 - thus inhibiting IRF3 and IRF7 phosphorylation. Although TRAF3 is known to participate in the activation of the non-canonical NF-κB pathway, DUBA did not affect the cleavage of the NF-κB precursor NF-κB2/p100 into the active p52 subunit.

The ubiquitous NF-κB inducible editing protein A20 (or TNFα-induced protein (TNFAIP3)) possesses both a deubiquitinase and an E3 ubiquitin ligase activity. The N-terminal domain of A20 contains an OTU domain responsible for its deubiquitinase activity [412-413]. The C-terminal domain of A20 is composed of seven zinc finger and functions as an E3 ubiquitin ligase [414]. A20 has been shown to affect both the NF-κB and IRF pathways by distinct and overlapping events, resulting in the inhibition of IFN-α/β production. Mice lacking A20 developed systemic inflammation following LPS or TNFα treatment and died prematurely. Overproduction of pro-inflammatory genes was due to constitutive activation of NF-κB pathway. A20 acts on several molecules such as TRAF6, RIP-1 and NEMO to disrupt NF-κB activation signals [415-416]. A20 abrogates NF-κB signaling using a two-step model. First, A20 removes the K63-linked ubiquitin chains, which serve as docking sites for protein-protein interaction, and then it mediates proteasomal degradation by adding K48-linked ubiquitin chains. The negative role of A20 on IRF3/7 activation in TLR3 and RIG-I signaling pathways has been reported by three independent groups [417-418]. Ectopic expression of A20 completely abolishes IRF3 phosphorylation and DNA binding mediated by Δ-RIG-I, an active form of RIG-I. Our group reported that TBK1 and IKK-ε interacted with A20, but are not degraded in its presence, suggesting that A20 inhibits RIG-I signaling by acting upstream of the kinases TBK-1/IKKε [418].

The tumor suppressor cylindromatosis protein CYLD has been shown to abrogate NF-κB activation by interacting with NEMO and promoting its deubiquitination [419]. CYLD also associates with both MAVS and RIG-I, inhibiting RIG-I signaling and thereby blocking type I IFN production [420].

TRIAD3A is a ubiquitous RING finger type E3 ubiquitin-protein ligase, which promotes Lys48-linked ubiquitination and proteasomal degradation of TLR9 and possibly of TLRs 3, 4, and 5 [421]. Recently, our group showed that Triad3A negatively regulates the RIG-I signaling pathway by targeting the adapter TRAF3 for Lys48-linked polyubiquitination and proteasomal degradation [422]. Treatment with dsRNA or virus infection induced Triad3A but decreased TRAF3 levels in a dose-dependent manner; moreover, Triad3A expression blocked IRF3 activation by Ser-396 phosphorylation and inhibited the expression of type I IFN and antiviral genes.

RNF125 (also termed TRAC-1: T-cell RING protein in activation 1) is member of a family of small single RING E3 ligases known as RNF (RING finger protein). This family is characterized by an N-terminal RING domain followed by three atypical zinc finger motifs and a C-terminal UIM-type domain that binds Lys48-linked polyubiquitin chains. RNF125 is involved in the negative regulation of RIG-I signaling pathway [423]. IFN-α or poly(I:C) treatment increased RNF125 expression and leads to the polyubiquitination and degradation of RIG-I, MDA5, and MAVS.

### *2.3.3. Limiting IFN response by induction of inhibitors of cytokine signaling*

Cells of the innate and adaptive immune response release cytokines that act in an autocrine/paracrine fashion to coordinate a coherent and integrated antiviral response. Cytokines contribute not only to the initiation, amplification and execution of the inflammatory response but also play an important role in the development and control of many lineages of immune cell populations. Cytokines regulate behaviour by stimulating a

panel of signal transduction cascades such as the JAK/STAT pathway via binding to receptors on the plasma membrane of target cells. Thus, JAKs and STATs are crucial intracellular mediators of immune cytokine function. Tight control of the initiation, magnitude and duration of cytokine receptor signals is necessary to prevent excessive signaling and abnormal cellular activation that can lead to the development of pathologies including hematopoietic, autoimmune, inflammatory diseases and interferon (IFN)-resistance, as well as cancer [424-427].

Mechanisms involved in the negative regulation of cytokine-mediated activation of the JAK/STAT pathway include i) receptors internalization via endocytosis followed by their subsequent lysosomal or proteasomal degradation; 2) dephosphorylation and inhibition of JAKs kinase activity by the SH2-containing protein tyrosine phosphatases (SHPs); 3) negative regulation of activated STATs via interaction with the protein inhibitors of activated STATs (PIAS), preventing activated STATs to translocate into the nucleus, binding to specific promoter sequences and activating cytokine responsive genes; 4) suppression of cytokine signaling either by induction of suppressor of cytokine signaling - SOCS - protein inhibiting JAKs activity, or by competition with STATs for phosphorylated docking sites on the receptors, or by interacting with and targeting signaling proteins for proteasomal degradation. The following section will focus on the role of SOCS proteins, in particular SOCS1, in regulating cytokine induced signaling.

#### *2.3.3.1. Suppressors of Cytokine Signaling: SOCS1*

The mammalian SOCS family contains eight members including the cytokine-inducible Src-homology 2 (SH2) protein (CIS) and SOCS1-SOCS7, of which SOCS1 and SOCS3 are the best characterized. CIS was first discovered and identified as an immediate early gene induced by numerous cytokines [428]. SOCS1, was later characterized by three independent groups and referred to different names: JAB (JAK binding protein), SOCS1 (suppressor of cytokine signaling) and SSI (STAT-induced STAT inhibitor), each indicating how it was identified [429-431]. SOCS proteins are

labile and are expressed as early response genes to a panel of external stimuli such as cytokines, growth factors, Toll receptors ligands, statins, cyclic adenosine monophosphate (cAMP) and isoproterenol [432].

All SOCS proteins harbour a three part architecture composed of 1) a central Src homology 2 (SH2) domain involved in substrate binding through recognition of specific phospho-tyrosine residues 2) a N-terminal region variable in length (and nucleotide sequence) also composed of an extended SH2 domain (ESS) contributing to substrate interaction, and 3) the N-terminal region of SOCS1 and SOCS3 contain an additional 12 amino acid sequence designated as kinase inhibitory region (KIR), adjacent to the SH2 region. All SOCS proteins also contain a conserved C-terminal region known as SOCS box, which assemble an E3 ubiquitin ligase complex (**Figure 20**). The SOCS box is not only conserved within the members of the SOCS family but is also shared by 32 other proteins, which are regrouped in 8 different families based on their structural characteristics [433].


**Figure 20. Structural domains of suppressor of cytokine signaling 1 (SOCS1).** SOCS1 contains a central SH2 domain, an extended SH2 subdomain (ESS) and a C-terminal SOCS box. The kinase inhibitory region (KIR) serves as a pseudo-substrate for JAKs, blocking JAK function.

2.3.3.1.1. Mechanisms of action of SOCS1

2.3.3.1.1.1. Inhibition of kinase activity via the SH2-domain

Expression of SOCS1 is first regulated at the transcriptional level and requires activated STATs. Binding sites for STAT1, STAT3 and STAT6 have been found in the promoter region of SOCS1 and explain why this gene is induced by a panel of cytokines. STAT1 has been shown to act indirectly by stimulating the expression of the transcription factor - IRF1 - which in turn induces the transcriptional activation of SOCS1 [434]. Overexpression studies reveal that SOCS1 inhibits most cytokines that regulate its own expression [435]. Disruption of signaling by different families of cytokines is due to the ability of SOCS1 to interact with and inhibit all the JAK family members (JAK-1, -2, -3 and TYK2) (reviewed in [436]). SOCS proteins exert their negative feedback function via

several distinct mechanisms. SOCS1 inhibits JAK2 activity by interacting exclusively with a phosphorylated tyrosine residue (Y1007), known to be critical for catalytic activation of JAK2. Mutation or dephosphorylation of this tyrosine residue disrupts the interaction between SOCS1 and JAK2. Structure-function studies using truncated or chimeric forms of SOCS1 have led to identification the regions involved in SOCS1-JAKs interaction and the inhibition of JAKs activity. Narazaki *et al.* demonstrated that deletion of the SH2 domain of SOCS1 results in the loss of SOCS1-TYK2 association, suggesting that SOCS1 recognizes and interacts with JAK family members via its SH2 domain [437]. However, higher affinity binding and functional inhibition of JAKs activity requires an additional 24 amino-acid present at the N-terminus composed of ESS and KIR domains. Yasukawa *et al.* proposed a two steps model where the KIR domain of SOCS1, at first, mimics the activation loop of JAK leading to a conformational change of the protein required for the binding of potential substrates such as STATs to the catalytic site of JAKs; second, SOCS1 through its KIR domain acts has a pseudosubstrate inhibitor for JAKs thus preventing the substrate access to the enzyme and blocking signaling. In this model, SOCS1 associates with JAK directly after JAK autophosphorylates Y1007, inhibits JAK kinase activity via its KIR domain thereby preventing further intra- and intermolecular phosphorylation [438]. In addition to its direct interaction with JAKs, SOCS1 has been reported to associate with the type I IFN alpha receptor (IFNAR) and the IFNγ receptor (IFNGR), which might ensure a very efficient suppressive effect of SOCS1 on IFN signaling. SOCS1 regulates IFNAR1-specific but not IFNAR2-specific signals, inhibits tyrosine phosphorylation of STAT1 and reduces the duration of antiviral gene expression [439]. Similarly, SOCS1 has been reported to bind to a phosphotyrosine residue (Tyrosine 441) on the IFNG1R to mediate attenuation of STAT1 activation [440], indicating that SOCS1 first binds to the IFN receptors, then interacts to JAKs and subsequently inhibits kinase activity.

#### 2.3.3.1.1.2. Ubiquitination through the SOCS-box

Another mechanism used by SOCS1 to modulate cytokine signaling is to link its substrates to the ubiquitin machinery via the SOCS-box. Genetic studies have

demonstrated the essential role of the SOCS-box for *in vivo* functions of SOCS1. Zhang *et al.* reported that mice lacking the SOCS-box show prolonged JAK/STAT signaling following cytokine exposure, present a hypomorphic phenotype and slowly developed a fatal inflammatory disease [441]. The discovery that SOCS1 induces ubiquitination of its target protein arises from the similarities found between the SOCS-box and the α-domain of the Von-Hippel Lidau (VHL) tumor suppressor protein [433, 442-443]. The N-terminal region of VHL (β-domain) identified as a protein-protein interaction domain, binds to the transcription factor - hypoxia-inducible factor-1α (HIF-1α) - whereas its α-domain interacts with elongins B/C to form a complex with an E3 ubiquitin ligase targeting HIF-1α for degradation. Protein ubiquitination is mediated by the subsequent cooperation of an E1 ubiquitin-activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ligating enzyme. The E3 ligase defines substrate specificity and covalently adds ubiquitin to lysine side chains on the target protein. Polyubiquitination of a specific protein leads to its recognition and degradation by the proteasome. Based on the VHL model, SOCS1 protein acts as substrate recognition factor by interacting with an E3 ligase through the SOCS-box and mediates ubiquitination of target proteins associates through its N-terminal domain (**Figure 21**). Thus, SOCS1 appears to combine specific inhibition (kinase inhibition by the KIR domain) with a generic mechanism of targeting bound proteins for ubiquitin-induced proteasomal degradation [444]. The SOCS-box of SOCS1 contains a 10 amino acid BC-box sequence that mediates interaction with both elongins B and C (**Figure 21**). Elongins B/C were initially identified as components of the mammalian transcription factor SIII; in the presence of a third protein - elongin A – the elongins B/C complex increases the overall elongation rate of RNA polymerase II [445-446]. Elongin B interacts with elongin C to form a dimer acting as a linker that bridges the target protein recognized by the SOCS box to a Cullin. Cullins are a family of scaffold proteins for the Cullin-Ring type E3 ligases (CRLs). This association with a specific Cullin protein - Cul5 - is further supported by a conserved Cullin-box motif found downstream of the B/C-box sequence in the SOCS-box [447]. SOCS1-containing E3 ligase has been shown to preferentially associate with Cul5, rather than Cul2, suggesting that Cullins may determine the SOCS-box specificity [448]. Then, the C-

terminal of Cul5 associates with the RING finger-containing protein 2 (Rbx2), which acts as a docking site for the E2 ubiquitin-conjugating enzyme. The SOCS/elongins B/C/Cul5/Rbx2/E2 complex in turn serves as a functional E3 ubiquitin ligase that allows proteasomal degradation of tyrosine phosphorylated signaling molecules bound to SOCS [449]. SOCS1 has been reported to mediate the ubiquitination/turnover of JAK2, as well as the TEL-JAK2 oncogene in a SOCS box-dependent fashion [450-452]. SOCS1 induced proteolytic degradation is not only a process restricted to JAKs family members. Indeed, SOCS1 can also target diverse proteins such as the guanine nucleotide exchange factor VAV, the p65/RelA subunit of NF-κB, the TLR adaptor Mal, the E7 protein of human papilloma virus (HPV), insulin receptor substrate (IRS) 1 or IRS2 and focal adhesion kinase (FAK), as well as several other targets [453-457]. By controlling a panel of intracellular signaling components in addition to those interacting with cytokine receptors, SOCS1 has the capacity to have a broad influence on cell fate.

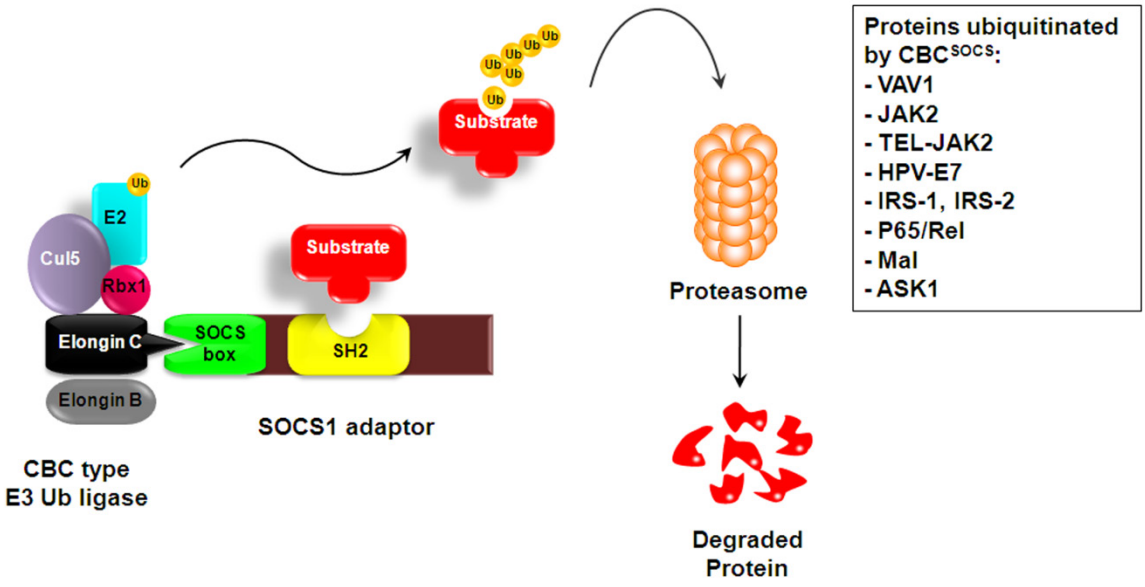



**Figure 21. Substrate-adaptor function of SOCS box in protein ubiquitination.** The SOCS1 containing E3–ligase ($CBC^{SOCS1}$) preferentially interacts with Cul5 to mediate proteasomal degradation of VAV1, JAK2, E7 protein from Human Papilloma Virus, IRS-1/IRS-2, p65, Mal, ASK1. Reproduced with permission from [458].

#### 2.3.3.1.2. Regulation of SOCS1 protein expression

SOCS1 protein expression is tightly controlled by translational initiation and post-translational regulatory mechanisms [459]. Several studies suggest that SOCS1 protein is unstable and is degraded by proteasomal pathways [460-461]; however, the precise mechanism by which SOCS1 protein level is regulated remains controversial. Kamura *et al.* reported that the interaction between SOCS-box and Elongin BC is essential for SOCS1 stability. Indeed, association of SOCS1 with Elongin BC complex increased the stability of the wild-type SOCS1, compared to the SOCS1 box-deleted SOCS1 which was rapidly degraded [462]. In contrast to these reports, Rothman *et al.* suggested that Elongin BC target SOCS1 to proteasomal degradation rather than stabilizing it. Pim-1, a Ser/Thr

kinase that phosphorylates SOCS1, destabilizes the SOCS1/Elongin BC complex and prevents the degradation of SOCS1. Destabilization of the SOCS1/Elongin BC complex by introducing mutations within the SOCS-box also prevented SOCS1 degradation, supporting the idea that association of Elongin BC with the SOCS-box alters the stability of the SOCS1 protein [463]. In addition, Toniato *et al.* showed that TRIM8/GERP - a Ring finger protein - binds to SOCS1 *in vitro* and *in vivo*; this interaction decreases the stability and thus the levels of SOCS1 [464]. Although SOCS1 degradation can be inhibited by proteasomal inhibitors, it is currently not known whether TRIM8 is a component of a unique E3–ligase. Further studies are required to determine whether the degradation of SOCS1 is dependent on its "self" ubiquitination, or whether SOCS1 is dragged to proteasomes by proteins interacting with SOCS1 and become ubiquitinated by the SOCS1 containing E3-ligase [458]. Interestingly, SOCS1 has been reported to colocalize with the microtubule organizing complex (MTOC) associated with the 20S proteasome. In overexpression systems, SOCS1 targets JAK1 via its SH2-domain, to a perinuclear location resembling the MTOC-associated 20S proteasome [465].

#### 2.3.3.1.3. Physiological function of SOCS1

The *in vivo* function of SOCS1 has been reported by three independent groups [466-468]. Mice lacking SOCS1 died rapidly after birth (within 2–3 weeks), due to myelomonocytic infiltration of visceral organs, fatty degeneration of the liver and lymphopenia. Naka *et al.* reported that the lymphopenia phenotype observed in SOCS1-/- mice was due to the upregulation of the proapoptotic protein Bax resulting in a high proportion of lymphocytes undergoing apoptosis. Thus, SOCS1 limits apoptosis in lymphocytes by downregulating Bax expression; however the exact mechanism by which this event occurs has not been determined [466]. The pathologies associated with SOCS1-/- mice can be attributed to IFN-γ dysregulation since SOCS1-/- mice phenotype can be reproduced in wild-type neonatal mice by simple administration of IFN-γ [467]. Injection of neutralizing anti-IFN-γ antibodies delayed the death of SOCS1-/- mice, and crossing them with an IFN-γ-/- background eradicated the lethal phenotype. Further characterization of SOCS1-/- mice phenotype by Marine *et al.* demonstrated that SOCS1,

expressed in the thymocytes is essential for the regulation of T-cell differentiation and prevents the development of a spontaneous IFN-γ-secreting T-cell population [468]. This group showed that thymic T cell development is perturbed and peripheral T-cells are highly activated in SOCS1-/- mice. In addition, IFN-γ levels were detected in the serum of these mice but not in the wild-type phenotype. Since T-cells are the major source of IFN-γ, they hypothesized that the population of activated T-cells in SOCS1 -/- mice is responsible for the high level of IFN-γ in the serum. Interestingly, mice deficient for SOCS1 and RAG2 (recombinant activating gene) - a key gene in lymphoid maturation - present normal levels of IFN-γ and survive. Although part of the SOCS1-/- phenotype might be explained by abnormal signaling by IFNγ, dysregulation of other inflammatory cytokines including IL-2, IL-6, IL-12, IL-15 and TNF have been also shown to contribute to development of pathologies associated to SOCS1 deficiency [444].

#### 2.3.3.1.4. SOCS1 and immune regulation

##### 2.3.3.1.4.1. SOCS1 and TLR4 signaling

Although it was initially accepted that SOCS1 induction and action is restricted to JAK/STAT-dependent cytokines and signaling pathways, recent studies suggest that SOCS1 is also induced by a broad variety of stimuli and even targets signaling pathways distinct from JAK/STAT, such as TLR signaling. As described previously, stimulation of TLR signaling by PAMPs is important for initiating the innate immune response and enhancing the adaptive immune response. Excessive activation of TLRs can lead to the establishment of chronic inflammation or the development of autoimmune diseases, while insufficient response can increase susceptibility to the infection or fail to control it [469]. Thus, TLR signaling must be tightly controlled to keep equilibrium between activation and inhibition. Interestingly, Dalpke *et al.* reported that both LPS- mediated activation of TLR4 and CpG-DNA-mediated activation of TLR9 induce expression of SOCS1 in macrophages [470]. Activation of TLR4 by LPS stimulates the synthesis of a panel of proinflammatory cytokines as well as nitric oxide (NO) and reactive oxygen species. SOCS1 has been identified as the factor responsible for the hypo-responsiveness of

macrophages to cytokines following LPS treatment. Genetic studies performed by Nakagawa *et al.* demonstrated that SOCS1-/- mice and mice deficient for both IFNγ and SOCS1 are hyper-responsive to LPS and also sensitive to LPS-induced lethality [471]. Kinjyo *et al.* further supported this study by showing that mice and macrophages deficient for SOCS1 produce high levels of nitric oxide and pro-inflammatory cytokines (TNF-α, IL-12) in response to LPS and thereby are intolerant to LPS [472]. SOCS1 has been shown to negatively regulate LPS-induced activation of macrophages by targeting different components of both the MyD88-dependent and -independent TLR4 pathway (**Figure 22**).

It is well established that IRAK is rapidly phosphorylated and degraded through the proteasome following LPS stimulation; however, the protein responsible for these post-translational modification has not yet been identified [473]. Nakagawa *et al.* demonstrated that SOCS1 suppress MyD88-dependent pathway by binding to IRAK. Overexpression studies revealed that SOCS1 interacts with IRAK via its SH2 domain resulting in the inhibition of LPS-induced NF-κB reporter gene activity [471, 474]. Based on these observations, it is possible that SOCS1 binds to phosphorylated IRAK and promotes its proteasomal degradation upon LPS treatment, thus inhibiting the NF-κB signaling pathway. However, more studies are required to determine whether IRAK is targeted by SOCS1 for ubiquitin- mediated degradation upon LPS treatment.

SOCS1 also binds to the TIR domain-containing adapter protein/Mal (TIRAP/Mal) - another component of LPS-activated TLR4-NF-κB signaling pathway. TIRAP/Mal is an adapter that specifically mediates the MyD88-dependent pathway via TLR2 and TLR4. Mansell *et al.* showed that SOCS1 suppresses Mal-dependent p65 phosphorylation and transactivation of NF-κB. Overexpressed SOCS1 impairs the TLR4 signaling pathway by interacting with phosphorylated Mal via its SH2 domain and thus promoting ubiquitination and degradation of Mal through the SOCS-box region [475]. Following TLR stimulation, Mal undergoes tyrosine phosphorylation by Bruton's

tyrosine kinase (Btk) suggesting the requirement for Btk to trigger tyrosine phosphorylation of Mal in order to be targeted by SOCS1.

Ryo *et al.* showed that in murine splenocytes treated with LPS, SOCS1 interacts with p65 - a subunit of NF-κB. NF-κB is heterodimer complex composed of p50 and p65 subunits; in unstimulated cells NF-κB is kept in an inactive state by IκB, which undergoes proteasomal degradation following LPS or TNF-α stimulation. During NF-κB activation, the peptidyl prolyl isomerase - Pin-1 - binds to phosphorylated p65 and prevents its association with IκB, thereby increasing stability and nuclear localization of NF-κB. In absence of Pin-1, ectopic expression of SOCS1 inhibits NF-κB signaling pathway by promoting ubiquitin-mediated proteolysis of endogenous p65 [457].

SOCS1 has been also reported to inhibit TNF-induced activation of the stress-activated MAPKs - JNK and p38 - which are involved in inflammatory signaling by inducing proteolytic degradation of the Apoptosis Signal-regulating Kinase 1 (ASK1) [476].

Another important inhibitory mechanism of macrophage activation by SOCS1 is the suppression of the secondary activated JAK/STAT pathway. In LPS-induced TLR4 MyD88-independent signaling, TRIF stimulates the synthesis of type I IFN through IRF3 activation. Ectopic expression of SOCS1 reduced STAT1 phosphorylation induced by binding of IFN-β to the IFNAR [471]. Furthermore, macrophages-derived from SOCS1-/- mice present prolonged STAT1 phosphorylation in response to IFN-β stimulation suggesting that SOCS1 has a direct inhibitory role in IFN-β signaling. Since the absence of a single allele encoding TYK2, a JAK family member essential for IFNAR signaling is sufficient to overcome the lethal effect of SOCS1 deficiency, it has been proposed that Tyk2 is targeted by SOCS1 thereby inhibiting IFN-β signaling [477].

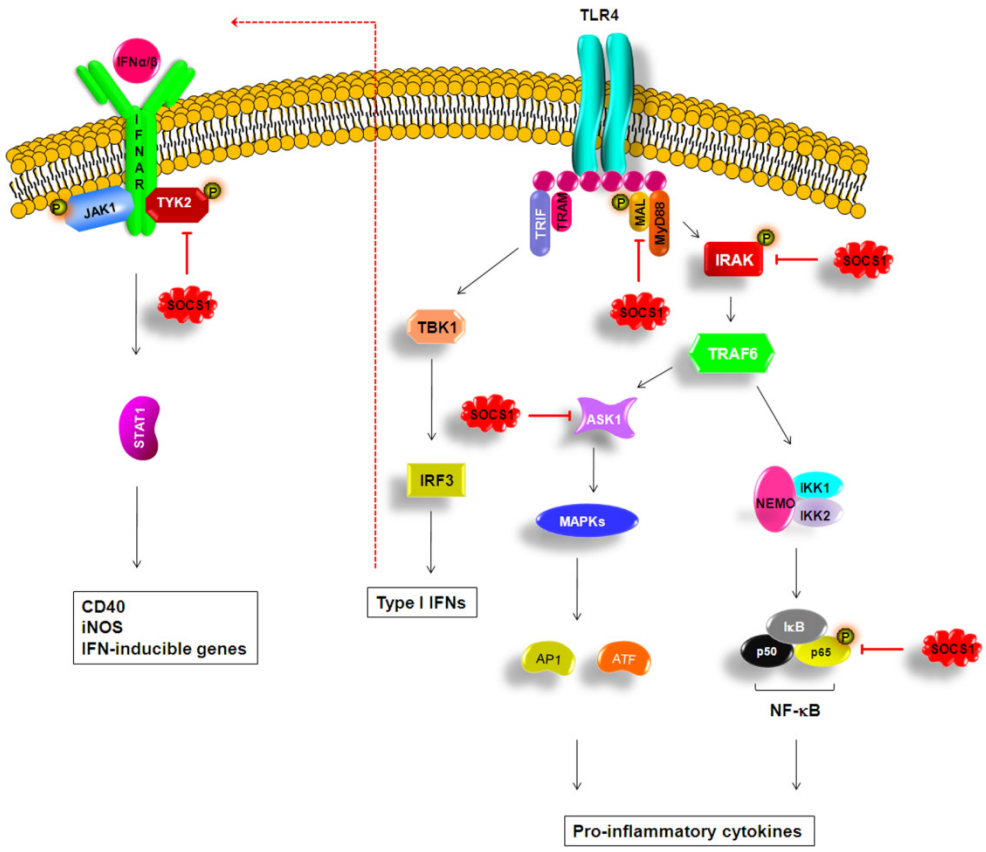



**Figure 22. Suppression of TLR4 signaling pathway by SOCS1.** Activation of TLR4 by lipopolysaccharide (LPS) transmits signals through adaptor proteins MyD88, MAL, TRIF and TRAM. NF-κB and MAPKs are activated by TRAF6, and TAK1 through MyD88 and MAL, whereas IRF3 is activated by TRIF and TRAM. IFN-β is rapidly induced through the TRIF–IRF3 signaling pathway and activates the JAK/STAT signaling pathway. SOCS1 inhibits JAK/STAT signaling pathway. Phosphorylated MAL interacts with SOCS1, which results in MAL polyubiquitylation and its subsequent degradation. SOCS1 also binds to the p65 subunit of NF-κB and induces its degradation. Adapted with permission from [432].

#### 2.3.3.1.5. SOCS1 and pathogens

SOCS1 is an essential regulator of both type I and type II IFN signaling *in vivo* and participates in the equilibrium of the positive antiviral and the negative proinflammatory effects of IFN signaling. Interfering with cytokine receptor signaling is a promising strategy used by various incoming pathogens to escape otherwise detrimental immune responses. Recent studies indicate that several pathogens hijack the host's inhibitory SOCS proteins for manipulating cytokine receptor signaling, especially to circumvent the actions of interferon. Fenner *et al.* reported that SOCS1-/- cells and mice are resistant to Semliki Forest Virus infection and survive longer than wild-type mice [439]. Another study from Yasukawa *et al*. suggested that transgenic expression of SOCS1 in cardiac myocytes inhibits enterovirus-induced JAK-STAT signaling pathway, enhances viral replication leading to cardiomyopathy and mortality of coxsackie virus-infected mice. Furthermore, expression of a dominant-negative SOCS1 vector increases myocyte resistance to acute cardiac injury provoked by enteroviral infection. Therefore, SOCS1 constitutes a novel therapeutic target for enterovirus-induced cardiac injury [478]. Recently, Potlichet *et al.* identified SOCS1 and SOCS3 as negative regulators of Influenza A virus-mediated lung mucosal innate immune response. Influenza A virus (IAV) upregulates SOCS1 and SOCS3 expression in human lung epithelial cells in a RIG-I/MAVS/IFNAR1-dependent but TLR3-independent manner [479]. SOCS1 expression is induced early in the infection whereas SOCS3 expression increases at later time points. Both SOCS proteins act in concert to inhibit type I IFN antiviral signaling; in IAV infected cells overepressing SOCS1, IRF3-dependent and IFN-β antiviral signaling pathways are reduced whereas IAV infected cells overexpressing SOCS3, NF-κB-dependent pro-inflammatory pathway is inhibited. However, the precise mechanism by which SOCS molecules inhibit the antiviral signaling pathway during IAV infection is still unknown.

It has been reported that HIV induces SOCS1 protein expression in different immune cells in order to negatively modulate several aspects of the immune response to

HIV. For example, a study from Song *et al.* showed that SOCS1-silenced dendritic cells enhance the number of HIV-Env specific CD8+ cytotoxic cells and CD4+ T helper cells, as well as the antibody response to HIV [480]. Immunoglobulin class switching is an important mechanism to mount appropriate immunity against viruses and involves the transition from the membrane form of immunoglobulin M (IgM) on B-cells to the production of secreted IgG and IgA. The HIV negative factor Nef – important for efficient viral replication and pathogenicity *in vivo* – has been reported to penetrate and accumulate in B lymphocytes *in vivo* and *in vitro*. Within B cells, Nef enhances expression of SOCS1, SOCS3, and IκBα, which blocks co-stimulatory and cytokine signals necessary for class switching, thus allowing HIV to evade antibody-mediated viral clearance [481].

Another aspect of SOCS1 function during HIV-1 infection has been proposed recently by Ryo *et al.*, who demonstrated that SOCS1 positively regulates the late stages of HIV-1 replication by enhancing Gag stability, trafficking to the plasma membrane, and viral egress [482]. Nishi *et al.* further demonstrated that SOCS1 co localizes with Gag along the microtubule network and promotes microtubule stability by inducing Gag ubiquitination [483]. In summary, SOCS1 plays an important role in innate immune signaling and is therefore targeted by invading viruses to enhance their replication.

## 3. Vesicular Stomatitis Virus as an oncolytic agent

An ideal cancer therapeutic will selectively kill malignant cells while leaving normal tissues unaffected. Unfortunately, the current standards of care for cancer - chemotherapy and radiation therapy - fall well short of this goal. It is imperative that emerging knowledge of the molecular biology of cancer be used to generate novel therapeutics that are targeted specifically to cancer cells. Recent basic, pre-clinical and clinical studies have demonstrated that several innocuous, non-disease causing attenuated strains of replication competent RNA and DNA viruses - Adenovirus, Coxsackievirus, Herpes Simplex Virus type 1 (HSV-1), Measles Virus (MV), Myxoma Virus, Newcastle-Disease Virus (NDV), Reovirus (Respiratory Enteric Orphan viruses), and Vaccinia Virus (VV) - can selectively replicate in and kill a large panel of human tumor cells, clear bone marrow of leukemic cells and effectively arrest metastatic spread of tumors [484-486]. These competent viruses are termed oncolytic (onco=cancer; lytic=killing) viruses. Vesicular Stomatitis Virus (VSV) is a nonpathogenic virus currently being developed as an oncolytic agent for anti-tumor therapies. Viral oncolysis is first achieved through selective infection, viral replication, spread of progeny viruses and ultimately virus induced-cell lysis of cancer cells. Moreover, oncolytic viruses can also act through direct induction of apoptosis, inhibition of tumor angiogenesis and activation of anti-tumoral immune responses. Interestingly, these viruses are unable to replicate as efficiently in normal tissues and thus have a superior therapeutic index. Although it is still unclear why oncolytic viruses preferentially target and kill tumor cells, it appears that during the evolution of malignancies, genetic abnormalities accumulate that, while providing the cancer cells with growth and survival advantages, compromise the normal antiviral program of transformed cells. As an example, NDV, MV and VSV have been shown to replicate to high titers and induce lysis of tumor cells containing defects in the IFN system [487-488]. Reovirus, influenza virus, and HSV-1 exert a preferential tropism

towards cancer cells with overactive RAS-dependent signaling [489-491]. Thus, given the limited therapeutic indices and often debilitating side effects of conventional cancer treatments, and the wide array of tumor cells which appear to be susceptible to oncolytic viruses, virotherapy represents an exciting and promising experimental strategy for cancer treatment. In the context of this thesis, we will focus only on the oncolytic properties of VSV.

## 3.1. Molecular virology of VSV

### *3.1.1. Immunity to VSV*

VSV is an arthropod borne virus that primarily infects cattle, swine and horses, although infection of humans and other species can also occur [492]. VSV is considered enzootic in different regions of the Americas, such as Panama. VSV produces an acute disease in cattle characterized by ulceration of the oral cavity and feet. The pathology mimics the early symptoms of foot and mouth disease virus [493]. VSV has also been reported to cause neuropathy in mice following infection at high doses [494-495]. Animals infected with VSV for the first time develop a strong humoral response within a week after their exposure. The strong antibody response and cytotoxic T-lymphocytes are directed against VSV N and G proteins, although VSV-specific neutralizing antibodies – responsible in majority for the elimination of the disease – target only the VSV G protein [496]. Thomsen *et al.* reported that mice deficient for B-cells are extremely susceptible to VSV and die from encephalitis within 9 days following infection whereas mice lacking T-cells develop neuropathy and die 30 days post-infection, suggesting that T-cells are necessary for long-term survival after VSV infection. Naturally occurring human infections with VSV are extremely rare, except in cases where individuals are exposed to infected livestock or medical researchers exposed within the laboratory environment. Most VSV infections are asymptomatic in humans or cause mild flu-like symptoms.

### *3.1.2. VSV life cycle*

VSV is a negative-stranded RNA virus belonging to the Rhabdoviridae family and Vesiculovirus genus. VSV is characterized by a typical bullet-shaped structure of approximatively 45-100 nm in diameter and 100-400 nm long which contains a single-stranded RNA genome of negative polarity (~11kb) which is completely protected by viral nucleoproteins. VSV synthesizes five subgenomic mRNAs that encode five distinct proteins: the nucleoprotein (N) in conjunction with the phosphoprotein (P), the large polymerase protein (L), the viral glycoprotein (G), and the multifunction matrix protein (M) (**Figure 23**) [497-498]. The VSV G protein is found on the outer surface of the envelope and is responsible for binding to the host cell surface receptor and initiates the infectious process. The host cellular receptor for VSV has not yet been identified; however, several lines of evidence suggest that VSV G protein binds to phosphatidylserine - a universal component of the cell surface membranes - thus enabling VSV to infect virtually all animal cells [497]. Although normal tissues can also be infected, this extensive tissue tropism allows VSV to be used as anti-cancer agent for all types of tumours. Binding of VSV G protein to the target cells initiates penetration via endocytosis [499]. Low pH within the endosome induces fusion of viral and cellular membranes, allowing the release of the viral genome and polymerase into the cytoplasm where it undergoes transcription to initiate production of viral mRNAs. Indeed, the existing polymerase, which consists of the L and P proteins, recognizes and binds to the viral nucleotide sequence found at the 3'-end of the genome and synthesizes in a sequential fashion five capped and polyadenylated mRNAs encoding the N, P, M, G and L genes products [500]. Once mRNAs and viral protein are synthesized, the parental genome is replicated to make negative-sense progeny genomes that can either be packaged or can undergo secondary transcription to increase the expression of viral proteins. The assembly and encapsidation process of the genomic RNA takes place at the inner surface of the host plasma membrane and is mediated by the ribonucleocapsid particles (RNP) constituted of VSV N, P and L proteins followed by further condensation of the cores by VSV M protein. G protein is also found at the plasma membrane where

VSV virions are formed and released [501].

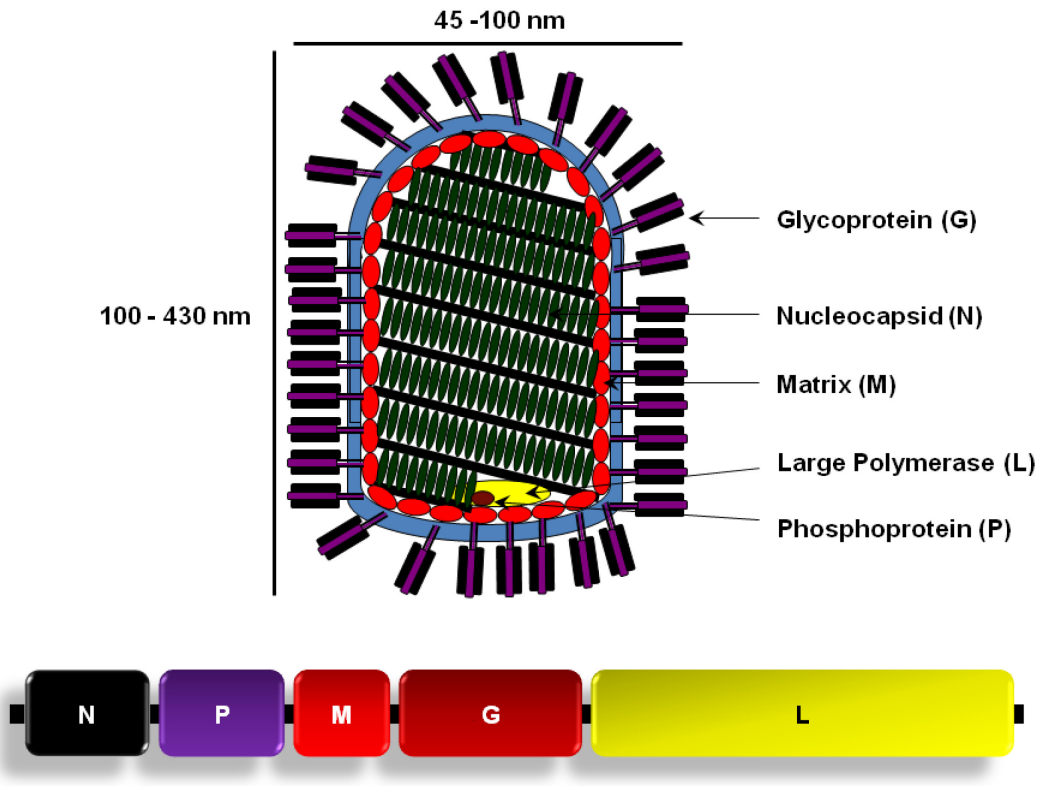



**Figure 23. Structure of VSV genome and viral particles.** VSV mRNAs encode for five distinct proteins: nucleocapsid (N), phosphoprotein (P), Matrix (M), Glycoprotein (G), and large (L) polymerase.

### *3.1.3. Functions of VSV M protein*

The VSV M protein has diverse functions which control VSV replication and pathogenesis: one is related to structure and virus assembly, where the other consist of the inhibition of the ongoing host gene expression. M protein partially regulates transcription of VSV genes by the virally encoded polymerase, and in late infection, it catalyzes the generation of the inactive RNA genome/nucleocapsid core (RNP) cores by preparing them for packaging into virions [502-503]. In addition to its role in budding, M protein participates in the early phases of viral infection by helping VSV to avoid cellular antiviral programs. The interruption of cellular transcription programs and the blockade of mRNA export from the nucleus are both mediated by M protein [504]. The inhibition of cellular transcription by VSV M protein has been demonstrated in several ways. First, M protein shuts off transcription of host genes by inhibiting initiation of the three host RNA polymerases - RNA polymerase I, II or III [505-506]. M protein also blocks the nucleocytoplasmic transport of host mRNAs by interacting with the cellular nucleoporins Nup98 and Rae1 - a shuttling mRNA nuclear export factor - thereby disrupting their function in mRNA export [507-508]. Interestingly, Nup98 is an interferon responsive gene and pre-treatment of cells with interferon increases Nup98 expression and reduces the ability of VSV matrix protein to inhibit nucleocytoplasmic transport [498, 509]. Mutations in VSV M protein abolish the ability of this protein to block host cell transcription and restore nucleocytoplasmic transport, suggesting M protein functions are not mutually exclusive [508]. In addition to its role in the suppression of host transcription, VSV M protein participates in the regulation of apoptosis. Ectopic expression of VSV M protein in absence of other viral components induces apoptosis in Hela and BHK, cells whereas a mutated form of the matrix protein defective in its ability to inhibit host gene expression does not [510-511]. Similar results were obtained in the context of infection with recombinant viruses mutated (or not) in their M protein. VSV M protein induced apoptosis likely results from the blockage of host cell gene expression, since mutations in M protein that abrogate this blockade also reduce VSV cytotoxicity.

### *3.1.4. Inhibition of the antiviral signaling by VSV M*

Several reports demonstrated that M protein participates in the early phases of viral infection by helping VSV to evade cellular antiviral programs. As explained in details in a previous section (refer to section 2.1.2), cellular antiviral programs are initiated by activation of latent transcription factors such as NF-κB, IRF3 and c-JUN/ATF2. Upon viral infection, c-JUN and IRF3 are phosphorylated by JNK and the upstream kinases TBK and IKKε whereas NF-κB is released from its inhibitor IκB by the action of upstream IKKs [375-376, 512]. Then, the activated transcription factors translocate to the nucleus and stimulate the synthesis of IFN-β. Autocrine stimulation of the JAK/STAT signaling pathways by IFN-β triggers the production of interferon responsive genes such as IRF7, which in turn are responsible for the synthesis of hundreds of genes including IFN-α [293]. Thus, IRF3 and IRF7 are essential activators of IFN gene induction and ultimately responsible for the induction of the host antiviral state following virus infection. Stojdl *et al.* showed that VSV triggers a primary antiviral response, but expression of M protein blunts the subsequent secondary and tertiary responses by blocking nuclear export of critical antiviral mRNAs. Comparison analysis of the cytoplasmic versus the nuclear fraction of cells infected with wild-type VSV or VSV mutated in the M protein revealed that IFN-β, although induced in nuclear fraction by all viruses, was not found in the cytoplasmic pool of mRNAs in wild-type infected cells. Thus, by blocking the nuclear export of IFN-β, VSV M protein inhibits the JAK/STAT signaling pathway and suppresses the antiviral response to facilitate its own replication [513].

## 3.2. Molecular basis for the selective permissiveness of cancer cells to VSV

### *3.2.1. Importance of the IFN system in VSV replication*

VSV infection can selectively kill a large panel of human tumor cell lines including 80% of the NCI 60 tumour cell bank**,** *ex vivo* primary leukemic AML cells [514] and effectively arrests metastatic spread of CT26 lung metastases in immunocompetent animals [513]. Although VSV fails to replicate efficiently in primary cells that contain a functional interferon system [515], this virus replicates to high titers in the majority of *ex vivo* transformed cell from patients. In addition, normal cells treated with IFN are protected from VSV infection, whereas transformed cells are much less resistant [516]. The current hypothesis is that aspects of IFN signaling and the action of downstream effectors are compromised in malignant cells, thus affording a cellular environment that facilitates viral replication - uninterrupted by the host antiviral response – and finally resulting in virus induced cell lysis (**Figure 24**). Balachandra *et al.* demonstrated that MEFs lacking the death domain containing protein (FADD) or the receptor interacting protein 1 (RIP1) - factors essential for the optimal intracellular induction of IFN – are extremely susceptible to VSV replication and cytolysis when compared to wild-type cells [517]. Other studies have demonstrated that translation control downstream of PKR activation, frequently deregulated in transformed cells, can cooperate with the attenuated IFN antiviral activity to facilitate VSV oncolysis. IFN-induced antiviral response is mediated through a variety of proteins including the IFN-inducible serine threonine protein kinase PKR. Following interaction with dsRNA, PKR autophosphorylates and activated PKR, in turn, phosphorylates the alpha subunit of eukaryotic initiation factor 2 (elF2α) resulting in the inhibition of viral protein synthesis [518]. It has been reported that PKR is an important and non-redundant component of VSV antiviral host defense. VSV replicates at high levels in PKR -/- cells compared to wild-type cells and PKR-deficient animals are extremely sensitive to VSV [519]. However, PKR is not sufficient by itself to clear the virus. Mice and cells lacking STAT1 but not PKR, die from VSV infection suggesting that the main function of PKR is to

moderate virus replication by inhibiting translation [520]. This event would give enough time to the host cell to mount an appropriate antiviral response against the virus. Therefore, defects in any of these innate components allow VSV to rapidly replicate and subsequently disseminate. Interestingly, Balachandra *et al.* showed that VSV replicates faster in immortalized PKR-/- or STAT1-/- cells than in their non-immortalized counterparts, suggesting that while PKR and IFN-signaling pathways are important in preventing VSV replication, other factors must help enhance VSV replication [517]. While defects in the IFN response may certainly provide one explanation for VSV replication and cytolysis, it is likely that a combination of unknown factors and/or pathways facilitate viral spread in tumors (**Figure 24**).

Stodjl *et al.,* identified what may be considered "second generation" oncolytic variants of VSV – naturally occurring, interferon inducing VSV mutants originally identified by reduced plaque size on cells monolayers which were able to produce and respond to interferon. Mutations to the matrix protein render these viruses interferon-inducing and prevent the matrix protein from blocking nucleocytoplasmic transport and inhibiting host cell transcription. Wild-type and mutant strains of VSV are both able to induce the expression of the gene encoding interferon, but the mutant viruses fail to block the export and translation of the interferon mRNA [506, 513]. The induction of interferon and other antiviral genes by these viruses generates what is been termed a 'cytokine cloud' that protects the host not only from the mutant virus but also from any wild-type virus present in the innoculum. The strategy produces an effective oncolytic virus that is less toxic than a recombinant virus engineered to express interferon.

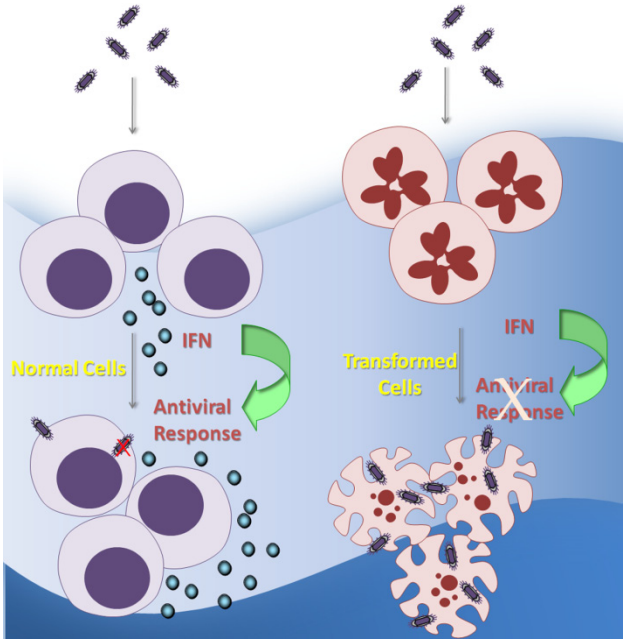



**Figure 24. Disruption of IFN antiviral response predisposes transformed cells to oncolysis by VSV.** Other defects such as PKR-mediated translational control, c-myc or Ras overexpression have been reported in transformed cells.

### *3.2.2. Importance of translation in VSV replication*

The translation of cellular mRNA to protein is a tightly controlled process that is often deregulated in cancer cells. Translation of most cellular mRNAs are regulated at the rate-limiting step of initiation, requiring the formation of an elongation-competent 80S ribosome composed of 40S and 60S subunits [521]. The translation process is initiated by the formation of a pre-initiation complex - 43S ribosome - constituted of the 40S small ribosomal subunit that binds the initiating methionyl tRNA (Met-tRNA$_i$) and a group of eukaryotic initiation factors (eIFs) including eIF5. Then, the 43S ribosome complex associates with a special tag - the m$^7$GpppN cap - bound to the 5'end of an mRNA molecule via eIF3 complex. The cap structure serves as a tag to identify where the 40S ribosomal subunit has to be recruited. This structure is involved in many cellular functions such as RNA splicing, transport, stabilization and translation. Binding of the

43S ribosome complex to the cap is mediated by eIF4F, a cap-binding initiation complex. eIF4F is composed of at least three subunits: eIF4G, eIF4E and eIF4A [522]. eIF4G is a scaffolding protein important for translation assembly machinery and contains three separate domains which bind eIF4E, eIF4A as well as eIF3, and the poly(A) tail binding protein (PABP). eIF4E is a cap-binding protein - since its function is to bind a cap mRNA and ultimately bring it to the ribosome - it is considered as the rate-limiting component of the eukaryotic translation apparatus. eIF4A is an ATP-dependent RNA helicase, of which the activity is stimulated by eIF4B. eIF4A unwinds the mRNA secondary structures thereby allowing ribosome scanning to progress. eIF4F function is regulated by eIF4E inhibitory binding proteins (4E-BPs); when dephosphorylated, eIF4E-BP prevents the formation of the eIF4F complex by binding and sequestering eIF4E [523-524]. In addition to its interaction with the cap mRNA, eIF4F complex associates also with the 3'-poly(A) tail of mRNA. eIF4F complex associates with the 5' cap mRNA via eIF4E and the 3'-poly(A) tail via the PABP resulting in the circulization of the mRNA. The circular conformation of the targeted mRNA protects the ends from degradation and brings the start and stop codons in close proximity to enhance the efficiency of ribosome association with the 5' end of mRNA. Thus, translation is a coordinate process where the ternary complex constituted of the translation factor eIF2 (composed of α, β and γ subunits), GTP and the initiating methionyl-tRNA$_i$ (Met-tRNA$_i$) binds to the ribosome, while eIF4F mediates the association of mRNA with the ribosome.

The 43S pre-initiation complex is therefore able to move along the mRNA in a 5'-3' direction and scan for an initiating AUG codon (or start codon), which indicates where the mRNA will start to code for the protein. Following AUG codon recognition, the GTP bound to eIF2 undergoes hydrolysis resulting in the recruitment of the 60S ribosome and release of eIF2-GDP. The exchange of GDP with GTP is catalyzed by the guanine nucleotide exchange factor (GEF) eIF2B [525]. Then, the complete ribosome (80S) initiates translation during which the sequence between the "start" and "stop" codons is translated from mRNA into an amino acid sequence. To induce a subsequent round of

translation initiation, eIF2-GDP must be converted to eIF2-GTP. Phosphorylation of eIF2α inhibits eIF2B-mediated recycling of GDP for GTP [526-527].

Although PKR has been reported as being defective in tumors such as chronic lymphocytic leukemia, some evidence indicates that PKR can be also overexpressed in certain tumors [528-530]. Balachandra *et al.* reported that normally resistant MEFs become highly susceptible to VSV mediated-oncolysis following cellular transformation, a process that compromises the antiviral effects of IFN. In these cells, PKR is functional and phosphorylates eIF2α. Surprisingly, phosphorylated eIF2α has no apparent effects on the rate of host protein synthesis and VSV replication induced cytolysis [531]. Interestingly, a report from Pavitt *et al.* showed that several mutations in subunits of eIF2B can occur in yeast and abolish the inhibitory effects of eIF2α phosphorylation on protein synthesis [532]. Balachandra *et al.* demonstrated that the catalytic subunit of eIF2B - eIF2Bε - is overexpressed in a panel of transformed MEFs cells, enhancing translation rate by neutralizing the consequence of eIF2α phosphorylation. Depletion of eIF2Bε by using siRNA technology protects transformed MEFs cells from VSV oncolysis 16 hours post-infection, suggesting that defects in the translational machinery can cooperate with impaired IFN signaling to facilitate VSV oncolysis. Another study from Connor *et al.* showed that a second translation initiation factor - eIF4F - is also altered in VSV infected cells [533]. VSV induces dephosphorylation of eIF4E and 4E-BP1 in Hela cells. This effect correlated with the dissociation of eIF4E from eIF4F, subsequent to the formation of the eIF4E/eIF4E-BP1 complex, and thus inhibition of host protein synthesis. These observation are in agreement with a previous study from Dratewka-Kos *et al.* showing that host protein synthesis in extracts from VSV-infected cells can be recovered by addition of either purified eIF4F or eIF2 [534]. As opposed to host cellular mRNAs, translation of VSV mRNAs remains unaffected by eIF4E-BP1. Furthermore, rapamycin, an inhibitor of eIF4E-BP1 phosphorylation does not block VSV protein synthesis indicating that dephosphorylation of eIF4E could be a strategy used by VSV to prevent host - but not its own - protein synthesis. Although VSV infection alters eIF4F complex formation, it is still unclear why translation of cellular and not viral mRNAs is affected. One hypothesis is that the short 5' untranslated regions found in VSV mRNAs confer

some advantage for translation, potentially making use of the remaining non-dissociated eIF4F complexes. Connor *et al*. proposed that the analysis of eIF2α phosphorylation, and findings that the eIF4F complex is rapidly altered during VSV infection and supports a temporal/kinetic model of translation control. At early time points post-infection, changes in the eIF4F complex result in the inhibition of host protein synthesis, while at later times inactivation of the eIF2 complex blocks VSV protein synthesis [535].

### 3.3. Oncolytic properties of VSV

VSV has many virtues that make it an excellent therapeutic candidate: (1) VSV rapidly and effectively kills a wide range of tumour cells, (2) from a commercial point of view, VSV is easy to produce, is physically stable, can be purified in high concentrations, and is very well characterized at the molecular level in the mammalian cell cultures, (3) VSV is not a human pathogen and most humans have never been infected, nor have preexisting neutralizing antibodies that could limit its clinical application, the virus replicates quickly *in vivo* and maybe be able to mediate a significant or complete tumor response before the patient develops an acquired immunity to the virus, and (4) cytoplasmic replication and genetic stability also preclude problems associated with integrating viral vectors. The virus is amenable to genetic manipulation because of a relatively efficient recombinant system, a feature that can be exploited in the generation of novel recombinant VSV vectors.

Several OVs are in early-phase clinical trials (**Table 1**). Based on the therapeutic potential observed in several studies, both naturally occurring replicating oncolytic viruses - NDV, Reovirus or MV - and genetically manipulated viruses - Adenovirus and HSV - are the subject of clinical trials. Phase I and II clinical trials using attenuated strains of these viruses have shown low toxicity, clinical efficacy and high tolerability in patients [536-538]. Despite encouraging pre-clinical and clinical studies, OV therapy requires further improvement in order to become an established cancer-fighting strategy

[539-540]. Many difficulties remain in the development of OVs [541], including poor penetration into the tumour mass, inefficient virus replication in primary cancers [542] and tumour-specific resistance to OV-mediated killing. The combination of OVs with cytotoxic agents, such as small molecule inhibitors of signaling or immunomodulators have shown promise as novel experimental strategies to overcome resistance to viral oncolysis [543-544].

| Oncolytic virus | Company | Combination | Cancer indication | Clinical phase |
|---|---|---|---|---|
| Herpes virus | OncoVEXGM-CSF | – | Melanoma | III |
| | (BioVex) | – | Head and neck cancer | III |
| | | – | Pancreatic and colo-rectal cancer | I |
| Reovirus | REOLYSIN® | – | Recurrent malignant gliomas | I/II |
| | | Paclitaxel and carboplatin | Head and neck carcinoma | II |
| | | Paclitaxel and carboplatin | Non-small cell lung cancer | II |
| | | Paclitaxel and carboplatin | Metastatic melanoma | II |
| | | Paclitaxel and carboplatin | Squamous cell carcinoma of the lung | II |
| | | Gemcitabine | Advanced pancreatic adenocarcinoma | II |
| Vaccinia virus: JX-594 (thymidine kinase-deleted and GM-CSF) | Jennerex Biotherapeutics | – | Liver (HCC) colon head and neck, melanoma lung | I/II |
| Vaccinia virus: JX-929 (double deleted and cytosine deaminase) | | – | Lung, colon, ovarian breast | I |
| Adenovirus 5 (dl1520 derivative) | | – | Head and neck carcinoma | Approved in China |
| Adenovirus 5 (PSE-E1A and E3 deleted) | | – | Prostate cancer | I |
| Newcastle disease virus (PV701) | NCI | – | Advanced or recurrent unresectable squamous cell carcinoma of the head and neck | I |
| | | | Advanced or recurrent peritoneal cancer | I |
| Measles virus (V-deleted and CEA-expressing) | Mayo Clinic | – | Ovarian epithelial cancer or primary peritoneal cancer | I |
| | | | Glioblastoma | I |

**Table1. Oncolytic viruses in clinical trial.** Reproduced with permission from [544].

## References


1. Poiesz, B.J., et al., *Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma.* Proc Natl Acad Sci U S A, 1980. **77**(12): p. 7415-9.
2. Uchiyama, T., et al., *Adult T-cell leukemia: clinical and hematologic features of 16 cases.* Blood, 1977. **50**(3): p. 481-92.
3. Osame, M., et al., *Blood transfusion and HTLV-I associated myelopathy.* Lancet, 1986. **2**(8498): p. 104-5.
4. Gessain, A., et al., *Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.* Lancet, 1985. **2**(8452): p. 407-10.
5. Gessain, A. and O. Gout, *Chronic myelopathy associated with human T-lymphotropic virus type I (HTLV-I).* Ann Intern Med, 1992. **117**(11): p. 933-46.
6. Bucher, B., et al., *Tropical neuromyelopathies and retroviruses: a review.* Rev Infect Dis, 1990. **12**(5): p. 890-9.
7. Yakova, M., et al., *Increased proviral load in HTLV-1-infected patients with rheumatoid arthritis or connective tissue disease.* Retrovirology, 2005. **2**: p. 4.
8. Verdonck, K., et al., *Human T-lymphotropic virus 1: recent knowledge about an ancient infection.* Lancet Infect Dis, 2007. **7**(4): p. 266-81.
9. Ohshima, K., *Pathological features of diseases associated with human T-cell leukemia virus type I.* Cancer Sci, 2007. **98**(6): p. 772-8.
10. Ohtsuki, Y., et al., *Ultrastructural study on type C virus particles in a human cord T-cell line established by co-cultivation with adult T-cell leukemia cells.* Arch Virol, 1982. **73**(1): p. 69-73.
11. Poiesz, B.J., M.J. Poiesz, and D. Choi, *The human T-cell lymphoma/leukemia viruses.* Cancer Invest, 2003. **21**(2): p. 253-77.
12. Nagai, M., et al., *CD8(+) T cells are an in vivo reservoir for human T-cell lymphotropic virus type I.* Blood, 2001. **98**(6): p. 1858-61.
13. Grant, C., et al., *Human T cell leukemia virus type I and neurologic disease: events in bone marrow, peripheral blood, and central nervous system during normal immune surveillance and neuroinflammation.* J Cell Physiol, 2002. **190**(2): p. 133-59.
14. Manel, N., et al., *HTLV-1 tropism and envelope receptor.* Oncogene, 2005. **24**(39): p. 6016-25.
15. Clapham, P., et al., *Productive infection and cell-free transmission of human T-cell leukemia virus in a nonlymphoid cell line.* Science, 1983. **222**(4628): p. 1125-7.
16. Jones, K.S., et al., *Cell-free HTLV-1 infects dendritic cells leading to transmission and transformation of CD4(+) T cells.* Nat Med, 2008. **14**(4): p. 429-36.
17. Manel, N., et al., *The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV.* Cell, 2003. **115**(4): p. 449-59.
18. Lambert, S., et al., *HTLV-1 uses HSPG and neuropilin-1 for entry by molecular mimicry of VEGF165.* Blood, 2009. **113**(21): p. 5176-85.

19. Jones, K.S., et al., *Human T-cell leukemia virus type 1 (HTLV-1) and HTLV-2 use different receptor complexes to enter T cells.* J Virol, 2006. **80**(17): p. 8291-302.
20. Ohshima, K., et al., *Random integration of HTLV-1 provirus: increasing chromosomal instability.* Cancer Lett, 1998. **132**(1-2): p. 203-12.
21. Garzino-Demo, A., R.C. Gallo, and S.K. Arya, *Human immunodeficiency virus type 2 (HIV-2): packaging signal and associated negative regulatory element.* Hum Gene Ther, 1995. **6**(2): p. 177-84.
22. Pais-Correia, A.M., et al., *Biofilm-like extracellular viral assemblies mediate HTLV-1 cell-to-cell transmission at virological synapses.* Nat Med, 2010. **16**(1): p. 83-9.
23. Matsuoka, M. and K.T. Jeang, *Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation.* Nat Rev Cancer, 2007. **7**(4): p. 270-80.
24. Gaudray, G., et al., *The complementary strand of the human T-cell leukemia virus type 1 RNA genome encodes a bZIP transcription factor that down-regulates viral transcription.* J Virol, 2002. **76**(24): p. 12813-22.
25. Kashanchi, F. and J.N. Brady, *Transcriptional and post-transcriptional gene regulation of HTLV-1.* Oncogene, 2005. **24**(39): p. 5938-51.
26. Kusuhara, K., et al., *Human T-cell leukemia virus type 2 Rex protein increases stability and promotes nuclear to cytoplasmic transport of gag/pol and env RNAs.* J Virol, 1999. **73**(10): p. 8112-9.
27. Ye, J., et al., *HTLV-1 Rex is required for viral spread and persistence in vivo but is dispensable for cellular immortalization in vitro.* Blood, 2003. **102**(12): p. 3963-9.
28. Nicot, C., et al., *HTLV-1-encoded p30II is a post-transcriptional negative regulator of viral replication.* Nat Med, 2004. **10**(2): p. 197-201.
29. Sinha-Datta, U., et al., *Human T-cell lymphotrophic virus type I rex and p30 interactions govern the switch between virus latency and replication.* J Biol Chem, 2007. **282**(19): p. 14608-15.
30. Grone, M., C. Koch, and R. Grassmann, *The HTLV-1 Rex protein induces nuclear accumulation of unspliced viral RNA by avoiding intron excision and degradation.* Virology, 1996. **218**(2): p. 316-25.
31. Bogerd, H.P., et al., *The type I human T-cell leukemia virus (HTLV-I) Rex trans-activator binds directly to the HTLV-I Rex and the type 1 human immunodeficiency virus Rev RNA response elements.* Proc Natl Acad Sci U S A, 1991. **88**(13): p. 5704-8.
32. Nosaka, T., et al., *Nucleolar targeting signal of human T-cell leukemia virus type I rex-encoded protein is essential for cytoplasmic accumulation of unspliced viral mRNA.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 9798-802.
33. Bohnlein, S., et al., *Transdominant repressors for human T-cell leukemia virus type I rex and human immunodeficiency virus type 1 rev function.* J Virol, 1991. **65**(1): p. 81-8.
34. Younis, I. and P.L. Green, *The human T-cell leukemia virus Rex protein.* Front Biosci, 2005. **10**: p. 431-45.
35. Fornerod, M., et al., *CRM1 is an export receptor for leucine-rich nuclear export signals.* Cell, 1997. **90**(6): p. 1051-60.

36. Zhang, X., et al., *CRM1, an RNA transporter, is a major species-specific restriction factor of human T cell leukemia virus type 1 (HTLV-1) in rat cells.* Microbes Infect, 2006. **8**(3): p. 851-9.
37. Hakata, Y., et al., *Involvement of human CRM1 (exportin 1) in the export and multimerization of the Rex protein of human T-cell leukemia virus type 1.* J Virol, 1998. **72**(8): p. 6602-7.
38. Seiki, M., et al., *Two cis-acting elements responsible for posttranscriptional trans-regulation of gene expression of human T-cell leukemia virus type I.* Proc Natl Acad Sci U S A, 1988. **85**(19): p. 7124-8.
39. Toyoshima, H., et al., *Secondary structure of the human T-cell leukemia virus type 1 rex-responsive element is essential for rex regulation of RNA processing and transport of unspliced RNAs.* J Virol, 1990. **64**(6): p. 2825-32.
40. Ballaun, C., et al., *Functional analysis of human T-cell leukemia virus type I rex-response element: direct RNA binding of Rex protein correlates with in vivo activity.* J Virol, 1991. **65**(8): p. 4408-13.
41. Ahmed, Y.F., et al., *The HTLV-I Rex response element mediates a novel form of mRNA polyadenylation.* Cell, 1991. **64**(4): p. 727-37.
42. Baydoun, H.H., M. Bellon, and C. Nicot, *HTLV-1 Yin and Yang: Rex and p30 master regulators of viral mRNA trafficking.* AIDS Rev, 2008. **10**(4): p. 195-204.
43. Kesic, M., et al., *Phosphorylation regulates human T-cell leukemia virus type 1 Rex function.* Retrovirology, 2009. **6**: p. 105.
44. Kesic, M., et al., *Site-specific phosphorylation regulates human T-cell leukemia virus type 2 Rex function in vivo.* J Virol, 2009. **83**(17): p. 8859-68.
45. Adachi, Y., et al., *Phosphorylation of the Rex protein of human T-cell leukemia virus type I.* J Biol Chem, 1992. **267**(30): p. 21977-81.
46. Kubota, S., M. Hatanaka, and R.J. Pomerantz, *Nucleo-cytoplasmic redistribution of the HTLV-I Rex protein: alterations by coexpression of the HTLV-I p21x protein.* Virology, 1996. **220**(2): p. 502-7.
47. Arnold, J., et al., *Enhancement of infectivity and persistence in vivo by HBZ, a natural antisense coded protein of HTLV-1.* Blood, 2006. **107**(10): p. 3976-82.
48. Lemasson, I., et al., *Human T-cell leukemia virus type 1 (HTLV-1) bZIP protein interacts with the cellular transcription factor CREB to inhibit HTLV-1 transcription.* J Virol, 2007. **81**(4): p. 1543-53.
49. Larocca, D., et al., *Human T-cell leukemia virus minus strand transcription in infected T-cells.* Biochem Biophys Res Commun, 1989. **163**(2): p. 1006-13.
50. Cavanagh, M.H., et al., *HTLV-I antisense transcripts initiating in the 3'LTR are alternatively spliced and polyadenylated.* Retrovirology, 2006. **3**: p. 15.
51. Murata, K., et al., *A novel alternative splicing isoform of human T-cell leukemia virus type 1 bZIP factor (HBZ-SI) targets distinct subnuclear localization.* J Virol, 2006. **80**(5): p. 2495-505.
52. Hivin, P., et al., *Nuclear localization of HTLV-I bZIP factor (HBZ) is mediated by three distinct motifs.* J Cell Sci, 2005. **118**(Pt 7): p. 1355-62.
53. Matsuoka, M., *HTLV-1 bZIP factor gene: Its roles in HTLV-1 pathogenesis.* Mol Aspects Med, 2010.

54. Zhao, T., et al., *Human T-cell leukemia virus type 1 bZIP factor selectively suppresses the classical pathway of NF-kappaB.* Blood, 2009. **113**(12): p. 2755-64.
55. Thebault, S., et al., *HBZ interacts with JunD and stimulates its transcriptional activity.* FEBS Lett, 2004. **562**(1-3): p. 165-70.
56. Kuhlmann, A.S., et al., *HTLV-1 HBZ cooperates with JunD to enhance transcription of the human telomerase reverse transcriptase gene (hTERT).* Retrovirology, 2007. **4**: p. 92.
57. Barbeau, B. and J.M. Mesnard, *Does the HBZ gene represent a new potential target for the treatment of adult T-cell leukemia?* Int Rev Immunol, 2007. **26**(5-6): p. 283-304.
58. Basbous, J., et al., *The HBZ factor of human T-cell leukemia virus type I dimerizes with transcription factors JunB and c-Jun and modulates their transcriptional activity.* J Biol Chem, 2003. **278**(44): p. 43620-7.
59. Matsumoto, J., et al., *HTLV-1 HBZ suppresses AP-1 activity by impairing both the DNA-binding ability and the stability of c-Jun protein.* Oncogene, 2005. **24**(6): p. 1001-10.
60. Jeang, K.T., et al., *Induction of the HTLV-I LTR by Jun occurs through the Tax-responsive 21-bp elements.* Virology, 1991. **181**(1): p. 218-27.
61. Koiwa, T., et al., *5'-long terminal repeat-selective CpG methylation of latent human T-cell leukemia virus type 1 provirus in vitro and in vivo.* J Virol, 2002. **76**(18): p. 9389-97.
62. Satou, Y., et al., *HTLV-I basic leucine zipper factor gene mRNA supports proliferation of adult T cell leukemia cells.* Proc Natl Acad Sci U S A, 2006. **103**(3): p. 720-5.
63. Miyazaki, M., et al., *Preferential selection of human T-cell leukemia virus type 1 provirus lacking the 5' long terminal repeat during oncogenesis.* J Virol, 2007. **81**(11): p. 5714-23.
64. Burton, M., et al., *Human T-cell leukemia virus type 1 Tax shuttles between functionally discrete subcellular targets.* J Virol, 2000. **74**(5): p. 2351-64.
65. Durkin, S.S., et al., *Site-specific phosphorylation differentiates active from inactive forms of the human T-cell leukemia virus type 1 Tax oncoprotein.* J Biol Chem, 2006. **281**(42): p. 31705-12.
66. Bex, F., et al., *Phosphorylation of the human T-cell leukemia virus type 1 transactivator tax on adjacent serine residues is critical for tax activation.* J Virol, 1999. **73**(1): p. 738-45.
67. Gitlin, S.D., et al., *Transdominant human T-cell lymphotropic virus type I TAX1 mutant that fails to localize to the nucleus.* J Virol, 1991. **65**(5): p. 2612-21.
68. Smith, M.R. and W.C. Greene, *Identification of HTLV-I tax trans-activator mutants exhibiting novel transcriptional phenotypes.* Genes Dev, 1990. **4**(11): p. 1875-85.
69. Alefantis, T., et al., *Characterization of a nuclear export signal within the human T cell leukemia virus type I transactivator protein Tax.* J Biol Chem, 2003. **278**(24): p. 21814-22.

70. Gachon, F., et al., *Molecular interactions involved in the transactivation of the human T-cell leukemia virus type 1 promoter mediated by Tax and CREB-2 (ATF-4).* Mol Cell Biol, 2000. **20**(10): p. 3470-81.
71. Xiao, G. and S.C. Sun, *Activation of IKKalpha and IKKbeta through their fusion with HTLV-I tax protein.* Oncogene, 2000. **19**(45): p. 5198-203.
72. Jeang, K.T., *Functional activities of the human T-cell leukemia virus type I Tax oncoprotein: cellular signaling through NF-kappa B.* Cytokine Growth Factor Rev, 2001. **12**(2-3): p. 207-17.
73. Semmes, O.J. and K.T. Jeang, *HTLV-I Tax is a zinc-binding protein: role of zinc in Tax structure and function.* Virology, 1992. **188**(2): p. 754-64.
74. Boxus, M., et al., *The HTLV-1 Tax interactome.* Retrovirology, 2008. **5**: p. 76.
75. Basbous, J., et al., *The central region of human T-cell leukemia virus type 1 Tax protein contains distinct domains involved in subunit dimerization.* J Virol, 2003. **77**(24): p. 13028-35.
76. Jin, D.Y. and K.T. Jeang, *HTLV-I Tax self-association in optimal trans-activation function.* Nucleic Acids Res, 1997. **25**(2): p. 379-87.
77. Xie, L., et al., *PDZ binding motif of HTLV-1 Tax promotes virus-mediated T-cell proliferation in vitro and persistence in vivo.* Blood, 2006. **107**(5): p. 1980-8.
78. Vendel, A.C., S.J. McBryant, and K.J. Lumb, *KIX-mediated assembly of the CBP-CREB-HTLV-1 tax coactivator-activator complex.* Biochemistry, 2003. **42**(43): p. 12481-7.
79. Semmes, O.J. and K.T. Jeang, *Definition of a minimal activation domain in human T-cell leukemia virus type I Tax.* J Virol, 1995. **69**(3): p. 1827-33.
80. Jeang, K.T., et al., *Characterization of cellular factors that interact with the human T-cell leukemia virus type I p40x-responsive 21-base-pair sequence.* J Virol, 1988. **62**(12): p. 4499-509.
81. Yao, J. and B. Wigdahl, *Human T cell lymphotropic virus type I genomic expression and impact on intracellular signaling pathways during neurodegenerative disease and leukemia.* Front Biosci, 2000. **5**: p. D138-68.
82. Gitlin, S.D., et al., *Transcriptional activation of the human T-lymphotropic virus type I long terminal repeat by functional interaction of Tax1 and Ets1.* J Virol, 1993. **67**(12): p. 7307-16.
83. Giebler, H.A., et al., *Anchoring of CREB binding protein to the human T-cell leukemia virus type 1 promoter: a molecular mechanism of Tax transactivation.* Mol Cell Biol, 1997. **17**(9): p. 5156-64.
84. Harrod, R., et al., *p300 and p300/cAMP-responsive element-binding protein associated factor interact with human T-cell lymphotropic virus type-1 Tax in a multi-histone acetyltransferase/activator-enhancer complex.* J Biol Chem, 2000. **275**(16): p. 11852-7.
85. Higuchi, M. and M. Fujii, *Distinct functions of HTLV-1 Tax1 from HTLV-2 Tax2 contribute key roles to viral pathogenesis.* Retrovirology, 2009. **6**: p. 117.
86. Uchiumi, F., T. Ohta, and S. Tanuma, *Replication factor C recognizes 5'-phosphate ends of telomeres.* Biochem Biophys Res Commun, 1996. **229**(1): p. 310-5.

87. Hall, W.W. and M. Fujii, *Deregulation of cell-signaling pathways in HTLV-1 infection.* Oncogene, 2005. **24**(39): p. 5965-75.
88. Kwok, R.P., et al., *Control of cAMP-regulated enhancers by the viral transactivator Tax through CREB and the co-activator CBP.* Nature, 1996. **380**(6575): p. 642-6.
89. Pomerantz, J.L. and D. Baltimore, *Two pathways to NF-kappaB.* Mol Cell, 2002. **10**(4): p. 693-5.
90. Yoshida, M., *Multiple viral strategies of HTLV-1 for dysregulation of cell growth control.* Annu Rev Immunol, 2001. **19**: p. 475-96.
91. Hiscott, J., et al., *Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses.* Oncogene, 2006. **25**(51): p. 6844-67.
92. Kfoury, Y., et al., *Proapoptotic regimes for HTLV-I-transformed cells: targeting Tax and the NF-kappaB pathway.* Cell Death Differ, 2005. **12 Suppl 1**: p. 871-7.
93. Xiao, G., et al., *Retroviral oncoprotein Tax induces processing of NF-kappaB2/p100 in T cells: evidence for the involvement of IKKalpha.* EMBO J, 2001. **20**(23): p. 6805-15.
94. O'Mahony, A.M., et al., *Human T-cell lymphotropic virus type 1 tax induction of biologically Active NF-kappaB requires IkappaB kinase-1-mediated phosphorylation of RelA/p65.* J Biol Chem, 2004. **279**(18): p. 18137-45.
95. Harhaj, N.S., S.C. Sun, and E.W. Harhaj, *Activation of NF-kappa B by the human T cell leukemia virus type I Tax oncoprotein is associated with ubiquitin-dependent relocalization of I kappa B kinase.* J Biol Chem, 2007. **282**(6): p. 4185-92.
96. Harhaj, E.W., et al., *Somatic mutagenesis studies of NF-kappa B signaling in human T cells: evidence for an essential role of IKK gamma in NF-kappa B activation by T-cell costimulatory signals and HTLV-I Tax protein.* Oncogene, 2000. **19**(11): p. 1448-56.
97. Xiao, G., E.W. Harhaj, and S.C. Sun, *NF-kappaB-inducing kinase regulates the processing of NF-kappaB2 p100.* Mol Cell, 2001. **7**(2): p. 401-9.
98. Qu, Z., et al., *Tax deregulation of NF-kappaB2 p100 processing involves both beta-TrCP-dependent and -independent mechanisms.* J Biol Chem, 2004. **279**(43): p. 44563-72.
99. Gerritsen, M.E., et al., *CREB-binding protein/p300 are transcriptional coactivators of p65.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 2927-32.
100. Perkins, N.D., et al., *Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator.* Science, 1997. **275**(5299): p. 523-7.
101. Fujii, M., et al., *HTLV-1 Tax induces expression of various immediate early serum responsive genes.* Oncogene, 1991. **6**(6): p. 1023-9.
102. Fujii, M., et al., *Activation of oncogenic transcription factor AP-1 in T cells infected with human T cell leukemia virus type 1.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1603-6.
103. Azran, I., Y. Schavinsky-Khrapunsky, and M. Aboud, *Role of Tax protein in human T-cell leukemia virus type-I leukemogenicity.* Retrovirology, 2004. **1**: p. 20.

104. Jeang, K.T., et al., *Life, death, and tax: role of HTLV-I oncoprotein in genetic instability and cellular transformation.* J Biol Chem, 2004. **279**(31): p. 31991-4.
105. Wano, Y., et al., *Stable expression of the tax gene of type I human T-cell leukemia virus in human T cells activates specific cellular genes involved in growth.* Proc Natl Acad Sci U S A, 1988. **85**(24): p. 9733-7.
106. Akagi, T., H. Ono, and K. Shimotohno, *Characterization of T cells immortalized by Tax1 of human T-cell leukemia virus type 1.* Blood, 1995. **86**(11): p. 4243-9.
107. Hinrichs, S.H., et al., *A transgenic mouse model for human neurofibromatosis.* Science, 1987. **237**(4820): p. 1340-3.
108. Nerenberg, M., et al., *The tat gene of human T-lymphotropic virus type 1 induces mesenchymal tumors in transgenic mice.* Science, 1987. **237**(4820): p. 1324-9.
109. Grossman, W.J., et al., *Development of leukemia in mice transgenic for the tax gene of human T-cell leukemia virus type I.* Proc Natl Acad Sci U S A, 1995. **92**(4): p. 1057-61.
110. Marriott, S.J. and O.J. Semmes, *Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response.* Oncogene, 2005. **24**(39): p. 5986-95.
111. Brooks, G., *Cyclins, cyclin-dependent kinases, and cyclin-dependent kinase inhibitors: detection methods and activity measurements.* Methods Mol Biol, 2005. **296**: p. 291-8.
112. Lemoine, F.J., D.R. Wycuff, and S.J. Marriott, *Transcriptional activity of HTLV-I Tax influences the expression of marker genes associated with cellular transformation.* Dis Markers, 2001. **17**(3): p. 129-37.
113. Santiago, F., et al., *Transcriptional up-regulation of the cyclin D2 gene and acquisition of new cyclin-dependent kinase partners in human T-cell leukemia virus type 1-infected cells.* J Virol, 1999. **73**(12): p. 9917-27.
114. Huang, Y., et al., *Direct trans-activation of the human cyclin D2 gene by the oncogene product Tax of human T-cell leukemia virus type I.* Oncogene, 2001. **20**(9): p. 1094-102.
115. Iwanaga, R., et al., *Molecular mechanism of cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I.* Oncogene, 2001. **20**(17): p. 2055-67.
116. Haller, K., et al., *Physical interaction of human T-cell leukemia virus type 1 Tax with cyclin-dependent kinase 4 stimulates the phosphorylation of retinoblastoma protein.* Mol Cell Biol, 2002. **22**(10): p. 3327-38.
117. Akagi, T., H. Ono, and K. Shimotohno, *Expression of cell-cycle regulatory genes in HTLV-I infected T-cell lines: possible involvement of Tax1 in the altered expression of cyclin D2, p18Ink4 and p21Waf1/Cip1/Sdi1.* Oncogene, 1996. **12**(8): p. 1645-52.
118. Low, K.G., et al., *Human T-cell leukemia virus type 1 Tax releases cell cycle arrest induced by p16INK4a.* J Virol, 1997. **71**(3): p. 1956-62.
119. Ohtani, K., et al., *Cell type-specific E2F activation and cell cycle progression induced by the oncogene product Tax of human T-cell leukemia virus type I.* J Biol Chem, 2000. **275**(15): p. 11154-63.

120. Lemasson, I., et al., *Activation of E2F-mediated transcription by human T-cell leukemia virus type I Tax protein in a p16(INK4A)-negative T-cell line.* J Biol Chem, 1998. **273**(36): p. 23598-604.
121. Kehn, K., et al., *The HTLV-I Tax oncoprotein targets the retinoblastoma protein for proteasomal degradation.* Oncogene, 2005. **24**(4): p. 525-40.
122. Kehn, K., et al., *Mechanisms of HTLV-1 transformation.* Front Biosci, 2004. **9**: p. 2347-72.
123. Tabakin-Fix, Y., et al., *Functional inactivation of p53 by human T-cell leukemia virus type 1 Tax protein: mechanisms and clinical implications.* Carcinogenesis, 2006. **27**(4): p. 673-81.
124. Pise-Masison, C.A., et al., *Inhibition of p53 transactivation function by the human T-cell lymphotropic virus type 1 Tax protein.* J Virol, 1998. **72**(2): p. 1165-70.
125. de La Fuente, C., et al., *Overexpression of p21(waf1) in human T-cell lymphotropic virus type 1-infected cells and its association with cyclin A/cdk2.* J Virol, 2000. **74**(16): p. 7270-83.
126. Sherr, C.J. and J.M. Roberts, *CDK inhibitors: positive and negative regulators of G1-phase progression.* Genes Dev, 1999. **13**(12): p. 1501-12.
127. Kibler, K.V. and K.T. Jeang, *CREB/ATF-dependent repression of cyclin a by human T-cell leukemia virus type 1 Tax protein.* J Virol, 2001. **75**(5): p. 2161-73.
128. Park, H.U., et al., *Human T-cell leukemia virus type 1 Tax interacts with Chk1 and attenuates DNA-damage induced G2 arrest mediated by Chk1.* Oncogene, 2004. **23**(29): p. 4966-74.
129. Liang, M.H., et al., *Human T-lymphotropic virus type 1 oncoprotein tax promotes S-phase entry but blocks mitosis.* J Virol, 2002. **76**(8): p. 4022-33.
130. Haoudi, A., et al., *Human T-cell leukemia virus-I tax oncoprotein functionally targets a subnuclear complex involved in cellular DNA damage-response.* J Biol Chem, 2003. **278**(39): p. 37736-44.
131. Jin, D.Y., F. Spencer, and K.T. Jeang, *Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1.* Cell, 1998. **93**(1): p. 81-91.
132. Liu, B., et al., *HTLV-I Tax directly binds the Cdc20-associated anaphase-promoting complex and activates it ahead of schedule.* Proc Natl Acad Sci U S A, 2005. **102**(1): p. 63-8.
133. Tanaka, A., et al., *Oncogenic transformation by the tax gene of human T-cell leukemia virus type I in vitro.* Proc Natl Acad Sci U S A, 1990. **87**(3): p. 1071-5.
134. Kao, S.Y. and S.J. Marriott, *Disruption of nucleotide excision repair by the human T-cell leukemia virus type 1 Tax protein.* J Virol, 1999. **73**(5): p. 4299-304.
135. Maruyama, K., et al., *Chromosome and gene rearrangements in immortalized human lymphocytes infected with human T-lymphotropic virus type I.* Cancer Res, 1990. **50**(17 Suppl): p. 5697S-5702S.
136. Philpott, S.M. and G.C. Buehring, *Defective DNA repair in cells with human T-cell leukemia/bovine leukemia viruses: role of tax gene.* J Natl Cancer Inst, 1999. **91**(11): p. 933-42.
137. Wycuff, D.R. and S.J. Marriott, *The HTLV-I Tax oncoprotein: hyper-tasking at the molecular level.* Front Biosci, 2005. **10**: p. 620-42.

138. Jeang, K.T., et al., *HTLV-I trans-activator protein, tax, is a trans-repressor of the human beta-polymerase gene.* Science, 1990. **247**(4946): p. 1082-4.
139. Mozzherin, D.J. and P.A. Fisher, *Human DNA polymerase epsilon: enzymologic mechanism and gap-filling synthesis.* Biochemistry, 1996. **35**(11): p. 3572-7.
140. Ng, P.W., et al., *Genome-wide expression changes induced by HTLV-1 Tax: evidence for MLK-3 mixed lineage kinase involvement in Tax-mediated NF-kappaB activation.* Oncogene, 2001. **20**(33): p. 4484-96.
141. Chlichlia, K. and K. Khazaie, *HTLV-1 Tax: Linking transformation, DNA damage and apoptotic T-cell death.* Chem Biol Interact, 2010.
142. Vajente, N., R. Trevisan, and D. Saggioro, *HTLV-1 Tax protein cooperates with Ras in protecting cells from apoptosis.* Apoptosis, 2009. **14**(2): p. 153-63.
143. Trevisan, R., et al., *Antiapoptotic effect of human T-cell leukemia virus type 1 tax protein correlates with its creb transcriptional activity.* Exp Cell Res, 2006. **312**(8): p. 1390-400.
144. Okamoto, K., et al., *Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kappaB.* Genes Cells, 2006. **11**(2): p. 177-91.
145. Krueger, A., et al., *HTLV-1 Tax protects against CD95-mediated apoptosis by induction of the cellular FLICE-inhibitory protein (c-FLIP).* Blood, 2006. **107**(10): p. 3933-9.
146. de La Fuente, C., et al., *Gene expression array of HTLV type 1-infected T cells: Up-regulation of transcription factors and cell cycle genes.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1695-700.
147. Inoue, J., et al., *Induction of interleukin 2 receptor gene expression by p40x encoded by human T-cell leukemia virus type 1.* EMBO J, 1986. **5**(11): p. 2883-8.
148. Migone, T.S., et al., *Constitutively activated Jak-STAT pathway in T cells transformed with HTLV-I.* Science, 1995. **269**(5220): p. 79-81.
149. Mariner, J.M., et al., *Human T cell lymphotropic virus type I Tax activates IL-15R alpha gene expression through an NF-kappa B site.* J Immunol, 2001. **166**(4): p. 2602-9.
150. Azimi, N., et al., *Involvement of IL-15 in the pathogenesis of human T lymphotropic virus type I-associated myelopathy/tropical spastic paraparesis: implications for therapy with a monoclonal antibody directed to the IL-2/15R beta receptor.* J Immunol, 1999. **163**(7): p. 4064-72.
151. Grassmann, R., M. Aboud, and K.T. Jeang, *Molecular mechanisms of cellular transformation by HTLV-1 Tax.* Oncogene, 2005. **24**(39): p. 5976-85.
152. Harhaj, E.W., et al., *Gene expression profiles in HTLV-I-immortalized T cells: deregulated expression of genes involved in apoptosis regulation.* Oncogene, 1999. **18**(6): p. 1341-9.
153. Nicot, C., et al., *Bcl-X(L) is up-regulated by HTLV-I and HTLV-II in vitro and in ex vivo ATLL samples.* Blood, 2000. **96**(1): p. 275-81.
154. Nicot, C. and R. Harrod, *Distinct p300-responsive mechanisms promote caspase-dependent apoptosis by human T-cell lymphotropic virus type 1 Tax protein.* Mol Cell Biol, 2000. **20**(22): p. 8580-9.

155. Brauweiler, A., et al., *Repression of bax gene expression by the HTLV-1 Tax protein: implications for suppression of apoptosis in virally infected cells.* Virology, 1997. **231**(1): p. 135-40.
156. Suemori, K., et al., *HBZ is an immunogenic protein, but not a target antigen for human T-cell leukemia virus type 1-specific cytotoxic T lymphocytes.* J Gen Virol, 2009. **90**(Pt 8): p. 1806-11.
157. Boxus, M. and L. Willems, *Mechanisms of HTLV-1 persistence and transformation.* Br J Cancer, 2009. **101**(9): p. 1497-501.
158. Fresno Vara, J.A., et al., *PI3K/Akt signalling pathway and cancer.* Cancer Treat Rev, 2004. **30**(2): p. 193-204.
159. Peloponese, J.M., Jr. and K.T. Jeang, *Role for Akt/protein kinase B and activator protein-1 in cellular proliferation induced by the human T-cell leukemia virus type 1 tax oncoprotein.* J Biol Chem, 2006. **281**(13): p. 8927-38.
160. Ikezoe, T., et al., *Longitudinal inhibition of PI3K/Akt/mTOR signaling by LY294002 and rapamycin induces growth arrest of adult T-cell leukemia cells.* Leuk Res, 2007. **31**(5): p. 673-82.
161. Jeong, S.J., et al., *Activated AKT regulates NF-kappaB activation, p53 inhibition and cell survival in HTLV-1-transformed cells.* Oncogene, 2005. **24**(44): p. 6719-28.
162. Kazanji, M. and A. Gessain, *Human T-cell Lymphotropic Virus types I and II (HTLV-I/II) in French Guiana: clinical and molecular epidemiology.* Cad Saude Publica, 2003. **19**(5): p. 1227-40.
163. Proietti, F.A., et al., *Global epidemiology of HTLV-I infection and associated diseases.* Oncogene, 2005. **24**(39): p. 6058-68.
164. Gessain, A. and G. de The, *Geographic and molecular epidemiology of primate T lymphotropic retroviruses: HTLV-I, HTLV-II, STLV-I, STLV-PP, and PTLV-L.* Adv Virus Res, 1996. **47**: p. 377-426.
165. Sabouri, A.H., et al., *Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8+ T cells in HTLV-1-associated neurologic disease.* Blood, 2008. **112**(6): p. 2411-20.
166. Vrielink, H. and H.W. Reesink, *HTLV-I/II prevalence in different geographic locations.* Transfus Med Rev, 2004. **18**(1): p. 46-57.
167. Hollsberg, P. and D.A. Hafler, *Seminars in medicine of the Beth Israel Hospital, Boston. Pathogenesis of diseases induced by human lymphotropic virus type I infection.* N Engl J Med, 1993. **328**(16): p. 1173-82.
168. Li, H.C., et al., *The presence of ancient human T-cell lymphotropic virus type I provirus DNA in an Andean mummy.* Nat Med, 1999. **5**(12): p. 1428-32.
169. Sonoda, S., et al., *Ancient HTLV type 1 provirus DNA of Andean mummy.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1753-6.
170. Weiss, R.A., *Retroviral zoonoses.* Nat Med, 1998. **4**(4): p. 391-2.
171. Gessain, A. and R. Mahieux, *[Epidemiology, origin and genetic diversity of HTLV-1 retrovirus and STLV-1 simian affiliated retrovirus]*. Bull Soc Pathol Exot, 2000. **93**(3): p. 163-71.
172. Gessain, A. and R. Mahieux, *[A virus called HTLV-1. Virological and molecular aspects]*. Presse Med, 2000. **29**(40): p. 2228-32.

173. Gessain, A. and R. Mahieux, *[A virus called HTLV-1. Epidemiological aspects]*. Presse Med, 2000. **29**(40): p. 2233-9.
174. Gessain, A., R.C. Gallo, and G. Franchini, *Low degree of human T-cell leukemia/lymphoma virus type I genetic drift in vivo as a means of monitoring viral transmission and movement of ancient human populations.* J Virol, 1992. **66**(4): p. 2288-95.
175. Gallo, R.C., A. Sliski, and F. Wong-Staal, *Origin of human T-cell leukaemia-lymphoma virus.* Lancet, 1983. **2**(8356): p. 962-3.
176. Liu, H.F., et al., *The three human T-lymphotropic virus type I subtypes arose from three geographically distinct simian reservoirs.* J Gen Virol, 1996. **77 ( Pt 2 )**: p. 359-68.
177. Bastian, I., et al., *Isolation of a human T-lymphotropic virus type I strain from Australian aboriginals.* J Virol, 1993. **67**(2): p. 843-51.
178. Watanabe, T., et al., *Sequence homology of the simian retrovirus genome with human T-cell leukemia virus type I.* Virology, 1985. **144**(1): p. 59-65.
179. Saksena, N.K., et al., *LTR sequence and phylogenetic analyses of a newly discovered variant of HTLV-I isolated from the Hagahai of Papua New Guinea.* Virology, 1992. **189**(1): p. 1-9.
180. Slattery, J.P., G. Franchini, and A. Gessain, *Genomic evolution, patterns of global dissemination, and interspecies transmission of human and simian T-cell leukemia/lymphotropic viruses.* Genome Res, 1999. **9**(6): p. 525-40.
181. Hinuma, Y., et al., *Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera.* Proc Natl Acad Sci U S A, 1981. **78**(10): p. 6476-80.
182. Stewart, P.S. and M.J. Franklin, *Physiological heterogeneity in biofilms.* Nat Rev Microbiol, 2008. **6**(3): p. 199-210.
183. Matsuoka, M. and K.T. Jeang, *Human T-cell leukemia virus type I at age 25: a progress report.* Cancer Res, 2005. **65**(11): p. 4467-70.
184. Iga, M., et al., *Genetic evidence of transmission of human T cell lymphotropic virus type 1 between spouses.* J Infect Dis, 2002. **185**(5): p. 691-5.
185. Manns, A., et al., *Detection of early human T-cell lymphotropic virus type I antibody patterns during seroconversion among transfusion recipients.* Blood, 1991. **77**(4): p. 896-905.
186. Ureta-Vidal, A., et al., *Mother-to-child transmission of human T-cell-leukemia/lymphoma virus type I: implication of high antiviral antibody titer and high proviral load in carrier mothers.* Int J Cancer, 1999. **82**(6): p. 832-6.
187. Roucoux, D.F., et al., *A prospective study of sexual transmission of human T lymphotropic virus (HTLV)-I and HTLV-II.* J Infect Dis, 2005. **191**(9): p. 1490-7.
188. Kondo, T., et al., *Age- and sex-specific cumulative rate and risk of ATLL for HTLV-I carriers.* Int J Cancer, 1989. **43**(6): p. 1061-4.
189. Shimoyama, M., *Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984-87).* Br J Haematol, 1991. **79**(3): p. 428-37.

190. White, J.D., et al., *Infectious complications and immunodeficiency in patients with human T-cell lymphotropic virus I-associated adult T-cell leukemia/lymphoma.* Cancer, 1995. **75**(7): p. 1598-607.
191. Tsukasaki, K., P. Koeffler, and M. Tomonaga, *Human T-lymphotropic virus type 1 infection.* Baillieres Best Pract Res Clin Haematol, 2000. **13**(2): p. 231-43.
192. Nicot, C., *Current views in HTLV-I-associated adult T-cell leukemia/lymphoma.* Am J Hematol, 2005. **78**(3): p. 232-9.
193. Raphael, M., *[T-cell leukemia-lymphoma in adults and other blood diseases in HTLV-I infection]*. Ann Med Interne (Paris), 1996. **147**(8): p. 582-5.
194. Mahieux, R. and A. Gessain, *HTLV-1 and associated adult T-cell leukemia/lymphoma.* Rev Clin Exp Hematol, 2003. **7**(4): p. 336-61.
195. Chen, S., et al., *Regulatory T cell-like activity of Foxp3+ adult T cell leukemia cells.* Int Immunol, 2006. **18**(2): p. 269-77.
196. Yano, H., et al., *Regulatory T-cell function of adult T-cell leukemia/lymphoma cells.* Int J Cancer, 2007. **120**(9): p. 2052-7.
197. Matsuoka, M., *Human T-cell leukemia virus type I (HTLV-I) infection and the onset of adult T-cell leukemia (ATL).* Retrovirology, 2005. **2**: p. 27.
198. Yamaguchi, K., et al., *Lymphoma type adult T-cell leukemia--a clinicopathologic study of HTLV related T-cell type malignant lymphoma.* Hematol Oncol, 1986. **4**(1): p. 59-65.
199. Yamaguchi, K., et al., *A proposal for smoldering adult T-cell leukemia: a clinicopathologic study of five cases.* Blood, 1983. **62**(4): p. 758-66.
200. Kaplan, J.E., et al., *The risk of development of HTLV-I-associated myelopathy/tropical spastic paraparesis among persons infected with HTLV-I.* J Acquir Immune Defic Syndr, 1990. **3**(11): p. 1096-101.
201. Matsuzaki, T., et al., *HTLV-I-associated myelopathy (HAM)/tropical spastic paraparesis (TSP) with amyotrophic lateral sclerosis-like manifestations.* J Neurovirol, 2000. **6**(6): p. 544-8.
202. Lepoutre, V., et al., *Role of resident CNS cell populations in HTLV-1-associated neuroinflammatory disease.* Front Biosci, 2009. **14**: p. 1152-68.
203. Kannagi, M., et al., *Target epitope in the Tax protein of human T-cell leukemia virus type I recognized by class I major histocompatibility complex-restricted cytotoxic T cells.* J Virol, 1992. **66**(5): p. 2928-33.
204. Umehara, F., et al., *Immunocytochemical analysis of the cellular infiltrate in the spinal cord lesions in HTLV-I-associated myelopathy.* J Neuropathol Exp Neurol, 1993. **52**(4): p. 424-30.
205. Jacobson, S., et al., *Isolation of an HTLV-1-like retrovirus from patients with tropical spastic paraparesis.* Nature, 1988. **331**(6156): p. 540-3.
206. Mogensen, T.H. and S.R. Paludan, *Molecular pathways in virus-induced cytokine production.* Microbiol Mol Biol Rev, 2001. **65**(1): p. 131-50.
207. Sakai, J.A., et al., *In vitro spontaneous lymphoproliferation in patients with human T-cell lymphotropic virus type I-associated neurologic disease: predominant expansion of CD8+ T cells.* Blood, 2001. **98**(5): p. 1506-11.
208. Cartier, L. and E. Ramirez, *Presence of HTLV-I Tax protein in cerebrospinal fluid from HAM/TSP patients.* Arch Virol, 2005. **150**(4): p. 743-53.

209. Jacobson, S., et al., *HTLV-I-specific cytotoxic T lymphocytes in the cerebrospinal fluid of patients with HTLV-I-associated neurological disease.* Ann Neurol, 1992. **32**(5): p. 651-7.
210. Barmak, K., E.W. Harhaj, and B. Wigdahl, *Mediators of central nervous system damage during the progression of human T-cell leukemia type I-associated myelopathy/tropical spastic paraparesis.* J Neurovirol, 2003. **9**(5): p. 522-9.
211. Kubota, R., et al., *HTLV-I proviral DNA amount correlates with infiltrating CD4+ lymphocytes in the spinal cord from patients with HTLV-I-associated myelopathy.* J Neuroimmunol, 1994. **53**(1): p. 23-9.
212. Lehky, T.J., et al., *Detection of human T-lymphotropic virus type I (HTLV-I) tax RNA in the central nervous system of HTLV-I-associated myelopathy/tropical spastic paraparesis patients by in situ hybridization.* Ann Neurol, 1995. **37**(2): p. 167-75.
213. Parker, C.E., et al., *Activated, HTLV-1-specific cytotoxic T-lymphocytes are found in healthy seropositives as well as in patients with tropical spastic paraparesis.* Virology, 1992. **188**(2): p. 628-36.
214. Biddison, W.E., et al., *Human T cell leukemia virus type I (HTLV-I)-specific CD8+ CTL clones from patients with HTLV-I-associated neurologic disease secrete proinflammatory cytokines, chemokines, and matrix metalloproteinase.* J Immunol, 1997. **159**(4): p. 2018-25.
215. Selmaj, K., et al., *Cytokine cytotoxicity against oligodendrocytes. Apoptosis induced by lymphotoxin.* J Immunol, 1991. **147**(5): p. 1522-9.
216. Afonso, P.V., et al., *Alteration of blood-brain barrier integrity by retroviral infection.* PLoS Pathog, 2008. **4**(11): p. e1000205.
217. Levin, M.C., et al., *Autoimmunity due to molecular mimicry as a cause of neurological disease.* Nat Med, 2002. **8**(5): p. 509-13.
218. Nagai, M., et al., *Analysis of HTLV-I proviral load in 202 HAM/TSP patients and 243 asymptomatic HTLV-I carriers: high proviral load strongly predisposes to HAM/TSP.* J Neurovirol, 1998. **4**(6): p. 586-93.
219. Olindo, S., et al., *HTLV-1 proviral load in peripheral blood mononuclear cells quantified in 100 HAM/TSP patients: a marker of disease progression.* J Neurol Sci, 2005. **237**(1-2): p. 53-9.
220. Lezin, A., et al., *Human T lymphotropic virus type I (HTLV-I) proviral load in cerebrospinal fluid: a new criterion for the diagnosis of HTLV-I-associated myelopathy/tropical spastic paraparesis?* J Infect Dis, 2005. **191**(11): p. 1830-4.
221. Barmak, K., et al., *Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration.* Virology, 2003. **308**(1): p. 1-12.
222. Furukawa, Y., et al., *Phylogenetic subgroups of human T cell lymphotropic virus (HTLV) type I in the tax gene and their association with different risks for HTLV-I-associated myelopathy/tropical spastic paraparesis.* J Infect Dis, 2000. **182**(5): p. 1343-9.
223. Yashiki, S., et al., *HLA-A*26, HLA-B*4002, HLA-B*4006, and HLA-B*4801 alleles predispose to adult T cell leukemia: the limited recognition of HTLV type 1 tax peptide anchor motifs and epitopes to generate anti-HTLV type 1 tax CD8(+) cytotoxic T lymphocytes.* AIDS Res Hum Retroviruses, 2001. **17**(11): p. 1047-61.

224. Jeffery, K.J., et al., *The influence of HLA class I alleles and heterozygosity on the outcome of human T cell lymphotropic virus type I infection.* J Immunol, 2000. **165**(12): p. 7278-84.
225. Jeffery, K.J., et al., *HLA alleles determine human T-lymphotropic virus-I (HTLV-I) proviral load and the risk of HTLV-I-associated myelopathy.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3848-53.
226. Osame, M., et al., *Nationwide survey of HTLV-I-associated myelopathy in Japan: association with blood transfusion.* Ann Neurol, 1990. **28**(1): p. 50-6.
227. Kannagi, M., et al., *Immunological aspects of rat models of HTLV type 1-infected T lymphoproliferative disease.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1737-40.
228. Saito, M., *Immunogenetics and the Pathological Mechanisms of Human T-Cell Leukemia VirusType 1- (HTLV-1-)Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP).* Interdiscip Perspect Infect Dis, 2010. **2010**: p. 478461.
229. La Grenade, L., et al., *Clinical, pathologic, and immunologic features of human T-lymphotrophic virus type I-associated infective dermatitis in children.* Arch Dermatol, 1998. **134**(4): p. 439-44.
230. Merle, H., et al., *Ocular lesions in 200 patients infected by the human T-cell lymphotropic virus type 1 in martinique (French West Indies).* Am J Ophthalmol, 2002. **134**(2): p. 190-5.
231. Iwakura, Y., et al., *Induction of inflammatory arthropathy resembling rheumatoid arthritis in mice transgenic for HTLV-I.* Science, 1991. **253**(5023): p. 1026-8.
232. Nascimento, M.C., et al., *Infective dermatitis has similar immunological features to human T lymphotropic virus-type 1-associated myelopathy/tropical spastic paraparesis.* Clin Exp Immunol, 2009. **156**(3): p. 455-62.
233. Primo, J.R., et al., *Infective dermatitis and human T cell lymphotropic virus type 1-associated myelopathy/tropical spastic paraparesis in childhood and adolescence.* Clin Infect Dis, 2005. **41**(4): p. 535-41.
234. Trojano, M. and D. Paolicelli, *The differential diagnosis of multiple sclerosis: classification and clinical features of relapsing and progressive neurological syndromes.* Neurol Sci, 2001. **22 Suppl 2**: p. S98-102.
235. Fox, R.I., et al., *Sjogren's syndrome.* Clin Dermatol, 2000. **18**(5): p. 589-600.
236. Kalyanaraman, V.S., et al., *A new subtype of human T-cell leukemia virus (HTLV-II) associated with a T-cell variant of hairy cell leukemia.* Science, 1982. **218**(4572): p. 571-3.
237. Levine, P.H., et al., *HTLV-II infection in Florida Indians.* AIDS Res Hum Retroviruses, 1993. **9**(2): p. 123-7.
238. Gessain, A., et al., *HTLV-1 but not HTLV-2 found in pygmies from Central African Republic.* J Acquir Immune Defic Syndr, 1993. **6**(12): p. 1373-4.
239. Goubau, P., et al., *HTLV-II seroprevalence in pygmies across Africa since 1970.* AIDS Res Hum Retroviruses, 1993. **9**(8): p. 709-13.
240. Khabbaz, R.F., et al., *Human T lymphotropic virus type II (HTLV-II) infection in a cohort of New York intravenous drug users: an old infection?* J Infect Dis, 1991. **163**(2): p. 252-6.

241. Toro, C., et al., *Molecular epidemiology of HTLV-2 infection among intravenous drug users in Spain.* J Clin Virol, 2005. **33**(1): p. 65-70.
242. Feuer, G. and P.L. Green, *Comparative biology of human T-cell lymphotropic virus type 1 (HTLV-1) and HTLV-2.* Oncogene, 2005. **24**(39): p. 5996-6004.
243. Lal, R.B., et al., *Infection with human T-lymphotropic viruses leads to constitutive expression of leukemia inhibitory factor and interleukin-6.* Blood, 1993. **81**(7): p. 1827-32.
244. Ye, J., L. Xie, and P.L. Green, *Tax and overlapping rex sequences do not confer the distinct transformation tropisms of human T-cell leukemia virus types 1 and 2.* J Virol, 2003. **77**(14): p. 7728-35.
245. Gatza, M.L., J.C. Watt, and S.J. Marriott, *Cellular transformation by the HTLV-I Tax protein, a jack-of-all-trades.* Oncogene, 2003. **22**(33): p. 5141-9.
246. Mahieux, R. and A. Gessain, *The human HTLV-3 and HTLV-4 retroviruses: new members of the HTLV family.* Pathol Biol (Paris), 2009. **57**(2): p. 161-6.
247. Carneiro-Proietti, A.B., et al., *Presence of human immunodeficiency virus (HIV) and T-lymphotropic virus type I and II (HTLV-I/II) in a haemophiliac population in Belo Horizonte, Brazil, and correlation with additional serological results.* Haemophilia, 1998. **4**(1): p. 47-50.
248. Wiktor, S.Z., et al., *Infection with human T lymphotropic virus types I and II in sexually transmitted disease clinics in Baltimore and New Orleans.* J Infect Dis, 1992. **165**(5): p. 920-4.
249. Gallo, R.C., *Human retroviruses after 20 years: a perspective from the past and prospects for their future control.* Immunol Rev, 2002. **185**: p. 236-65.
250. Moriuchi, H., M. Moriuchi, and A.S. Fauci, *Factors secreted by human T lymphotropic virus type I (HTLV-I)-infected cells can enhance or inhibit replication of HIV-1 in HTLV-I-uninfected cells: implications for in vivo coinfection with HTLV-I and HIV-1.* J Exp Med, 1998. **187**(10): p. 1689-97.
251. Brites, C., J. Sampalo, and A. Oliveira, *HIV/human T-cell lymphotropic virus coinfection revisited: impact on AIDS progression.* AIDS Rev, 2009. **11**(1): p. 8-16.
252. Beilke, M.A., et al., *Laboratory study of HIV-1 and HTLV-I/II coinfection.* J Med Virol, 1994. **44**(2): p. 132-43.
253. Beilke, M.A., et al., *Clinical outcomes and disease progression among patients coinfected with HIV and human T lymphotropic virus types 1 and 2.* Clin Infect Dis, 2004. **39**(2): p. 256-63.
254. Bessinger, R., et al., *Retroviral coinfections at a New Orleans HIV outpatient clinic.* J Acquir Immune Defic Syndr Hum Retrovirol, 1997. **14**(1): p. 67-71.
255. Gudo, E.S., et al., *Co-infection by human immunodeficiency virus type 1 (HIV-1) and human T cell leukemia virus type 1 (HTLV-1): does immune activation lead to a faster progression to AIDS?* BMC Infect Dis, 2009. **9**: p. 211.
256. Roy, U., et al., *Upregulation of HTLV-1 and HTLV-2 expression by HIV-1 in vitro.* J Med Virol, 2008. **80**(3): p. 494-500.
257. Gudo, E.S., et al., *Serologic and molecular typing of human T-lymphotropic virus among blood donors in Maputo City, Mozambique.* Transfusion, 2009. **49**(6): p. 1146-50.

258. Andersson, S., et al., *Comparative evaluation of 14 immunoassays for detection of antibodies to the human T-lymphotropic virus types I and II using panels of sera from Sweden and West Africa.* Transfusion, 1999. **39**(8): p. 845-51.
259. Thorstensson, R., J. Albert, and S. Andersson, *Strategies for diagnosis of HTLV-I and -II.* Transfusion, 2002. **42**(6): p. 780-91.
260. Sabino, E.C., et al., *Evaluation of the INNO-LIA HTLV I/II assay for confirmation of human T-cell leukemia virus-reactive sera in blood bank donations.* J Clin Microbiol, 1999. **37**(5): p. 1324-8.
261. Zrein, M., et al., *Assessment of a new immunoassay for serological confirmation and discrimination of human T-cell lymphotropic virus infections.* Clin Diagn Lab Immunol, 1998. **5**(1): p. 45-9.
262. Varma, M., et al., *Enhanced specificity of truncated transmembrane protein for serologic confirmation of human T-cell lymphotropic virus type 1 (HTLV-1) and HTLV-2 infections by western blot (immunoblot) assay containing recombinant envelope glycoproteins.* J Clin Microbiol, 1995. **33**(12): p. 3239-44.
263. Roberts, B.D., et al., *Evaluation of an immunoblot assay for serological confirmation and differentiation of human T-cell lymphotropic virus types I and II.* J Clin Microbiol, 1993. **31**(2): p. 260-4.
264. Poiesz, B.J., et al., *Comparative performances of an HTLV-I/II EIA and other serologic and PCR assays on samples from persons at risk for HTLV-II infection.* Transfusion, 2000. **40**(8): p. 924-30.
265. Mangano, A.M., et al., *Human T lymphotropic virus types I and II proviral sequences in Argentinian blood donors with indeterminate Western blot patterns.* J Med Virol, 2004. **74**(2): p. 323-7.
266. Matsuzaki, T., et al., *HTLV-I proviral load correlates with progression of motor disability in HAM/TSP: analysis of 239 HAM/TSP patients including 64 patients followed up for 10 years.* J Neurovirol, 2001. **7**(3): p. 228-34.
267. Kamihira, S., et al., *Significance of HTLV-1 proviral load quantification by real-time PCR as a surrogate marker for HTLV-1-infected cell count.* Clin Lab Haematol, 2003. **25**(2): p. 111-7.
268. Yasunaga, J. and M. Matsuoka, *Human T-cell leukemia virus type I induces adult T-cell leukemia: from clinical aspects to molecular mechanisms.* Cancer Control, 2007. **14**(2): p. 133-40.
269. Bazarbachi, A., et al., *Evidence against a direct cytotoxic effect of alpha interferon and zidovudine in HTLV-I associated adult T cell leukemia/lymphoma.* Leukemia, 2000. **14**(4): p. 716-21.
270. Kchour, G., et al., *Zidovudine and interferon-alpha treatment induces a high response rate and reduces HTLV-1 proviral load and VEGF plasma levels in patients with adult T-cell leukemia from North East Iran.* Leuk Lymphoma, 2007. **48**(2): p. 330-6.
271. Hermine, O., et al., *A prospective phase II clinical trial with the use of zidovudine and interferon-alpha in the acute and lymphoma forms of adult T-cell leukemia/lymphoma.* Hematol J, 2002. **3**(6): p. 276-82.

272. Kchour, G., et al., *Phase 2 study of the efficacy and safety of the combination of arsenic trioxide, interferon alpha, and zidovudine in newly diagnosed chronic adult T-cell leukemia/lymphoma (ATL).* Blood, 2009. **113**(26): p. 6528-32.
273. Zhang, M., et al., *Activating Fc receptors are required for antitumor efficacy of the antibodies directed toward CD25 in a murine model of adult t-cell leukemia.* Cancer Res, 2004. **64**(16): p. 5825-9.
274. Waldmann, T.A., et al., *The interleukin-2 receptor: a target for monoclonal antibody treatment of human T-cell lymphotrophic virus I-induced adult T-cell leukemia.* Blood, 1993. **82**(6): p. 1701-12.
275. Zhang, Z., et al., *Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD2 monoclonal antibody, MEDI-507.* Blood, 2003. **102**(1): p. 284-8.
276. Maeda, N., et al., *Susceptibility of human T-cell leukemia virus type I-infected cells to humanized anti-CD30 monoclonal antibodies in vitro and in vivo.* Cancer Sci, 2010. **101**(1): p. 224-30.
277. Yamamoto, K., et al., *Phase I study of KW-0761, a defucosylated humanized anti-CCR4 antibody, in relapsed patients with adult T-cell leukemia-lymphoma and peripheral T-cell lymphoma.* J Clin Oncol, 2010. **28**(9): p. 1591-8.
278. Mitra-Kaushik, S., et al., *Effects of the proteasome inhibitor PS-341 on tumor growth in HTLV-1 Tax transgenic mice and Tax tumor transplants.* Blood, 2004. **104**(3): p. 802-9.
279. Satou, Y., et al., *Proteasome inhibitor, bortezomib, potently inhibits the growth of adult T-cell leukemia cells both in vivo and in vitro.* Leukemia, 2004. **18**(8): p. 1357-63.
280. Tan, C. and T.A. Waldmann, *Proteasome inhibitor PS-341, a potential therapeutic agent for adult T-cell leukemia.* Cancer Res, 2002. **62**(4): p. 1083-6.
281. Mori, N., et al., *Apoptosis induced by the histone deacetylase inhibitor FR901228 in human T-cell leukemia virus type 1-infected T-cell lines and primary adult T-cell leukemia cells.* J Virol, 2004. **78**(9): p. 4582-90.
282. Utsunomiya, A., et al., *Improved outcome of adult T cell leukemia/lymphoma with allogeneic hematopoietic stem cell transplantation.* Bone Marrow Transplant, 2001. **27**(1): p. 15-20.
283. Okamura, J., et al., *Allogeneic stem-cell transplantation with reduced conditioning intensity as a novel immunotherapy and antiviral therapy for adult T-cell leukemia/lymphoma.* Blood, 2005. **105**(10): p. 4143-5.
284. Oh, U. and S. Jacobson, *Treatment of HTLV-I-associated myelopathy/tropical spastic paraparesis: toward rational targeted therapy.* Neurol Clin, 2008. **26**(3): p. 781-97, ix-x.
285. Nakamura, T., Y. Nishiura, and K. Eguchi, *Therapeutic strategies in HTLV-I-associated myelopathy/tropical spastic paraparesis (HAM/TSP).* Cent Nerv Syst Agents Med Chem, 2009. **9**(2): p. 137-49.
286. Nakagawa, M., et al., *Therapeutic trials in 200 patients with HTLV-I-associated myelopathy/ tropical spastic paraparesis.* J Neurovirol, 1996. **2**(5): p. 345-55.
287. Feng, J., et al., *Interferon-alpha significantly reduces cerebrospinal fluid CD4 cell subsets in HAM/TSP.* J Neuroimmunol, 2003. **141**(1-2): p. 170-3.

288. Saito, M., et al., *Decreased human T lymphotropic virus type I (HTLV-I) provirus load and alteration in T cell phenotype after interferon-alpha therapy for HTLV-I-associated myelopathy/tropical spastic paraparesis.* J Infect Dis, 2004. **189**(1): p. 29-40.
289. Machuca, A., B. Rodes, and V. Soriano, *The effect of antiretroviral therapy on HTLV infection.* Virus Res, 2001. **78**(1-2): p. 93-100.
290. Lezin, A., et al., *Histone deacetylase mediated transcriptional activation reduces proviral loads in HTLV-1 associated myelopathy/tropical spastic paraparesis patients.* Blood, 2007. **110**(10): p. 3722-8.
291. Lindenmann, J., D.C. Burke, and A. Isaacs, *Studies on the production, mode of action and properties of interferon.* Br J Exp Pathol, 1957. **38**(5): p. 551-62.
292. Isaacs, A., J. Lindenmann, and R.C. Valentine, *Virus interference. II. Some properties of interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 268-73.
293. Malmgaard, L., *Induction and regulation of IFNs during viral infections.* J Interferon Cytokine Res, 2004. **24**(8): p. 439-54.
294. Pestka, S., C.D. Krause, and M.R. Walter, *Interferons, interferon-like cytokines, and their receptors.* Immunol Rev, 2004. **202**: p. 8-32.
295. Taniguchi, T., et al., *Human leukocyte and fibroblast interferons are structurally related.* Nature, 1980. **285**(5766): p. 547-9.
296. Weissmann, C. and H. Weber, *The interferon genes.* Prog Nucleic Acid Res Mol Biol, 1986. **33**: p. 251-300.
297. Randall, R.E. and S. Goodbourn, *Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures.* J Gen Virol, 2008. **89**(Pt 1): p. 1-47.
298. Farrar, M.A. and R.D. Schreiber, *The molecular cell biology of interferon-gamma and its receptor.* Annu Rev Immunol, 1993. **11**: p. 571-611.
299. Ank, N., et al., *Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo.* J Virol, 2006. **80**(9): p. 4501-9.
300. Akira, S., *TLR signaling.* Curr Top Microbiol Immunol, 2006. **311**: p. 1-16.
301. Hashimoto, C., K.L. Hudson, and K.V. Anderson, *The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein.* Cell, 1988. **52**(2): p. 269-79.
302. Lemaitre, B., *The road to Toll.* Nat Rev Immunol, 2004. **4**(7): p. 521-7.
303. Carpenter, S. and L.A. O'Neill, *How important are Toll-like receptors for antimicrobial responses?* Cell Microbiol, 2007. **9**(8): p. 1891-901.
304. Roach, J.C., et al., *The evolution of vertebrate Toll-like receptors.* Proc Natl Acad Sci U S A, 2005. **102**(27): p. 9577-82.
305. Xu, Y., et al., *Structural basis for signal transduction by the Toll/interleukin-1 receptor domains.* Nature, 2000. **408**(6808): p. 111-5.
306. Radons, J., et al., *Identification of essential regions in the cytoplasmic tail of interleukin-1 receptor accessory protein critical for interleukin-1 signaling.* J Biol Chem, 2002. **277**(19): p. 16456-63.
307. Murawski, M.R., et al., *Respiratory syncytial virus activates innate immunity through Toll-like receptor 2.* J Virol, 2009. **83**(3): p. 1492-500.

308. Jiang, Z., et al., *CD14 is required for MyD88-independent LPS signaling.* Nat Immunol, 2005. **6**(6): p. 565-70.
309. Georgel, P., et al., *Vesicular stomatitis virus glycoprotein G activates a specific antiviral Toll-like receptor 4-dependent pathway.* Virology, 2007. **362**(2): p. 304-13.
310. Kawai, T. and S. Akira, *TLR signaling.* Semin Immunol, 2007. **19**(1): p. 24-32.
311. Kawai, T. and S. Akira, *Antiviral signaling through pattern recognition receptors.* J Biochem, 2007. **141**(2): p. 137-45.
312. O'Neill, L.A., *The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress.* Immunol Rev, 2008. **226**: p. 10-8.
313. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3.* Nature, 2001. **413**(6857): p. 732-8.
314. Wang, T., et al., *Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis.* Nat Med, 2004. **10**(12): p. 1366-73.
315. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.
316. Meylan, E., et al., *RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation.* Nat Immunol, 2004. **5**(5): p. 503-7.
317. Oganesyan, G., et al., *Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response.* Nature, 2006. **439**(7073): p. 208-11.
318. Hoshino, K., et al., *IkappaB kinase-alpha is critical for interferon-alpha production induced by Toll-like receptors 7 and 9.* Nature, 2006. **440**(7086): p. 949-53.
319. Kawai, T., et al., *Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6.* Nat Immunol, 2004. **5**(10): p. 1061-8.
320. Tenoever, B.R., et al., *Multiple functions of the IKK-related kinase IKKepsilon in interferon-mediated antiviral immunity.* Science, 2007. **315**(5816): p. 1274-8.
321. Lee, H.K., et al., *Autophagy-dependent viral recognition by plasmacytoid dendritic cells.* Science, 2007. **315**(5817): p. 1398-401.
322. Takeuchi, O. and S. Akira, *Innate immunity to virus infection.* Immunol Rev, 2009. **227**(1): p. 75-86.
323. Yoneyama, M. and T. Fujita, *Structural mechanism of RNA recognition by the RIG-I-like receptors.* Immunity, 2008. **29**(2): p. 178-81.
324. Murali, A., et al., *Structure and function of LGP2, a DEX(D/H) helicase that regulates the innate immunity response.* J Biol Chem, 2008. **283**(23): p. 15825-33.
325. Kato, H., et al., *Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5.* J Exp Med, 2008. **205**(7): p. 1601-10.
326. Malathi, K., et al., *Small self-RNA generated by RNase L amplifies antiviral innate immunity.* Nature, 2007. **448**(7155): p. 816-9.
327. Takahasi, K., et al., *Nonself RNA-sensing mechanism of RIG-I helicase and activation of antiviral immune responses.* Mol Cell, 2008. **29**(4): p. 428-40.
328. Pichlmair, A., et al., *RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates.* Science, 2006. **314**(5801): p. 997-1001.

329. Schlee, M., et al., *Recognition of 5′ triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus.* Immunity, 2009. **31**(1): p. 25-34.
330. Schmidt, A., et al., *5′-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I.* Proc Natl Acad Sci U S A, 2009. **106**(29): p. 12067-72.
331. Rothenfusser, S., et al., *The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I.* J Immunol, 2005. **175**(8): p. 5260-8.
332. Saito, T., et al., *Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2.* Proc Natl Acad Sci U S A, 2007. **104**(2): p. 582-7.
333. Yoneyama, M., et al., *Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity.* J Immunol, 2005. **175**(5): p. 2851-8.
334. Pippig, D.A., et al., *The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA.* Nucleic Acids Res, 2009. **37**(6): p. 2014-25.
335. Cui, S., et al., *The C-terminal regulatory domain is the RNA 5′-triphosphate sensor of RIG-I.* Mol Cell, 2008. **29**(2): p. 169-79.
336. Li, X., et al., *The RIG-I-like receptor LGP2 recognizes the termini of double-stranded RNA.* J Biol Chem, 2009. **284**(20): p. 13881-91.
337. Moresco, E.M. and B. Beutler, *LGP2: positive about viral sensing.* Proc Natl Acad Sci U S A, 2010. **107**(4): p. 1261-2.
338. Gack, M.U., et al., *TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity.* Nature, 2007. **446**(7138): p. 916-920.
339. Sun, Q., et al., *The specific and essential role of MAVS in antiviral innate immune responses.* Immunity, 2006. **24**(5): p. 633-42.
340. Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses.* Nat Immunol, 2004. **5**(7): p. 730-7.
341. Lin, R., et al., *Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage.* J Virol, 2006. **80**(12): p. 6072-83.
342. Onomoto, K., M. Yoneyama, and T. Fujita, *Regulation of antiviral innate immune responses by RIG-I family of RNA helicases.* Curr Top Microbiol Immunol, 2007. **316**: p. 193-205.
343. Gatot, J.S., et al., *Lipopolysaccharide-mediated interferon regulatory factor activation involves TBK1-IKKepsilon-dependent Lys(63)-linked polyubiquitination and phosphorylation of TANK/I-TRAF.* J Biol Chem, 2007. **282**(43): p. 31131-46.
344. Zhao, T., et al., *The NEMO adaptor bridges the nuclear factor-kappaB and interferon regulatory factor signaling pathways.* Nat Immunol, 2007. **8**(6): p. 592-600.
345. Allan R. Brasier, A.G.-S., Stanley M. Lemon., *Cellular Signaling and Innate Immune Response to RNA Virus Infections.* . ASM Book. Vol. 5. 2009.

346. Mamane, Y., et al., *Interferon regulatory factors: the next generation.* Gene, 1999. **237**(1): p. 1-14.
347. Taniguchi, T., et al., *IRF family of transcription factors as regulators of host defense.* Annu Rev Immunol, 2001. **19**: p. 623-55.
348. Veals, S.A., et al., *Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins.* Mol Cell Biol, 1992. **12**(8): p. 3315-24.
349. Kim, T.K. and T. Maniatis, *The mechanism of transcriptional synergy of an in vitro assembled interferon-beta enhanceosome.* Mol Cell, 1997. **1**(1): p. 119-29.
350. Qin, B.Y., et al., *Crystal structure of IRF-3 reveals mechanism of autoinhibition and virus-induced phosphoactivation.* Nat Struct Biol, 2003. **10**(11): p. 913-21.
351. Takahasi, K., et al., *X-ray crystal structure of IRF-3 and its functional implications.* Nat Struct Biol, 2003. **10**(11): p. 922-7.
352. Qing, J., et al., *Transforming growth factor beta/Smad3 signaling regulates IRF-7 function and transcriptional activation of the beta interferon promoter.* Mol Cell Biol, 2004. **24**(3): p. 1411-25.
353. Honda, K. and T. Taniguchi, *IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors.* Nat Rev Immunol, 2006. **6**(9): p. 644-58.
354. Pernis, A.B., *The role of IRF-4 in B and T cell activation and differentiation.* J Interferon Cytokine Res, 2002. **22**(1): p. 111-20.
355. Ozato, K., H. Tsujimura, and T. Tamura, *Toll-like receptor signaling and regulation of cytokine gene expression in the immune system.* Biotechniques, 2002. **Suppl**: p. 66-8, 70, 72 passim.
356. Yoneyama, M., et al., *Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300.* EMBO J, 1998. **17**(4): p. 1087-95.
357. Miyamoto, M., et al., *Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements.* Cell, 1988. **54**(6): p. 903-13.
358. Matsuyama, T., et al., *Targeted disruption of IRF-1 or IRF-2 results in abnormal type I IFN gene induction and aberrant lymphocyte development.* Cell, 1993. **75**(1): p. 83-97.
359. Takaoka, A., et al., *Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors.* Nature, 2005. **434**(7030): p. 243-9.
360. Hiscott, J., *Triggering the innate antiviral response through IRF-3 activation.* J Biol Chem, 2007. **282**(21): p. 15325-9.
361. Au, W.C., et al., *Identification of a member of the interferon regulatory factor family that binds to the interferon-stimulated response element and activates expression of interferon-induced genes.* Proc Natl Acad Sci U S A, 1995. **92**(25): p. 11657-61.
362. Juang, Y.T., et al., *Primary activation of interferon A and interferon B gene transcription by interferon regulatory factor 3.* Proc Natl Acad Sci U S A, 1998. **95**(17): p. 9837-42.

363. Lin, R., Y. Mamane, and J. Hiscott, *Structural and functional analysis of interferon regulatory factor 3: localization of the transactivation and autoinhibitory domains.* Mol Cell Biol, 1999. **19**(4): p. 2465-74.
364. Hiscott, J., et al., *Triggering the interferon response: the role of IRF-3 transcription factor.* J Interferon Cytokine Res, 1999. **19**(1): p. 1-13.
365. Kumar, K.P., et al., *Regulated nuclear-cytoplasmic localization of interferon regulatory factor 3, a subunit of double-stranded RNA-activated factor 1.* Mol Cell Biol, 2000. **20**(11): p. 4159-68.
366. Goubau, D., et al., *Transcriptional re-programming of primary macrophages reveals distinct apoptotic and anti-tumoral functions of IRF-3 and IRF-7.* Eur J Immunol, 2009. **39**(2): p. 527-40.
367. Panne, D., T. Maniatis, and S.C. Harrison, *Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer.* EMBO J, 2004. **23**(22): p. 4384-93.
368. Nakaya, T., et al., *Gene induction pathways mediated by distinct IRFs during viral infection.* Biochem Biophys Res Commun, 2001. **283**(5): p. 1150-6.
369. Peters, R.T., S.M. Liao, and T. Maniatis, *IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex.* Mol Cell, 2000. **5**(3): p. 513-22.
370. Shimada, T., et al., *IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases.* Int Immunol, 1999. **11**(8): p. 1357-62.
371. Hemmi, H., et al., *The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection.* J Exp Med, 2004. **199**(12): p. 1641-50.
372. McWhirter, S.M., et al., *IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts.* Proc Natl Acad Sci U S A, 2004. **101**(1): p. 233-8.
373. tenOever, B.R., et al., *Activation of TBK1 and IKKvarepsilon kinases by vesicular stomatitis virus infection and the role of viral ribonucleoprotein in the development of interferon antiviral immunity.* J Virol, 2004. **78**(19): p. 10636-49.
374. Paz, S., et al., *Induction of IRF-3 and IRF-7 phosphorylation following activation of the RIG-I pathway.* Cell Mol Biol 2006. **52**(1): p. 17-28.
375. Fitzgerald, K.A., et al., *IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway.* Nat Immunol, 2003. **4**(5): p. 491-6.
376. Sharma, S., et al., *Triggering the interferon antiviral response through an IKK-related pathway.* Science, 2003. **300**(5622): p. 1148-51.
377. Hiscott, J., J. Lacoste, and R. Lin, *Recruitment of an interferon molecular signaling complex to the mitochondrial membrane: disruption by hepatitis C virus NS3-4A protease.* Biochem Pharmacol, 2006. **72**(11): p. 1477-84.
378. Mori, M., et al., *Identification of Ser-386 of interferon regulatory factor 3 as critical target for inducible phosphorylation that determines activation.* J Biol Chem, 2004. **279**(11): p. 9698-702.
379. Servant, M.J., et al., *Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA.* J Biol Chem, 2003. **278**(11): p. 9441-7.
380. Panne, D., et al., *Interferon regulatory factor 3 is regulated by a dual phosphorylation-dependent switch.* J Biol Chem, 2007. **282**(31): p. 22816-22.

381. Bergstroem, B., et al., *Identification of a novel in vivo virus-targeted phosphorylation site in IRF3.* J Biol Chem, 2010.
382. Uze, G., G. Lutfalla, and I. Gresser, *Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA.* Cell, 1990. **60**(2): p. 225-34.
383. Novick, D., B. Cohen, and M. Rubinstein, *The human interferon alpha/beta receptor: characterization and molecular cloning.* Cell, 1994. **77**(3): p. 391-400.
384. Stark, G.R., et al., *How cells respond to interferons.* Annu Rev Biochem, 1998. **67**: p. 227-64.
385. Stancato, L.F., et al., *Preassociation of STAT1 with STAT2 and STAT3 in separate signalling complexes prior to cytokine stimulation.* J Biol Chem, 1996. **271**(8): p. 4134-7.
386. Honda, K., et al., *Regulation of the type I IFN induction: a current view.* Int Immunol, 2005. **17**(11): p. 1367-78.
387. Honda, K., A. Takaoka, and T. Taniguchi, *Type I interferon gene induction by the interferon regulatory factor family of transcription factors.* Immunity, 2006. **25**(3): p. 349-60.
388. Osterlund, P.I., et al., *IFN regulatory factor family members differentially regulate the expression of type III IFN (IFN-lambda) genes.* J Immunol, 2007. **179**(6): p. 3434-42.
389. Marie, I., J.E. Durbin, and D.E. Levy, *Differential viral induction of distinct interferon-alpha genes by positive feedback through interferon regulatory factor-7.* EMBO J, 1998. **17**(22): p. 6660-9.
390. Marie, I., et al., *Phosphorylation-induced dimerization of interferon regulatory factor 7 unmasks DNA binding and a bipartite transactivation domain.* Mol Cell Biol, 2000. **20**(23): p. 8803-14.
391. Lin, R., et al., *Selective DNA binding and association with the CREB binding protein coactivator contribute to differential activation of alpha/beta interferon genes by interferon regulatory factors 3 and 7.* Mol Cell Biol, 2000. **20**(17): p. 6342-53.
392. Iwamura, T., et al., *Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways.* Genes Cells, 2001. **6**(4): p. 375-88.
393. Sato, M., et al., *Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction.* Immunity, 2000. **13**(4): p. 539-48.
394. Erlandsson, L., et al., *Interferon-beta is required for interferon-alpha production in mouse fibroblasts.* Curr Biol, 1998. **8**(4): p. 223-6.
395. Garcia-Sastre, A. and C.A. Biron, *Type 1 interferons and the virus-host relationship: a lesson in detente.* Science, 2006. **312**(5775): p. 879-82.
396. Ferrantini, M., I. Capone, and F. Belardelli, *Interferon-alpha and cancer: mechanisms of action and new perspectives of clinical use.* Biochimie, 2007. **89**(6-7): p. 884-93.
397. Hall, J.C. and A. Rosen, *Type I interferons: crucial participants in disease amplification in autoimmunity.* Nat Rev Rheumatol, 2010. **6**(1): p. 40-9.

398. Bhoj, V.G. and Z.J. Chen, *Ubiquitylation in innate and adaptive immunity.* Nature, 2009. **458**(7237): p. 430-7.
399. Liu, Y.C., *Ubiquitin ligases and the immune response.* Annu Rev Immunol, 2004. **22**: p. 81-127.
400. Saitoh, T., et al., *Negative regulation of interferon-regulatory factor 3-dependent innate antiviral response by the prolyl isomerase Pin1.* Nat Immunol, 2006. **7**(6): p. 598-605.
401. Higgs, R., et al., *The E3 ubiquitin ligase Ro52 negatively regulates IFN-beta production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3.* J Immunol, 2008. **181**(3): p. 1780-6.
402. Tian, Y., et al., *RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-kappaB activation by targeting TAB2/3 for degradation.* J Biol Chem, 2007. **282**(23): p. 16776-82.
403. Zhang, M., et al., *Negative feedback regulation of cellular antiviral signaling by RBCK1-mediated degradation of IRF3.* Cell Res, 2008. **18**(11): p. 1096-104.
404. Okumura, A., et al., *HIV-1 accessory proteins VPR and Vif modulate antiviral response by targeting IRF-3 for degradation.* Virology, 2008. **373**(1): p. 85-97.
405. Saira, K., Y. Zhou, and C. Jones, *The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) induces degradation of interferon response factor 3 and, consequently, inhibits beta interferon promoter activity.* J Virol, 2007. **81**(7): p. 3077-86.
406. Melroe, G.T., et al., *Recruitment of activated IRF-3 and CBP/p300 to herpes simplex virus ICP0 nuclear foci: Potential role in blocking IFN-beta induction.* Virology, 2007. **360**(2): p. 305-21.
407. Bauhofer, O., et al., *Classical swine fever virus Npro interacts with interferon regulatory factor 3 and induces its proteasomal degradation.* J Virol, 2007. **81**(7): p. 3087-96.
408. Hilton, L., et al., *The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation.* J Virol, 2006. **80**(23): p. 11723-32.
409. Zheng, D., et al., *PLP2, a potent deubiquitinase from murine hepatitis virus, strongly inhibits cellular type I interferon production.* Cell Res, 2008. **18**(11): p. 1105-13.
410. Kayagaki, N., et al., *DUBA: a deubiquitinase that regulates type I interferon production.* Science, 2007. **318**(5856): p. 1628-32.
411. Saha, S.K. and G. Cheng, *TRAF3: a new regulator of type I interferons.* Cell Cycle, 2006. **5**(8): p. 804-7.
412. Evans, P.C., et al., *Zinc-finger protein A20, a regulator of inflammation and cell survival, has de-ubiquitinating activity.* Biochem J, 2004. **378**(Pt 3): p. 727-34.
413. Boone, D.L., et al., *The ubiquitin-modifying enzyme A20 is required for termination of Toll-like receptor responses.* Nat Immunol, 2004. **5**(10): p. 1052-60.
414. Wertz, I.E., et al., *De-ubiquitination and ubiquitin ligase domains of A20 downregulate NF-kappaB signalling.* Nature, 2004. **430**(7000): p. 694-9.

415. Heyninck, K., et al., *Yeast two-hybrid screening for proteins interacting with the anti-apoptotic protein A20.* Methods Mol Biol, 2004. **282**: p. 223-41.
416. Mauro, C., et al., *ABIN-1 binds to NEMO/IKKgamma and co-operates with A20 in inhibiting NF-kappaB.* J Biol Chem, 2006. **281**(27): p. 18482-8.
417. Saitoh, T., et al., *A20 is a negative regulator of IFN regulatory factor 3 signaling.* J Immunol, 2005. **174**(3): p. 1507-12.
418. Lin, R., et al., *Negative regulation of the retinoic acid-inducible gene I-induced antiviral state by the ubiquitin-editing protein A20.* J Biol Chem, 2006. **281**(4): p. 2095-103.
419. Zhang, M., et al., *Regulation of IkappaB kinase-related kinases and antiviral responses by tumor suppressor CYLD.* J Biol Chem, 2008. **283**(27): p. 18621-6.
420. Friedman, C.S., et al., *The tumour suppressor CYLD is a negative regulator of RIG-I-mediated antiviral response.* EMBO Rep, 2008. **9**(9): p. 930-6.
421. Chuang, T.H. and R.J. Ulevitch, *Triad3A, an E3 ubiquitin-protein ligase regulating Toll-like receptors.* Nat Immunol, 2004. **5**(5): p. 495-502.
422. Nakhaei, P., et al., *RIG-I-like receptors: Sensing and responding to RNA virus infection.* Semin Immunol, 2009.
423. Arimoto, K., et al., *Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125.* Proc Natl Acad Sci U S A, 2007. **104**(18): p. 7500-5.
424. Duhe, R.J., L.H. Wang, and W.L. Farrar, *Negative regulation of Janus kinases.* Cell Biochem Biophys, 2001. **34**(1): p. 17-59.
425. Yasukawa, H., A. Sasaki, and A. Yoshimura, *Negative regulation of cytokine signaling pathways.* Annu Rev Immunol, 2000. **18**: p. 143-64.
426. Bromberg, J.F., et al., *Stat3 activation is required for cellular transformation by v-src.* Mol Cell Biol, 1998. **18**(5): p. 2553-8.
427. Turkson, J., et al., *Stat3 activation by Src induces specific gene regulation and is required for cell transformation.* Mol Cell Biol, 1998. **18**(5): p. 2545-52.
428. Yoshimura, A., et al., *A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors.* EMBO J, 1995. **14**(12): p. 2816-26.
429. Endo, T.A., et al., *A new protein containing an SH2 domain that inhibits JAK kinases.* Nature, 1997. **387**(6636): p. 921-4.
430. Naka, T., et al., *Structure and function of a new STAT-induced STAT inhibitor.* Nature, 1997. **387**(6636): p. 924-9.
431. Starr, R., et al., *A family of cytokine-inducible inhibitors of signalling.* Nature, 1997. **387**(6636): p. 917-21.
432. Yoshimura, A., T. Naka, and M. Kubo, *SOCS proteins, cytokine signalling and immune regulation.* Nat Rev Immunol, 2007. **7**(6): p. 454-65.
433. Kile, B.T., et al., *The SOCS box: a tale of destruction and degradation.* Trends Biochem Sci, 2002. **27**(5): p. 235-41.
434. Saito, H., et al., *IFN regulatory factor-1-mediated transcriptional activation of mouse STAT-induced STAT inhibitor-1 gene promoter by IFN-gamma.* J Immunol, 2000. **164**(11): p. 5833-43.
435. Alexander, W.S., *Suppressors of cytokine signalling (SOCS) in the immune system.* Nat Rev Immunol, 2002. **2**(6): p. 410-6.

436. Ilangumaran, S. and R. Rottapel, *Regulation of cytokine receptor signaling by SOCS1.* Immunol Rev, 2003. **192**: p. 196-211.
437. Narazaki, M., et al., *Three distinct domains of SSI-1/SOCS-1/JAB protein are required for its suppression of interleukin 6 signaling.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13130-4.
438. Yasukawa, H., et al., *The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop.* EMBO J, 1999. **18**(5): p. 1309-20.
439. Fenner, J.E., et al., *Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity.* Nat Immunol, 2006. **7**(1): p. 33-9.
440. Qing, Y., et al., *Role of tyrosine 441 of interferon-gamma receptor subunit 1 in SOCS-1-mediated attenuation of STAT1 activation.* J Biol Chem, 2005. **280**(3): p. 1849-53.
441. Zhang, J.G., et al., *The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(23): p. 13261-5.
442. Lisztwan, J., et al., *The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity.* Genes Dev, 1999. **13**(14): p. 1822-33.
443. Stebbins, C.E., W.G. Kaelin, Jr., and N.P. Pavletich, *Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function.* Science, 1999. **284**(5413): p. 455-61.
444. Kubo, M., T. Hanada, and A. Yoshimura, *Suppressors of cytokine signaling and immunity.* Nat Immunol, 2003. **4**(12): p. 1169-76.
445. Bradsher, J.N., et al., *RNA polymerase II transcription factor SIII. I. Identification, purification, and properties.* J Biol Chem, 1993. **268**(34): p. 25587-93.
446. Aso, T., et al., *Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II.* Science, 1995. **269**(5229): p. 1439-43.
447. Kamura, T., et al., *VHL-box and SOCS-box domains determine binding specificity for Cul2-Rbx1 and Cul5-Rbx2 modules of ubiquitin ligases.* Genes Dev, 2004. **18**(24): p. 3055-65.
448. Kamura, T., et al., *Muf1, a novel Elongin BC-interacting leucine-rich repeat protein that can assemble with Cul5 and Rbx1 to reconstitute a ubiquitin ligase.* J Biol Chem, 2001. **276**(32): p. 29748-53.
449. Mahrour, N., et al., *Characterization of Cullin-box sequences that direct recruitment of Cul2-Rbx1 and Cul5-Rbx2 modules to Elongin BC-based ubiquitin ligases.* J Biol Chem, 2008. **283**(12): p. 8005-13.
450. Kamizono, S., et al., *The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2.* J Biol Chem, 2001. **276**(16): p. 12530-8.
451. Frantsve, J., et al., *Socs-1 inhibits TEL-JAK2-mediated transformation of hematopoietic cells through inhibition of JAK2 kinase activity and induction of proteasome-mediated degradation.* Mol Cell Biol, 2001. **21**(10): p. 3547-57.

452. Ungureanu, D., et al., *Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1.* Mol Cell Biol, 2002. **22**(10): p. 3316-26.
453. De Sepulveda, P., S. Ilangumaran, and R. Rottapel, *Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation.* J Biol Chem, 2000. **275**(19): p. 14005-8.
454. Rui, L., et al., *SOCS-1 and SOCS-3 block insulin signaling by ubiquitin-mediated degradation of IRS1 and IRS2.* J Biol Chem, 2002. **277**(44): p. 42394-8.
455. Liu, E., J.F. Cote, and K. Vuori, *Negative regulation of FAK signaling by SOCS proteins.* EMBO J, 2003. **22**(19): p. 5036-46.
456. Kamio, M., et al., *SOCS1 [corrected] inhibits HPV-E7-mediated transformation by inducing degradation of E7 protein.* Oncogene, 2004. **23**(17): p. 3107-15.
457. Ryo, A., et al., *Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA.* Mol Cell, 2003. **12**(6): p. 1413-26.
458. Ilangumaran, S., S. Ramanathan, and R. Rottapel, *Regulation of the immune system by SOCS family adaptor proteins.* Semin Immunol, 2004. **16**(6): p. 351-65.
459. Gregorieff, A., et al., *Regulation of SOCS-1 expression by translational repression.* J Biol Chem, 2000. **275**(28): p. 21596-604.
460. Zhang, J.G., et al., *The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation.* Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2071-6.
461. Hanada, T., et al., *A mutant form of JAB/SOCS1 augments the cytokine-induced JAK/STAT pathway by accelerating degradation of wild-type JAB/CIS family proteins through the SOCS-box.* J Biol Chem, 2001. **276**(44): p. 40746-54.
462. Kamura, T., et al., *The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families.* Genes Dev, 1998. **12**(24): p. 3872-81.
463. Chen, X.P., et al., *Pim serine/threonine kinases regulate the stability of Socs-1 protein.* Proc Natl Acad Sci U S A, 2002. **99**(4): p. 2175-80.
464. Toniato, E., et al., *TRIM8/GERP RING finger protein interacts with SOCS-1.* J Biol Chem, 2002. **277**(40): p. 37315-22.
465. Vuong, B.Q., et al., *SOCS-1 localizes to the microtubule organizing complex-associated 20S proteasome.* Mol Cell Biol, 2004. **24**(20): p. 9092-101.
466. Naka, T., et al., *Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15577-82.
467. Alexander, W.S., et al., *SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine.* Cell, 1999. **98**(5): p. 597-608.
468. Marine, J.C., et al., *SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality.* Cell, 1999. **98**(5): p. 609-16.
469. Liew, F.Y., et al., *Negative regulation of toll-like receptor-mediated immune responses.* Nat Rev Immunol, 2005. **5**(6): p. 446-58.

470. Dalpke, A.H., et al., *Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs.* J Immunol, 2001. **166**(12): p. 7082-9.
471. Nakagawa, R., et al., *SOCS-1 participates in negative regulation of LPS responses.* Immunity, 2002. **17**(5): p. 677-87.
472. Kinjyo, I., et al., *SOCS1/JAB is a negative regulator of LPS-induced macrophage activation.* Immunity, 2002. **17**(5): p. 583-91.
473. Yamin, T.T. and D.K. Miller, *The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation.* J Biol Chem, 1997. **272**(34): p. 21540-7.
474. Dimitriou, I.D., et al., *Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins.* Immunol Rev, 2008. **224**: p. 265-83.
475. Mansell, A., et al., *Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation.* Nat Immunol, 2006. **7**(2): p. 148-55.
476. He, Y., et al., *SOCS1 inhibits tumor necrosis factor-induced activation of ASK1-JNK inflammatory signaling by mediating ASK1 degradation.* J Biol Chem, 2006. **281**(9): p. 5559-66.
477. Gingras, S., et al., *Re-examination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling.* J Biol Chem, 2004. **279**(52): p. 54702-7.
478. Yasukawa, H., et al., *The suppressor of cytokine signaling-1 (SOCS1) is a novel therapeutic target for enterovirus-induced cardiac injury.* J Clin Invest, 2003. **111**(4): p. 469-78.
479. Pothlichet, J., M. Chignard, and M. Si-Tahar, *Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway.* J Immunol, 2008. **180**(4): p. 2034-8.
480. Song, X.T., et al., *An alternative and effective HIV vaccination approach based on inhibition of antigen presentation attenuators in dendritic cells.* PLoS Med, 2006. **3**(1): p. e11.
481. Qiao, X., et al., *Human immunodeficiency virus 1 Nef suppresses CD40-dependent immunoglobulin class switching in bystander B cells.* Nat Immunol, 2006. **7**(3): p. 302-10.
482. Ryo, A., et al., *SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag.* Proc Natl Acad Sci U S A, 2008. **105**(1): p. 294-9.
483. Nishi, M., et al., *Requirement for microtubule integrity in the SOCS1-mediated intracellular dynamics of HIV-1 Gag.* FEBS Lett, 2009. **583**(8): p. 1243-50.
484. Bell, J.C., et al., *Oncolytic viruses: programmable tumour hunters.* Curr Gene Ther, 2002. **2**(2): p. 243-54.
485. Hawkins, L.K., N.R. Lemoine, and D. Kirn, *Oncolytic biotherapy: a novel therapeutic plafform.* Lancet Oncol, 2002. **3**(1): p. 17-26.

486. Kruyt, F.A. and D.T. Curiel, *Toward a new generation of conditionally replicating adenoviruses: pairing tumor selectivity with maximal oncolysis.* Hum Gene Ther, 2002. **13**(4): p. 485-95.
487. Nakamura, T.M., et al., *Histone H2A phosphorylation controls Crb2 recruitment at DNA breaks, maintains checkpoint arrest, and influences DNA repair in fission yeast.* Mol Cell Biol, 2004. **24**(14): p. 6215-30.
488. Krishnamurthy, S., et al., *Differentially regulated interferon response determines the outcome of Newcastle disease virus infection in normal and tumor cell lines.* J Virol, 2006. **80**(11): p. 5145-55.
489. Bergmann, M., et al., *A genetically engineered influenza A virus with ras-dependent oncolytic properties.* Cancer Res, 2001. **61**(22): p. 8188-93.
490. Varghese, S. and S.D. Rabkin, *Oncolytic herpes simplex virus vectors for cancer virotherapy.* Cancer Gene Ther, 2002. **9**(12): p. 967-78.
491. Figova, K., J. Hrabeta, and T. Eckschlager, *Reovirus - possible therapy of cancer.* Neoplasma, 2006. **53**(6): p. 457-62.
492. Mead, D.G., et al., *Transmission of vesicular stomatitis virus from infected to noninfected black flies co-feeding on nonviremic deer mice.* Science, 2000. **287**(5452): p. 485-7.
493. Flanagan, E.B., et al., *Rearrangement of the genes of vesicular stomatitis virus eliminates clinical disease in the natural host: new strategy for vaccine development.* J Virol, 2001. **75**(13): p. 6107-14.
494. Bi, Z., et al., *Vesicular stomatitis virus infection of the central nervous system activates both innate and acquired immunity.* J Virol, 1995. **69**(10): p. 6466-72.
495. Thomsen, A.R., et al., *Cooperation of B cells and T cells is required for survival of mice infected with vesicular stomatitis virus.* Int Immunol, 1997. **9**(11): p. 1757-66.
496. Plakhov, I.V., et al., *The earliest events in vesicular stomatitis virus infection of the murine olfactory neuroepithelium and entry of the central nervous system.* Virology, 1995. **209**(1): p. 257-62.
497. Barr, J.N., S.P. Whelan, and G.W. Wertz, *Transcriptional control of the RNA-dependent RNA polymerase of vesicular stomatitis virus.* Biochim Biophys Acta, 2002. **1577**(2): p. 337-53.
498. Enninga, J., et al., *Role of nucleoporin induction in releasing an mRNA nuclear export block.* Science, 2002. **295**(5559): p. 1523-5.
499. Carneiro, F.A., et al., *Membrane recognition by vesicular stomatitis virus involves enthalpy-driven protein-lipid interactions.* J Virol, 2002. **76**(8): p. 3756-64.
500. Ball, L.A., et al., *Phenotypic consequences of rearranging the P, M, and G genes of vesicular stomatitis virus.* J Virol, 1999. **73**(6): p. 4705-12.
501. Barber, G.N., *VSV-tumor selective replication and protein translation.* Oncogene, 2005. **24**(52): p. 7710-9.
502. Carroll, A.R. and R.R. Wagner, *Role of the membrane (M) protein in endogenous inhibition of in vitro transcription by vesicular stomatitis virus.* J Virol, 1979. **29**(1): p. 134-42.
503. Clinton, G.M., et al., *The matrix (M) protein of vesicular stomatitis virus regulates transcription.* Cell, 1978. **15**(4): p. 1455-62.

504. Desforges, M., et al., *Different host-cell shutoff strategies related to the matrix protein lead to persistence of vesicular stomatitis virus mutants on fibroblast cells.* Virus Res, 2001. **76**(1): p. 87-102.
505. Ahmed, M. and D.S. Lyles, *Effect of vesicular stomatitis virus matrix protein on transcription directed by host RNA polymerases I, II, and III.* J Virol, 1998. **72**(10): p. 8413-9.
506. Ahmed, M., et al., *Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis.* J Virol, 2003. **77**(8): p. 4646-57.
507. Faria, P.A., et al., *VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway.* Mol Cell, 2005. **17**(1): p. 93-102.
508. von Kobbe, C., et al., *Vesicular stomatitis virus matrix protein inhibits host cell gene expression by targeting the nucleoporin Nup98.* Mol Cell, 2000. **6**(5): p. 1243-52.
509. Petersen, J.M., et al., *The matrix protein of vesicular stomatitis virus inhibits nucleocytoplasmic transport when it is in the nucleus and associated with nuclear pore complexes.* Mol Cell Biol, 2000. **20**(22): p. 8590-601.
510. Kopecky, S.A., M.C. Willingham, and D.S. Lyles, *Matrix protein and another viral component contribute to induction of apoptosis in cells infected with vesicular stomatitis virus.* J Virol, 2001. **75**(24): p. 12169-81.
511. Kopecky, S.A. and D.S. Lyles, *The cell-rounding activity of the vesicular stomatitis virus matrix protein is due to the induction of cell death.* J Virol, 2003. **77**(9): p. 5524-8.
512. DiDonato, J.A., et al., *A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB.* Nature, 1997. **388**(6642): p. 548-54.
513. Stojdl, D.F., et al., *VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents.* Cancer Cell, 2003. **4**(4): p. 263-75.
514. Stojdl, D.F., et al., *Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus.* Nat Med, 2000. **6**(7): p. 821-5.
515. Stojdl, D.F., et al., *The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus.* J Virol, 2000. **74**(20): p. 9580-5.
516. Balachandran, S. and G.N. Barber, *Vesicular stomatitis virus (VSV) therapy of tumors.* IUBMB Life, 2000. **50**(2): p. 135-8.
517. Balachandran, S., E. Thomas, and G.N. Barber, *A FADD-dependent innate immune mechanism in mammalian cells.* Nature, 2004. **432**(7015): p. 401-5.
518. Meurs, E., et al., *Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon.* Cell, 1990. **62**(2): p. 379-90.
519. Balachandran, S., et al., *Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection.* Immunity, 2000. **13**(1): p. 129-41.
520. Meraz, M.A., et al., *Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway.* Cell, 1996. **84**(3): p. 431-42.

521. Sonenberg, N. and A.G. Hinnebusch, *Regulation of translation initiation in eukaryotes: mechanisms and biological targets.* Cell, 2009. **136**(4): p. 731-45.
522. Gingras, A.C., B. Raught, and N. Sonenberg, *eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation.* Annu Rev Biochem, 1999. **68**: p. 913-63.
523. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes Dev, 2004. **18**(16): p. 1926-45.
524. Richter, J.D. and N. Sonenberg, *Regulation of cap-dependent translation by eIF4E inhibitory proteins.* Nature, 2005. **433**(7025): p. 477-80.
525. Mohammad-Qureshi, S.S., M.D. Jennings, and G.D. Pavitt, *Clues to the mechanism of action of eIF2B, the guanine-nucleotide-exchange factor for translation initiation.* Biochem Soc Trans, 2008. **36**(Pt 4): p. 658-64.
526. Kimball, S.R., *Eukaryotic initiation factor eIF2.* Int J Biochem Cell Biol, 1999. **31**(1): p. 25-9.
527. Clemens, M.J. and A. Elia, *The double-stranded RNA-dependent protein kinase PKR: structure and function.* J Interferon Cytokine Res, 1997. **17**(9): p. 503-24.
528. Koromilas, A.E., et al., *Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase.* Science, 1992. **257**(5077): p. 1685-9.
529. Meurs, E.F., et al., *Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase.* Proc Natl Acad Sci U S A, 1993. **90**(1): p. 232-6.
530. Hii, S.I., et al., *Loss of PKR activity in chronic lymphocytic leukemia.* Int J Cancer, 2004. **109**(3): p. 329-35.
531. Balachandran, S. and G.N. Barber, *Defective translational control facilitates vesicular stomatitis virus oncolysis.* Cancer Cell, 2004. **5**(1): p. 51-65.
532. Pavitt, G.D., W. Yang, and A.G. Hinnebusch, *Homologous segments in three subunits of the guanine nucleotide exchange factor eIF2B mediate translational regulation by phosphorylation of eIF2.* Mol Cell Biol, 1997. **17**(3): p. 1298-313.
533. Connor, J.H. and D.S. Lyles, *Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1.* J Virol, 2002. **76**(20): p. 10177-87.
534. Dratewka-Kos, E., et al., *Catalytic utilization of eIF-2 and mRNA binding proteins are limiting in lysates from vesicular stomatitis virus infected L cells.* Biochemistry, 1984. **23**(25): p. 6184-90.
535. Connor, J.H. and D.S. Lyles, *Inhibition of host and viral translation during vesicular stomatitis virus infection. eIF2 is responsible for the inhibition of viral but not host translation.* J Biol Chem, 2005. **280**(14): p. 13512-9.
536. Liu, T.C., et al., *Development of targeted oncolytic virotherapeutics through translational research.* Expert Opin Biol Ther, 2008. **8**(9): p. 1381-91.
537. Liu, T.C., E. Galanis, and D. Kirn, *Clinical trial results with oncolytic virotherapy: a century of promise, a decade of progress.* Nat Clin Pract Oncol, 2007. **4**(2): p. 101-17.
538. Msaouel, P., A. Dispenzieri, and E. Galanis, *Clinical testing of engineered oncolytic measles virus strains in the treatment of cancer: an overview.* Curr Opin Mol Ther, 2009. **11**(1): p. 43-53.

539. Power, A.T. and J.C. Bell, *Taming the Trojan horse: optimizing dynamic carrier cell/oncolytic virus systems for cancer biotherapy.* Gene Ther, 2008. **15**(10): p. 772-9.
540. Qiao, J., et al., *Cyclophosphamide facilitates antitumor efficacy against subcutaneous tumors following intravenous delivery of reovirus.* Clin Cancer Res, 2008. **14**(1): p. 259-69.
541. Davis, J.J. and B. Fang, *Oncolytic virotherapy for cancer treatment: challenges and solutions.* J Gene Med, 2005. **7**(11): p. 1380-9.
542. Carey, B.L., et al., *Early steps of the virus replication cycle are inhibited in prostate cancer cells resistant to oncolytic vesicular stomatitis virus.* J Virol, 2008. **82**(24): p. 12104-15.
543. Nguyen, T.L., et al., *The emergence of combinatorial strategies in the development of RNA oncolytic virus therapies.* Cell Microbiol, 2009. **11**(6): p. 889-97.
544. Nguyen, T.L., M.G. Wilson, and J. Hiscott, *Oncolytic viruses and histone deacetylase inhibitors--a multi-pronged strategy to target tumor cells.* Cytokine Growth Factor Rev, 2010. **21**(2-3): p. 153-9.

# ~ CHAPTER II ~

## *RESULTS*

# Rationale, Global Hypothesis and Specific Aims

Infection with the Human T-cell Leukemia virus type I (HTLV-I) results in a number of diverse pathologies, including the aggressive, fatal T-cell malignancy - Adult T-cell Leukemia (ATL) - and the chronic, progressive neurologic disorder termed HTLV-1-associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). Although the majority of HTLV-1–infected individuals remain asymptomatic carriers (AC) during their lifetime, a small proportion (2 -5%) of AC develop either ATL or HAM/TSP, but never both. Since the discovery of HTLV-1, extensive studies on the molecular biology and virology of this retrovirus have been performed; however, the nature of HTLV-1 pathogenesis remains elusive and therefore represents a serious obstacle to establishing effective therapies for HTLV-1 associated diseases. **The first objective of this thesis was to better understand HTLV-1 pathogenesis by identifying candidate genes important for early diagnosis of HTLV-1- associated diseases.** The prognosis of ATL is poor with a median survival time for acute disease of less than 6 months. Standard chemotherapeutic regimens have failed miserably in the treatment of ATL. New combination therapies have shown to produce a synergistic response and induce a partial remission, with a maximum response reached after several months. However, these therapies are not a cure, since relapse occurred when patients in remission were taken off the treatment. **The failure of conventional therapies in the treatment of acute ATL led us to our second objective which was two pronged: 1) to use an oncolytic virus - VSV - as therapeutic approach to target the CD4+/CD25+ leukemic T-cells from ATL individuals and 2) to better understand the mechanism of VSV-mediated oncolysis in ATL cells.**

**Accordingly, we developed the 3 following specific aims:**

1- **Characterization of gene expression profiles in CD4+ T-lymphocytes from a unique cohort of HTLV-1 infected patients (AC, HAM/TSP, ATL).**
2- **Evaluation of VSV-induced oncolysis in normal versus CD4+ T-cells versus CD4+ leukemic cells derived from ATL individuals.**
3- **Since ATL cells are highly proliferating CD4+/CD25+ T-cells, we sought to characterize the signaling events governing VSV permissiveness in primary leukemic versus normal activated T-cells.**

## Preamble for Manuscript I

HTLV-1 is the etiological agent of ATL and HAM/TSP diseases. Although the majority of HTLV-1–infected individuals remain AC during their lifetime, a small proportion of patients develop either ATL or HAM/TSP, but never both. HAM/TSP is a systemic immune-mediated inflammatory disease characterized by demyelination of motor neurons in the spinal cord. High HTLV-1 proviral load is related to disease progression and a pro-inflammatory microenvironment triggered by invading lymphocytes within the affected tissue is a hallmark of the immunological profile. In contrast, ATL is characterized by low proviral load, yet progressive development of leukemogenesis, which occurs in two stages. Transformation of CD4+ T- lymphocytes by HTLV-1 starts by the induction of an IL-2-dependent, CD4+ T-cell proliferation phase. Over a period of decades, leukemogenesis progresses with the emergence of an IL-2-independent malignant clone(s) that accumulate multiple secondary genetic changes in growth regulatory and tumor suppressor genes. Despite extensive progress in the understanding of the molecular biology and virology of HTLV-1, the factors contributing to HTLV-1 pathogenesis are poorly defined and required further investigation. In this chapter, we sought to study gene expression differences between HTLV-1 associated diseases and ultimately identify genes that would serve as markers for early diagnosis of disease outcome.

# - MANUSCRIPT I -

## HTLV-1 Evades Type I Interferon Antiviral Signaling by Inducing the Suppressor of Cytokine Signaling 1 (SOCS1)

*In revision at: PLoS Pathogens*

**Stéphanie Olière[1,2], Eduardo Hernandez[1], Agnès Lézin[3], Meztli Arguello[1], Renée Douville[1,2], Thi Lien-Anh Nguyen[1,2], Stéphane Olindo[3], Gérard Panelatti[3], Mirdad Kazanji[4], Peter Wilkinson[5*], Rafick-Pierre Sékaly[5*], Raymond Césaire[3] and John Hiscott[1,2#].**

[1]Molecular Oncology Group, Lady Davis Institute for Medical Research, Jewish General Hospital and [2]Depts. of Microbiology and Medicine, McGill University, Montreal Canada H3T 1E2. [3]Laboratoire de Virologie-Immunologie, Service de Neurologie and JE2503, Université des Antilles et de la Guyane, Centre Hospitalier Universitaire de Fort-de-France, Martinique. [4]Unité de Rétrovirologie, Centre International de Recherches Médicales de Franceville (CIRMF), Franceville, Gabon. [5]Laboratoire d'Immunologie, Centre de Recherche du Centre Hospitalier de l'Université de Montréal Saint-Luc, Montréal, Québec, Canada.

* Present address : Vaccine and Gene Therapy Institute – Florida, Port St. Lucie FL.

[#] Corresponding author.

Running title: HTLV-1 inhibits IFN signaling by inducing SOCS1.

Key words: HTLV-1, HAM/TSP, ATL, SOCS1, interferon, IRF3, microarray

This research was supported by grants from the National Cancer Institute, the Canadian Cancer Society, the Canadian Institutes of Health Research, CANFAR, the Canadian Foundation for AIDS Research and Genome Quebec. SO and TLAN were supported by FRSQ and JH is a recipient of a CIHR Senior Investigator award.

**ABSTRACT**

Human T cell leukemia virus type 1 (HTLV-1) is the etiologic agent of Adult T cell Leukemia (ATL) and the neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). Although the majority of HTLV-1–infected individuals remain asymptomatic carriers (AC) during their lifetime, 2-5% will develop either ATL or HAM/TSP, but never both. To better understand the gene expression changes in HTLV-1-associated diseases, we examined the mRNA profiles of CD4+ T cells isolated from 7 ATL, 12 HAM/TSP, 11 AC and 8 non-infected controls. Using genomic approaches followed by bioinformatic analysis, we identified gene expression pattern characteristic of HTLV-1 infected individuals and particular disease states. Of particular interest, the suppressor of cytokine signaling 1 - SOCS1- was upregulated in HAM/TSP and AC patients but not in ATL. Moreover, SOCS1 was positively correlated with the expression of HTLV-1 mRNA in HAM/TSP patient samples. In primary PBMCs transfected with a HTLV-1 proviral clone and in HTLV-1-transformed MT-2 cells, HTLV-1 replication correlated with induction of SOCS1 and inhibition of IFN-α/β and IFN-stimulated gene expression. Targeting SOCS1 with siRNA restored type I IFN production and reduced HTLV-1 replication in MT-2 cells. Conversely, exogenous expression of SOCS1 resulted in enhanced HTLV-1 mRNA synthesis. In addition to inhibiting signaling downstream of the IFN receptor, SOCS1 inhibited IFN-β production by targeting IRF3 for ubiquitination and proteasomal degradation. These observations identify a novel SOCS1-driven mechanism of evasion of the type I IFN antiviral response against HTLV-1.

## AUTHOR'S SUMMARY

Infection with HTLV-1 leads to the development of Adult T cell Leukemia (ATL) or the neurological disorder HTLV-1-associated myelopathy / tropical spastic paraparesis (HAM/TSP). Although the majority of HTLV-1–infected individuals remain asymptomatic carriers (AC) during their lifetime, 2-5% will develop either ATL or HAM/TSP. Using gene expression profiling of CD4+ T lymphocytes from HTLV-1 infected patients, we identified Suppressor of cytokine signaling 1 (SOCS1) as being highly expressed in HAM/TSP and AC patients. SOCS1 expression positively correlated with the high HTLV-1 mRNA load that is characteristic of HAM/TSP patients. SOCS1 inhibited cellular antiviral signaling during HTLV-1 infection by degrading IRF3, an essential transcription factor in the interferon pathway. Our study reveals a novel evasion mechanism utilized by HTLV-1 that leads to increased retroviral replication, without triggering the innate immune response.

## INTRODUCTION

Infection with the Human T cell Leukemia Virus type I (HTLV-I) can result in a number of disorders, including the aggressive T cell malignancy Adult T cell Leukemia (ATL) and the chronic, progressive neurologic disorder termed HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) [1,2,3]. In endemic areas including Southern Japan, the Caribbean basin, Western Africa and Central/South America - where infection rates range from 2 to 30% - these diseases are major causes of mortality and morbidity [4]. The majority of HTLV-1–infected individuals remain asymptomatic (AC) during their lifetime and only ~ 2-5% of AC will develop either ATL or HAM/TSP [5,6]. Although the factors determining progression from AC to ATL or HAM/TSP remain unknown, it is well established that the risk of ATL vs. HAM/TSP development varies dramatically with the geographical distribution of HTLV-1-infected populations.

Clinically, acute ATL is characterized by abnormally elevated T cell counts, accompanied by readily observed 'flower cells' – multi-lobed, leukemic cells with highly condensed chromatin - hypercalcemia, prominent skin lesions, hepatosplenomegaly and suffer from serious bacterial, viral, fungal and protozoan infections. Most patients present at this final acute stage, often unaware of their HTLV-1 positive status and given a poor prognosis, with a survival estimate of 6-10 months [7]. Transformation of CD4+ T lymphocytes by HTLV-1 and the development of ATL leukemogenesis generally occur in two stages [8,9]. After infection with the blood borne pathogen, HTLV-1 induces IL-2-dependent, CD4+ T cell proliferation, that over a period of decades *in vivo*, progresses with the emergence of an IL-2-independent malignant clone that has accumulated multiple secondary genetic changes in growth regulatory and tumor suppressor genes [9,10]. HTLV-1 encodes the 40 kd nuclear oncoprotein Tax that promotes cellular transformation through dysregulation of mitotic checkpoints, activation of cellular signaling pathways and inactivation of tumor suppressors (reviewed in [11,12]).

HAM/TSP is a systemic immune-mediated inflammatory disease characterized by demyelination of motor neurons in the spinal cord, although other tissues can also be damaged [13]. HAM/TSP attacks in the prime of life (median age of onset: 35 years) and is associated with a clinical history that includes neurological symptoms in 80% of cases – gradual onset of leg weakness, paresthesis, and impairment of urinary or bowel function. Central nervous system (CNS) white matter lesions of the spinal cord harbor activated $CD4^+$ and $CD8^+$ T cells during early stages of disease, with a predominance of $CD8^+$ T cells later in disease. HTLV-1 viral RNA has been found associated with $CD4^+$ T cells and astrocytes in CNS lesions, suggesting that virus-infected cells migrate through the blood-brain barrier and infect CNS resident cells [14,15]. While the mechanisms resulting in HAM/TSP development remain unresolved, it has been suggested that Tax expression in CNS cells triggers a strong virus-specific $CD8^+$ (as well as $CD4^+$) T cell response leading to inflammation, myelin loss, and axonal damage [16,17]. Elevated levels of proinflammatory cytokines (IL-6, IFN-γ, IL-15, IL-1β, TNF-α and IL-12) have been detected in the serum and cerebrospinal fluid (CSF) of patients with HAM/TSP, corroborating the link between HAM/STP development and dysregulated inflammation [18,19].

It is widely accepted that type I interferon (IFN-α/β) has a negative impact on HIV-1 replication [20,21], and although few reports have documented the IFN antiviral effects during HTLV-1 infection, type I IFN constitutes a potent anti-retroviral mechanism that affects HTLV-1 replication [22,23]. In return, HTLV-1 infection of pDCs results in impaired IFN-α production, and correlates with elevated HTLV-1 proviral load in infected individuals [24]. Central to the establishment of an antiviral state is the activation of diverse IFN-stimulated genes (ISGs) which restrict viral replication [25]. Interferon regulatory factors IRF3 and IRF7 play essential roles in the early phase of IFN gene activation [26]. IRF3 is constitutively expressed and is activated by C-terminal phosphorylation by IKKε and TBK1, which promotes transactivation of downstream genes such as IFN-β and IFN-α [27,28]. In contrast, IRF7 protein is synthesized *de novo* upon IFN stimulation and contributes to the amplification of the IFN response, *via*

expression of multiple IFN-α subtypes [29]. IRF-driven IFN secretion acts in a paracrine fashion to induce the expression of hundreds of genes through engagement of the IFN receptors and activation of the JAK/STAT signaling pathway, which leads to the development of an antiviral state (reviewed in [30,31]).

IFN-induced JAK/STAT signaling is negatively regulated at different levels by several cellular factors to control the extent of the antiviral response and limit tissue damage [32,33]. Suppressor of cytokine signaling 1 (SOCS1) belongs to the SOCS protein family and is induced after virus infection [34]. SOCS1 suppresses IFN signaling by direct binding to phosphorylated type I IFN receptor and active JAK kinase, abrogating phosphorylation of STAT1 [35]. Through its SOCS-Box domain, SOCS1 targets various proteins such as JAK, MAL, p65, Steel, Vav for proteasomal degradation [36,37,38]. The SOCS-Box serves as a recruiting platform for the formation of a E3 ligase complex composed of elongin B/C-Cullin 2 and Rbx2 [39,40]. Thus, SOCS1 initiates and orchestrates the events leading to proteasomal degradation of target proteins [34]. Recently, virus-induced upregulation of SOCS1 protein has emerged as a novel mechanism employed by several viruses to evade the antiviral response [41,42,43]. In the present study, global gene expression profiles in CD4+ T lymphocytes were examined in a unique cohort of 30 HTLV-1 infected individuals from the Caribbean basin including ATL, HAM/TSP and asymptomatic carriers (AC) patients. Interestingly, among the many genes dysregulated in HTLV-1 infected patients, SOCS1 was highly expressed in CD4+ T cells from HAM/TSP and AC patients, but not in ATL. Subsequent biochemical analysis demonstrated that HTLV-1-induced SOCS1 expression played a positive role in viral replication through inhibition of the IFN response. SOCS1 directly interacted with IRF3 and promoted its proteasomal degradation in a SOCS-Box dependent manner, thus identifying a novel mechanism of HTLV-1-mediated evasion of the IFN response.

## MATERIAL AND METHODS

### *Ethics Statement.*

Blood samples from HTLV-1 infected patients and non-infected (NI) donors were obtained from the Centre Hospitalier Universitaire de Fort-de-France in Martinique and Institut Pasteur de Cayenne in French Guyana. Patients suffering from ATL, HAM/TSP or HTLV-1 asymptomatic carriers were recruited according to World Health Organization (WHO) criteria. According to the French Bioethics laws, the collection of samples from HAM/TSP, ATL, AC and NI has been declared to the French Ministry of Research and the study was reviewed and approved by the CPP (Comité de Protection des Personnes) Sud-Ouest/Outre-Mer III, as well as the ARH (Agence Régionale de l'Hospitalisation) from Martinique. Because the protocol is non-interventional (e.g. blood samples collected for routine health care with no additional samplings or specific procedures for subjects), no informed consent was provided by the patient, as stated by the French Public Health code and therefore the study was conducted anonymously. Clinical collection of samples for research purpose are stored at the Centre de Ressources Biologiques de Martinique (CeRBiM). The CeRBiM database has been approved by the CNIL (Commission nationale de l'informatique et des libertés). Leukophoresis from healthy donors were also obtained at the Royal Victoria Hospital, Montreal, Quebec, Canada. Informed consent were written and provided by study participants in accordance with the Declaration of Helsinki. The study was reviewed and approved by the Royal Victoria Hospital, the Jewish General Hospital, and McGill University Research Ethics Committee (REC) board of the SMBD-Jewish General Hospital.

### *Patient samples*

In total, we selected for study 12 HAM/TSP, 11 asymptomatics (AC), 7 ATL and 8 not infected individuals (NI). The diagnosis of the 7 ATL cases included in patient cohort number 1 respected the international consensus recently published by Tsukasaki et al. [7]. Diagnostic criteria for ATL included serologic evidence of HTLV-1 infection, and cytologically or histologically proven T-cell malignancy. Six ATL cases were classified

as acute leukemia type on the basis of leukemic manifestations, with >5% typical ATL cells in the peripheral blood, and immunologically confirmed mature CD4+ T cell phenotype. One case (HISS0023) was a lymphoma type, with <5% circulating abnormal cells, the ATL cell phenotype and clonal integration of HTLV-1 being confirmed on lymph node tissue. Diagnosis of HAM/TSP was in accordance with WHO criteria [67], which comprise (1) slowly progressive spastic paraparesis with symmetrical pyramidal signs, (2) disturbance of bladder function, (3) no radiologic evidence of significant spinal cord compression, and (4) intra-thecal synthesis of anti-HTLV-1 antibodies. The asymptomatic HTLV-1 carriers did not display any neurological symptoms (Tables S1 and S2). PBMCs were isolated by centrifugation (400g at 20°C for 25 min) on a Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences Inc., Oakville, Canada). CD4+ T lymphocytes were isolated using a negative selection CD4 enrichment cocktail with the high-speed autoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. In all cases, the purity of CD4+ T lymphocytes was between 90 and 95% as determined by flow cytometry. Cells were pelleted and kept at -80°C until all samples were ready for RNA extraction.

***Cell lines and reagents***

The HTLV-1-carrying T cell lines MT-2, MT-4, C8166, RMP and the HTLV-1-negative T cell lines CEM and Jurkat were used for experiments. MT-2, MT-4 and C8166 cells are derived from umbilical cord blood lymphocytes after cocultivation with leukemic cells from ATL patients [68]. MT-2 cells are reported to have integrated at least fifteen copies, including defective types, of HTLV-1 proviral DNA whereas C8166 cells have only one copy of proviral DNA integrated in the genome [69]. The interleukin (IL-2)-independent RMP cell line is derived from CD4+ T cell of a patient with acute ATL. All cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillinG, and 100 µg/ml streptomycin. HEK293T cells were used for transient transfection and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillinG, and 100 µg/ml streptomycin.

For proteasome inhibitor treatment, MG132 (Sigma-Aldrich) or Lactacystin (Boston Biochem) were used at 5 and 10 μM, respectively. Sendai virus CANTELL strain (SeV) was obtained from Charles River Laboratory (North Franklin, CT). Cells were infected with SeV at 20 hemagglutinating units (HAU) per $10^6$ cells in serum-free medium supplemented with 10% heat-inactivated fetal bovine serum 2h postinfection and harvested for whole cell extracts or RNA extraction at indicated times.

***Plasmids***

The HTLV-1 proviral clone pX1M-TM was kind gift from Dr David Derse (National Cancer Institute-Frederick, Frederick, USA). Myc-tagged SOCS1 full length and the deletion mutant SOCS1-ΔBCBox (amino acids 174-183) were kind gifts from Dr. Ferbeyre Gerardo (Departement de Biochimie, Universite de Montreal, Canada). Ha-Ub-K48R and Ha-Ub-KO were kind gifts from Dr. Zhijan Chen (Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas). Plasmid encoding for Flag-tagged IRF3 full length was described previously [27].

***RNA isolation, amplification, and hybridization***

Total RNA was extracted using Trizol Reagent (Invitrogen) or RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA integrity and purity was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies). Total RNA was amplified using the MessageAmp II mRNA kit (Ambion, Austin, USA). Sample and universal human RNA probes (Stratagene) for microarray hybridization were prepared by labeling the amplified RNA with Cy5 or Cy3, respectively, by reverse transcription, and hybridizing the labeled cDNA on the CANVAC (http://www.canvac.ca/) human Immunoarray version 2 manufactured by the Microarray Center (UHN, Toronto, ON, Canada) containing 7256 duplicate spots representing 3628 expressed sequence tags (ESTs). Details of the labeling and hybridization procedures can be obtained at http://transnet.uhnres.utoronto.ca

***Microarray analysis***

Microarrays were scanned using Scanarray Express Scanner (Packard Biosciences) or the Axon 4000B scanner at 10-μm resolution. Array images were inspected visually for poor quality spots and flagged for omission. Quantified raw data was acquired with QuantArray version 3 and saved as quantarray text files. The quantified raw data were managed and pre-processed in GeneTraffic (Iobion Informatic). Following background correction and removal of genes where both channels were less than 100 or represented by less than 90% of the samples and polished data was generated by normalization by Lowess sub-grid. The final data array was analyzed using JExpress Pro software (http://www.molmine). To establish differentially expressed genes, multi-class analysis was performed by one-way ANOVA on $Log_2$ fold change ($Log_2$Fc) data for ATL, HAM/TSP and NI groups. Genes with a $p$ value $\leq 0.01$ were selected as significant (1039 total). Visualization was produced by unsupervised clustering of the 1039 genes after re-introduction of final data for AC using Pearson correlation parameters. Pair wise correspondance analysis (PCA) was performed on the first 500 genes by Future subset Selection (FSS) t-test. Genes were selected based on false discovery rate (FDR) according to the Benjamini/Hochberg (BH) methods. Gene annotations were gathered using manual searches in NCBI as well as the ontology tools DAVID (http://david.abcc.ncifcrf.gov/) and BioRag (Bioresource for array genes, http://www.biorag.org). Fold change (Fc) for each gene was calculated as $2^{(Log_2X-Log_2NI)}$, where $Log_2$ X represents the $Log_2$ (Fc) for either ATL or HAM and $Log_2$ NI represents the $Log_2$ (Fc) for NI. Microarray data have been deposited in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). The results of the microarray experiment were confirmed by quantitative PCR (Q-PCR) on 47 genes chosen on the basis of a fold change of at least 2-fold, with RNA from 3 patients per group used for validation. A strong correlation between the average fold-change determined by microarray and the average of the qPCR results was observed, with 25/47 genes having a Pearson correlation value of at least 0.6 (Figure S1A, B, C) and 18/47 genes with a value of at least 0.9 (Figure S1A).

***Transfection of primary PBMCs with HTLV-1 proviral clone***

Leukophoresis from healthy donors were obtained at the Royal Victoria Hospital, Montreal, Quebec, Canada. PBMCs were isolated by Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences Inc., Oakville, Ontario, Canada) and activated for 4 days with 2 µg/ml of phytohemagglutinin-P (PHA-P) (Sigma Aldrich) and 50 U of interleukin 2 per ml (IL-2) (PBL Biomedical Laboratories). 5 µg of pX1M-TM was pulsed into $10 \times 10^6$ cells PBMCs in a 0.4-cm cuvette using a Gene Pulser II (Bio-Rad Laboratories) set at 0.25 kV and 0.95 µF. Cells were plated in six-well plates in complete medium and collected at indicated times for whole cell extracts or RNA extraction.

***Real time PCR analysis***

Validation of selected target genes was performed by relative quantification PCR (RQ-PCR) in 9 samples (3 NI, 3 ATL, 3 HAM). A total of 2 µg of amplified RNA from uninfected and HTLV-1-infected samples was converted to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. cDNA was amplified using SyBR Green I PCR master mix (Roche Applied Science, Germany) or TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Real-time PCR primers were designed using the primer3 website (primer3_www.cgiv. 0.2) and listed in Supporting information (Table S3). Some predesigned primers and probe sets from TaqMan (Applied Biosystems) were also used and listed in Table S3. Data were then collected using the AB 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and analyzed by comparative $C_T$ method using the SDS v1.3.1 Relative Quantification (RQ) Software where ddCT = dCT(Sample) – dCT (non-infected), dCT (Sample) = CT (Sample) - CT (GAPDH) and dCT (non-infected) = CT (non-infected) - CT (GAPDH).

***Immunoblot analysis and Dimerization assay***

Cells destined for immunoblotting were washed with PBS and lysed in lysis buffer (0.05% NP-40, 1% glycerol, 30 mMNaF, 40 mM β-glycerophosphate, 10 mM $Na_3VO_4$, 10 ng/ml of protease inhibitors cocktail (Sigma Aldrich, Oakville, Ontario, Canada). The

protein concentration was determined by using the Bradford assay (Bio-Rad, Mississauga, Canada). Whole-cell extracts (30 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 10%-acrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Mississauga, Canada). Membranes were blocked in 5% nonfat dried milk in Tris-buffered saline (TBS) plus 0.1% Tween 20 for 1 h at room temperature. Membranes were then probed overnight with antibodies against Stat 1 phosphorylated (Tyr701) (1:1000; Cell Signaling) and non-phosphorylated forms (p91) (1μg/ml; Santa Cruz); phosphorylated Jak1 (Tyr 1022/1023) (1:1000; Cell Signaling); total Jak 1 (1μg/ml Santa Cruz); SeV (1:10,000); SOCS1 (1μg/ml; Zymed laboratories), IRF3 (1:10,000; IBL, Japan) in 5% bovine serum albumin and PBS at 4°C. Incubation mixtures were washed in TBS-0.05% Tween 20 five times for a total of 25 min. Following washes, the membrane was incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (KPL, Gaithersburg, MD) at a dilution of 1:5,000 for 1 h at room temperature. Following the incubation with the secondary antibody, membranes were washed again (5 times, 5 min each) and then visualized with an enhanced chemiluminescence (detection system as recommended by the manufacturer (ECL; GE Healthcare Bio-Sciences Inc., Oakville, Ontario, Canada).

Native-PAGE was conducted as described [70]. Briefly, 10 μg WCE in native sample buffer (62.5 mMTris-HCl, pH 6.8, 15% glycerol, and bromophenol blue) were resolved by electrophoresis on a 7.5% acrylamide gel (without SDS) pre-runned for 30 min at 40 mA using 25 mMTris and 192 mM glycine, pH 8.4, with and without 1% deoxycholate in the cathode and anode chamber, respectively. After transferred into nitrocellulose membrane, IRF3 monomers and dimers were detected by immunoblot using an IRF3 anti-NES antibody (1:10, 000, IBL, Japan).

***Immunoprecipitation assay***

HEK293 cells (1 X $10^6$ cells/60-mm dish) were transiently transfected with equal amounts (5 μg) of IRF3 and MYC-tagged SOCS1 expression plasmids by using calcium phosphate precipitation method. Cells were harvested 24h post-transfection, washed with

1 X phosphate-buffered saline (PBS), and lysed in a 1% Triton X-100 lysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1% Triton X-100, 10% glycerol, 40 mM β-glycerophosphate, 0.1% protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, 1 mM $Na_3VO_4$, 5 mM NaF, 1 mM dithiothreitol). Immunoprecipitations were performed by incubating WCE (300 µg) with 1 µg of anti-MYC (9E10; Sigma-Aldrich, St. Louis, MO) or 1 µg of antiserum directed against IRF3 (rabbit polyclonal antibody, IBL, Japan) coupled to 50 µl of A/G Plus-agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C with constant agitation. Immunocomplexes were washed at least 3 times in lysis buffer eluted by boiling beads in 5 volumes SDS-PAGE sample buffer. The proteins were fractioned on 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed by immunoblot assay using anti-MYC (Sigma-Aldrich) or anti-IRF3 (IBL, Japan) antibodies.

***RNA interference***

Control and SOCS1-specific RNA interference sequences were described previously [71,72]. SOCS1 protein was knocked down using siSOCS (1), siSOCS (2) or a pool of the two siRNAs (siSOCS (1)+siSOCS (2)). siRNAs were pulsed into MT-2 cells in a 0.4-cm cuvette using a Gene Pulser II (Bio-Rad Laboratories) set at 0.25 kV and 0.95 µF. Cells were plated in six-well plates in complete medium, washed 4 and 12h later and collected at 72h post-transfection. RNA extinction efficiency was demonstrated by real time PCR and immunoblot assay.

***Statistical analysis***

Data are presented as the mean ± standard error of the mean (SEM). Statistical significance for comparison of gene expression was assessed by an unpaired Student's *t* test, with the expection of Figure 4, panels D and E where a two-way ANOVA with Bonferroni post-test was used. Analyses were performed using Prism 5 software (GraphPad). Statistical significance was evaluated using the following *p* values: $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

## RESULTS

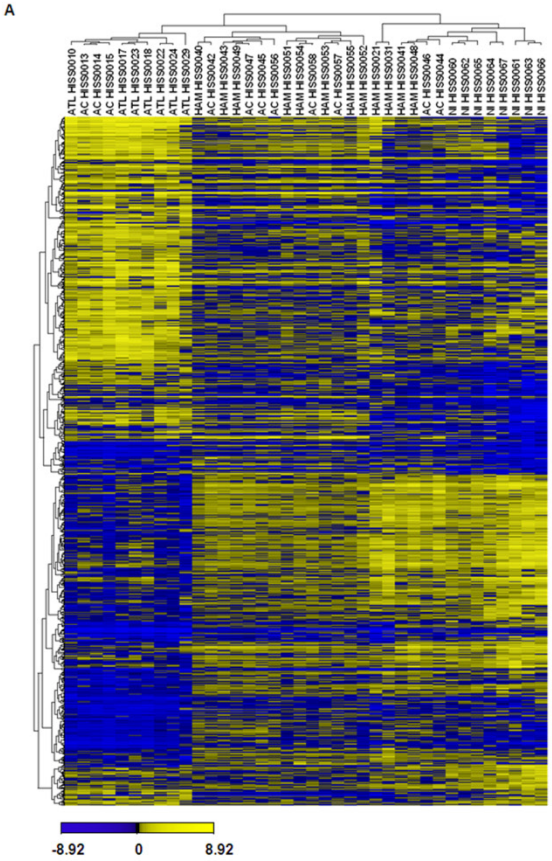
### Gene expression profiling of CD4+ T cells from ATL, HAM/TSP and AC patients.

To analyze gene expression profiles of CD4+ T cells isolated from HTLV-1 infected patients, we gathered a unique cohort of 30 HTLV-1 infected individuals from the Caribbean basin, including 11 AC, 7 ATL, 12 HAM/TSP and 8 healthy, non-infected donors (NI) (Table S1). Microarray experiments were performed using the human ImmuneArray cDNA array (UHN Microarray Center, University of Toronto), followed by higher order analysis. About three thousand genes were analyzed with Future Selection Subset/ANOVA on log-transformed data, followed by unsupervised hierarchical clustering on 1039 genes selected by Anova analysis ($p < 0.01$) (Figure 1A). These genes displayed differential expression patterns depending on the type of HTLV-1 associated disease. Unsupervised clustering based on the 1039 genes signature accurately discriminated between NI, HAM/TSP and ATL patients. AC samples however did not separate as an individual cluster, but rather distributed amongst HAM/TSP and ATL samples. Also, two of the HAM/TSP patients and two AC clustered with the NI group, suggesting that the profile of their circulating CD4+ T lymphocytes had not undergone significant variation compared to healthy donors.

Pair-wise correspondance analysis (PCA) was performed on the top 500 genes modulated in HTLV-1 infected and non-infected samples ($p$ value < 0.01, false discovery rate (FDR) = 0.17 %) (Figure 1B). PCA identified prevalent expression profiles among the three clinical groups, and confirmed significant class discrimination between non-HTLV-1-infected donors (NI) and each of the HTLV-1 associated diseases when plotted in two dimensions (Figure 1B). Gene clusters common to each HTLV-1 infected clinical group, and shared within pair-wise comparisons (AC-HAM/TSP, AC-ATL and ATL-HAM/TSP), could be identified and are presented in the adjoining Tables of Figure 1B. For each grouping, genes with a high differential expression are located with quantitative spacing from the center comparator (gene expression of NI group). Specifically, SOCS1 (green square) was identified as a strongly upregulated gene in both HAM/TSP and AC

patients, in agreement with the prior observation by Nishiura *et* al. [44]. Since SOCS1 is known to counter-regulate the anti-viral response, it was selected as a gene of interest for further study.

A

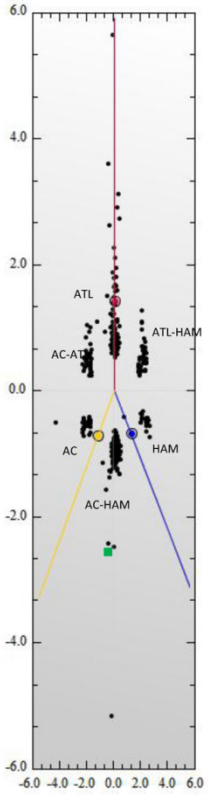



B

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| PPP1CB | 2.2 | -0.1 | 4.6 |
| MMP15 | 2.1 | 0.0 | 3.9 |
| PLA2G12A | 2.4 | 0.4 | 3.9 |
| CDC16 | 2.4 | 0.3 | 3.7 |
| HSPBAP1 | 2.2 | 0.1 | 3.5 |
| ACAD8 | 2.2 | 0.1 | 3.3 |
| COPS5 | 2.7 | 0.6 | 3.8 |
| PRKCB1 | 2.5 | 0.4 | 3.5 |
| CUBN | 2.2 | 0.1 | 3.0 |
| MAP2K1IP1 | 2.3 | 0.2 | 2.9 |
| TP53I11 | 3.2 | 1.0 | 3.6 |
| TRAF3 | 2.3 | 0.2 | 2.7 |
| DNAJA2 | 2.5 | 0.5 | 2.7 |
| IFITM1 | 2.7 | 0.5 | 2.6 |
| MEP1A | 3.1 | 1.0 | 3.2 |
| EFCBP2 | 2.7 | 0.6 | 2.7 |

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| NOC2L | 8.9 | 2.7 | 3.9 |
| FEZ1 | 2.6 | 0.5 | -0.5 |
| C6orf166 | 2.3 | 0.3 | -0.6 |
| SAMD1 | 2.5 | 0.2 | -0.2 |
| PDZD2 | 2.3 | 0.1 | -0.4 |
| EDN3 | 2.4 | 0.2 | -0.3 |
| CCL13 | 2.3 | 0.1 | -0.4 |
| CHUK | 2.2 | 0.1 | -0.5 |
| MARK2 | 2.6 | 0.4 | -0.1 |
| CRIP2 | 2.4 | 0.2 | -0.3 |
| SIAH2 | 2.3 | 0.2 | -0.4 |
| OXA1L | 2.3 | 0.2 | -0.3 |
| EPHB4 | 2.2 | 0.2 | -0.3 |
| MGEA5 | 2.2 | 0.1 | -0.3 |
| NUDT1 | 2.1 | 0.0 | -0.4 |
| CARTPT | 2.7 | 0.5 | 0.2 |
| CNP | 2.6 | 0.5 | 0.1 |
| BOK | 2.2 | 0.1 | -0.3 |
| TLR2 | 2.2 | 0.1 | -0.3 |
| COX7C | 3.4 | 1.2 | 1.0 |

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| ENG | 6.3 | 5.4 | -3.3 |
| LGALS3BP | 2.9 | 2.0 | -2.5 |
| SOCS1 | 5.0 | 3.8 | 0.3 |
| TBP | 4.2 | 4.0 | 0.4 |
| SPN | 3.0 | 3.0 | -0.6 |
| FOLR3 | 3.1 | 2.7 | -0.3 |
| GZMB | 3.2 | 3.1 | 0.3 |
| SPOCK1 | 2.6 | 2.5 | -0.2 |
| AXIN2 | 2.5 | 2.4 | -0.2 |
| SERPINE1 | 2.6 | 2.5 | -0.2 |
| HLF | 2.4 | 2.4 | -0.3 |
| CLIP1 | 2.5 | 2.5 | -0.1 |




| Symbol | AC | HAM | ATL |
|---|---|---|---|
| CD48 | 1.9 | 2.2 | 36.5 |
| CD2 | 2.5 | 2.1 | 20.1 |
| GDF10 | -0.2 | 0.4 | 12.9 |
| STK39 | 1.3 | 2.0 | 13.6 |
| KLRB1 | 0.3 | 0.8 | 12.6 |
| TRA@ | 1.1 | 0.7 | 11.8 |
| GTF2F2 | 0.1 | 0.1 | 8.9 |
| LRRFIP1 | 0.6 | 0.7 | 9.0 |
| TARP | 0.2 | 0.4 | 7.7 |
| SELPLG | 2.1 | 2.4 | 9.3 |
| CCL14 | 0.0 | -0.1 | 6.9 |
| CEP110 | 0.1 | 0.3 | 7.0 |
| CXCR4 | 2.5 | 1.9 | 8.5 |
| FNBP1L | -0.4 | -0.4 | 5.9 |
| DUSP1 | 0.1 | 0.2 | 6.3 |
| ITGA2 | -0.2 | 0.1 | 5.7 |
| CD63 | 3.4 | 1.7 | 7.1 |
| CFLAR | -2.1 | 0.0 | 3.1 |
| TNFSF11 | -2.1 | -2.1 | 3.0 |
| CDC7 | 0.1 | 0.0 | 5.1 |

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| CLEC4A | 0.0 | 2.1 | 3.0 |
| SETD2 | 0.1 | 2.3 | 3.0 |
| LASP1 | 0.8 | 3.0 | 3.6 |
| ARHGAP19 | 0.1 | 2.3 | 2.7 |
| DNAJB1 | 0.3 | 2.4 | 2.8 |
| USP16 | 0.1 | 2.1 | 2.5 |
| FIGF | 0.0 | 2.0 | 2.4 |
| IDE | -0.1 | 2.2 | 2.3 |
| WSB2 | 0.6 | 2.7 | 2.9 |
| UBE3C | 0.5 | 2.5 | 2.7 |
| AIM2 | 0.5 | 2.7 | 2.7 |

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| RABL4 | 0.6 | 2.6 | 0.1 |
| IKBKG | 0.1 | 2.2 | -0.1 |
| PHC2 | 0.0 | 2.0 | -0.3 |
| BMP4 | 0.1 | 2.4 | 0.3 |
| MKL1 | 0.4 | 2.5 | 0.3 |
| CBLB | 0.6 | 2.7 | 0.6 |
| IFNG | 1.3 | 2.0 | 0.4 |

| Symbol | AC | HAM | ATL |
|---|---|---|---|
| ERBB4 | 2.2 | 2.2 | -0.3 |
| MICA | 2.1 | 2.2 | -0.4 |
| IGHG1 | 2.7 | 2.6 | 0.2 |
| LAIR2 | 2.4 | 2.4 | -0.1 |
| PPIL2 | 2.1 | 2.1 | -0.4 |
| TCF1 | 2.4 | 2.5 | 0.0 |
| STAT3 | 2.4 | 2.3 | 0.0 |
| NXPH3 | 2.4 | 2.2 | -0.1 |

**Figure 1. Expression profiling of genes differentially expressed in HTLV-1 associated-diseases.** (A) Unsupervised hierarchical clustering of the 1039 genes differentially expressed in CD4+ T-lymphocytes from 7 ATL, 12 HAM/TSP, 11 AC and 8 NI donors. Significant variation in the expression pattern of 1039 genes was determined by ANOVA ($p < 0.01$). Each row represents the relative level of expression for a single gene; each column shows the expression level for a single sample. The yellow and blue colors indicate high and low expression, respectively. Genes were clustered into 3 groups using a complete linkage and Pearson correlation as distance metric. (B) Pair-wise contrast by Correspondance Analysis (PCA)**.** PCA analysis was performed on the 1039 genes selected by ANOVA. Correspondence analysis shows genes plotted in discriminate space and separated into 6 groups: genes associated specifically with ATL, HAM/TSP or AC, and genes commonly regulated in the three groups; AC-HAM/TSP, AC-ATL or ATL-HAM/TSP. Colored lines denote the direction of class medians and black dots correspond to the genes. Genes with high differential expression in a group are located far-off the center (gene expression of NI), in the direction determined by the group. The closest to the group line, the more evident is the associations of the genes with that group. Genes that are down-regulated in this group appear on the opposite site of the centroid. A short list of the top genes was created by selecting the genes having $\geq 2$ fold changes relative to NI. The genes lists are ranked by the absolute variation (Max-Min) in fold change expression among the three HTLV-1 infected groups.

**HTLV-1 infection induces the expression of SOCS1 mRNA.**

Efficient HTLV-1 spread must overcome cellular antiviral programs [45]; yet how HTLV-1 evades the host innate immune response is poorly understood. SOCS1 stood out among the many genes identified as having the potential to counteract the innate immune response against HTLV-1. HAM/TSP and AC patients exhibited a greater than two fold increase in *SOCS1* gene expression compared to NI individuals (Fisher's test, $p$ value = 0.054 and < 0.05, respectively) (Figure 2A). However, no significant difference in mRNA levels of SOCS1 was found between ATL and NI patients, suggesting that SOCS1 expression was uniquely upregulated in AC and HAM/TSP. This increase was specific for SOCS1, as SOCS3 mRNA was unchanged in HTLV-1 infected samples compared to control samples (fold change < 2) (Figure 2B). Using a separate cohort of patient samples (Table S2), we demonstrated that SOCS1 expression was strongly and positively correlated with HTLV-1 mRNA load in CD4+ T cells of HAM/TSP patients (Pearson's $p$<0.0001) (Figure 2C).

Since high proviral load is a hallmark of HAM/TSP pathology [46], we investigated the relationship between HTLV-1 replication and *SOCS1* gene expression. Initially, the level of SOCS1 mRNA was examined in HTLV-1-carrying T cell lines (MT-2, C8166, MT-4, RMP), control T cell lines (Jurkat and CEM), as well as PBMCs infected with the HTLV-1 infectious molecular clone pX1M-TM (Figure 2D and 2E). In MT-2 cells that carry an integrated replication-competent provirus and produce infectious HTLV-1 viral particles, a ~50-fold increase in SOCS1 mRNA was detected, as compared to non-infected CEM and Jurkat cells (< 5-fold). In MT-4 and C8166 cells, which carry a defective provirus, lower levels of SOCS1 mRNA were detected, suggesting that SOCS1 induction required an intact proviral genome (Figure 2D). The RMP cell line derived from an ATL patient which express low amount of HTLV-1 mRNA also displayed lower SOCS1 level (~10-fold). In order to determine whether *de novo* HTLV-1 infection induced SOCS1 expression, PBMCs expressing the HTLV-1 infectious molecular clone pX1M-TM (Figure 2E) were analyzed for the level of SOCS1 and HTLV-1 mRNA at different times post-transfection. HTLV-1 RNA expression was determined by amplifying the Px region

(*tax*/*rex*) of the HTLV-1 proviral genome; HTLV-1 RNA expression was modest between 24 and 72h (< 10-fold), but the viral mRNA load increased sharply at 96h (50-fold), concomitent with a dramatic increase in *SOCS1* gene expression (~24-fold).

The initial observation that SOCS1 was induced upon HTLV-1 infection prompted us to examine whether SOCS1 also influenced viral replication. To do so, the effect of SOCS1 expression on HTLV-1 provirus replication was examined in the CEM T cells, co-expressing a SOCS1 expression vector together with the HTLV-1 provirus. The level of HTLV-1 mRNA was consistently higher in SOCS1 expressing cells compared to CEM cells expressing HTLV-1 provirus alone (e.g. 55-fold vs. 10-fold at 24h) (Figure 3A). As a complementary strategy, SOCS1 expression was silenced in MT-2 cells (Figure 3B); siRNAs targeting SOCS1 (siSOCS1(1) siSOCS1(2) and siSOCS1(1)+siSOCS1(2)) inhibited SOCS1 levels by 50, 75 and 90%, respectively. Knock-down of SOCS1 protein expression was confirmed by immunoblot assay (Figure 3B, bottom panel). Real time PCR analysis of the HTLV-1 pX region demonstrated a significant reduction of HTLV-1 mRNA that directly correlated with the decrease in the observed SOCS1 levels (~27, 56 and 80% decrease, respectively). These data indicate that SOCS1 induction during HTLV-1 infection leads to enhanced HTLV-1 replication.

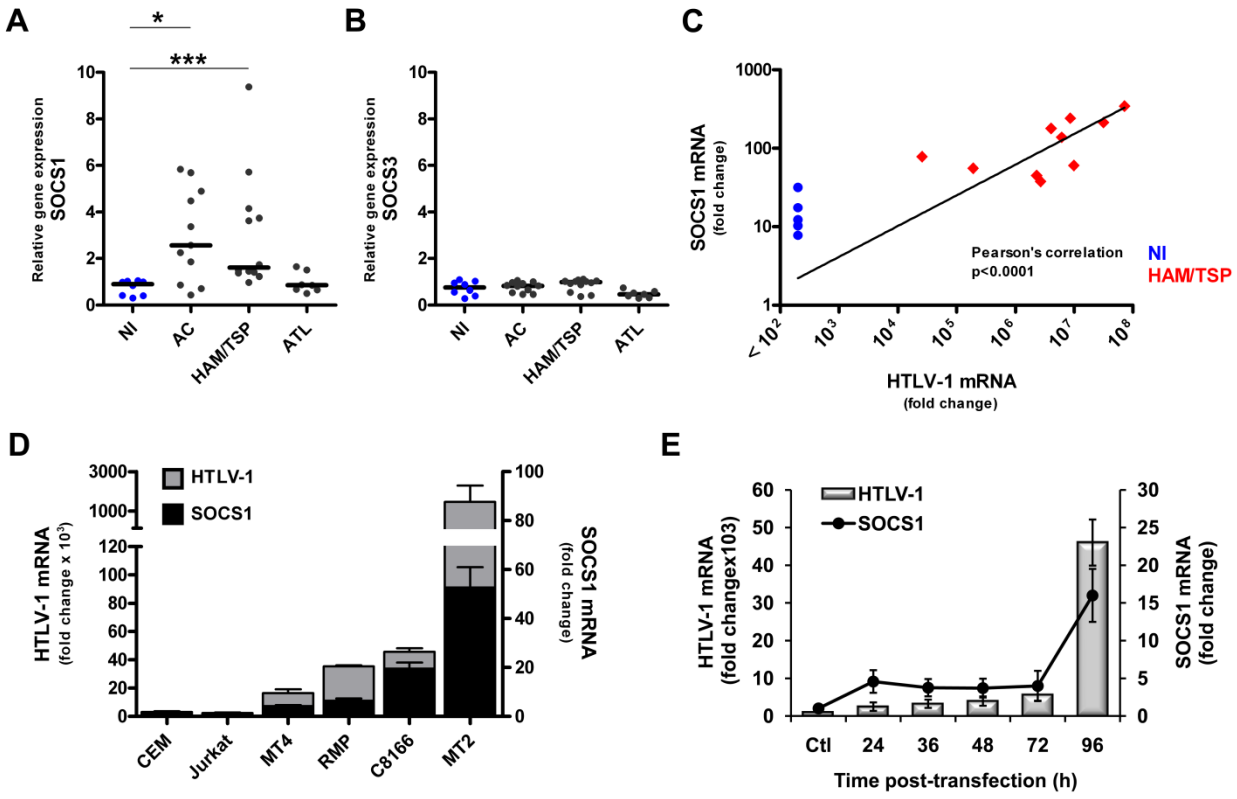

A
*
***
Relative gene expression
SOCS1
NI
AC
HAM/TSP
ATL
B
Relative gene expression
SOCS3
C
SOCS1 mRNA
(fold change)
Pearson's correlation
p<0.0001
NI
HAM/TSP
HTLV-1 mRNA
(fold change)
D
HTLV-1
SOCS1
HTLV-1 mRNA
(fold change x 10⁵)
SOCS1 mRNA
(fold change)
CEM
Jurkat
MT4
RMP
C8166
MT2
E
HTLV-1
SOCS1
HTLV-1 mRNA
(fold changex103)
SOCS1 mRNA
(fold change)
Ctl
24
36
48
72
96
Time post-transfection (h)

**Figure 2. HTLV-1 infection results in the induction of SOCS1 mRNA levels in *ex vivo* CD4+ T cells and correlates with HTLV-1 mRNA expression.** (A, B) Comparison of mRNA expression of SOCS1 and SOCS3 in CD4+ T cells from HTLV-1 infected and NI patients. CD4+ T cells were isolated from PBMCs of HTLV-1 infected and NI patients; SOCS1 (A) and SOCS3 (B) mRNA levels were assessed using a human cDNA array. *SOCS1* gene expression was stratified by clinical status forming 4 groups: NI, ATL, AC and HAM/TSP. Each point represents SOCS level from one individual, with black bars showing median value in each group. Mann-Whitney test was used to compare intensity of SOCS expression between groups (*, $p<0.05$; ***, $p<0.001$). (C) Expression of SOCS1 mRNA levels correlated with HTLV-1 mRNA load in HAM/TSP patients. CD4+ T cells from HAM/TSP and NI patients were lysed and total RNA was subjected to reverse transcription. cDNA was analyzed by quantitative real time PCR to assess mRNA levels of SOCS1 and HTLV-1 (Pearson's correlation $p<0.0001$, line represents log-log non-linear fit of the data) (D) Expression of SOCS1 and HTLV-1 mRNAs was measured in HTLV-1-carrying T cell lines (MT-2, MT-4, C8166, RMP) vs. HTLV-1-negative T cell lines (CEM, Jurkat). Cells were treated as previously and cDNA was analyzed by quantitative real time PCR to measured *SOCS1* and HTLV-1 gene expression. Equivalent mRNA amounts were normalized to *GAPDH* gene expression and calculated as fold change with the levels of uninfected CEM cells set arbitrarily as 1. (E) PBMCs from healthy individuals were electroporated with either HTLV-1 provirus (pX1M-TM) or control empty vector (ctl). At the indicated times, total RNA was extracted and analyzed for *SOCS1* and HTLV-1 gene expression. Equivalent mRNA amounts were normalized to GAPDH mRNA expression and calculated as fold change from the levels of control cells that were arbitrarily set as 1.

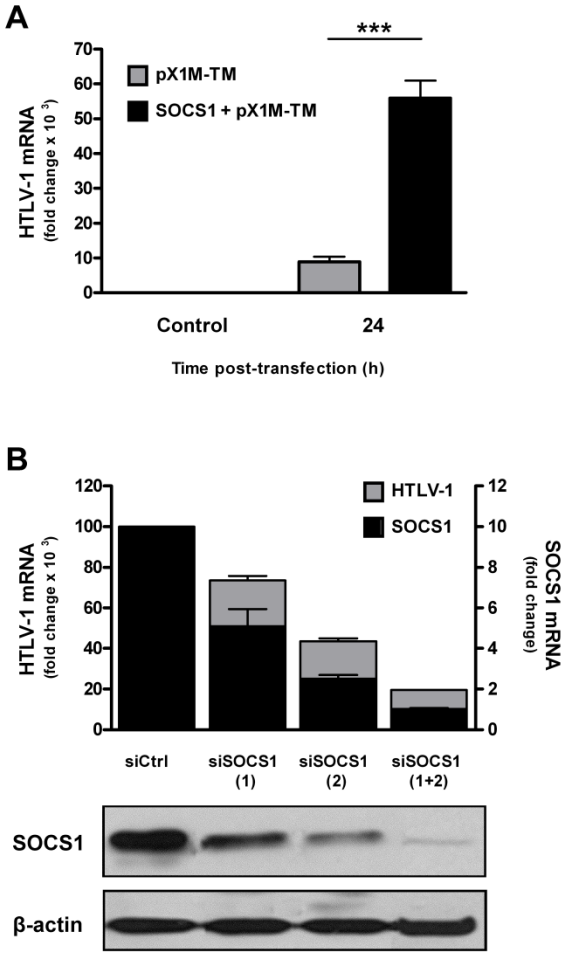

A
***
pX1M-TM
SOCS1 + pX1M-TM
HTLV-1 mRNA
(fold change x 10 3)
70
60
50
40
30
20
10
0
Control
24
Time post-transfection (h)
B
HTLV-1
SOCS1
HTLV-1 mRNA
(fold change x 10 3)
SOCS1 mRNA
(fold change)
120
100
80
60
40
20
0
12
10
8
6
4
2
0
siCtrl
siSOCS1 (1)
siSOCS1 (2)
siSOCS1 (1+2)
SOCS1
β-actin

**Figure 3. SOCS1 promotes HTLV-1 mRNA synthesis.** (A) CEM cells were transfected with pX1M-TM alone or co-transfected with pX1M-TM and Myc-tagged SOCS1 expression vectors. At 24h post-transfection, total RNA was extracted and evaluated for HTLV-1 (A) gene expression by real time PCR. Equivalent mRNA amounts were normalized to GAPDH mRNA expression and calculated as fold change of the levels of control cells which were arbitrarily set as 1 (*** $p< 0.001$). (B) Depletion of SOCS1 decreases HTLV-1 mRNA synthesis in MT-2 cells. MT-2 cells were electroporated with control or SOCS1 specific-siRNAs (siSOCS1 (1), siSOCS1 (2), or a pool of both siSOCS1 (1) and siSOCS (2)). At 72h post-transfection, total RNA was extracted and analyzed for HTLV-1 and SOCS1 mRNA levels. MT2 cells were treated as in B; cells lysates were prepared at 72h post-electroporation, and equal amounts of protein (20 μg) were resolved by SDS-PAGE followed by immunoblotting against SOCS1, with β-actin shown as a loading control (bottom panel).

**HTLV-1 suppresses type I IFN production.**

Since SOCS1 has been shown to negatively regulate type I IFN signaling [34,42], we sought to investigate the relationship between HTLV-1 infection, type I IFN response and SOCS1 gene expression. First, the profile of type I IFN (IFN-β and IFN-$\alpha_2$*)* and IFN-stimulated gene expression (IRF7 and CXCL10) was examined in PBMCs expressing the HTLV-1 provirus pX1M-TM (Figure 4A). IFN-β, IFN-$\alpha_2$ and CXCL10 mRNAs were induced (30, 3.5 and 35-fold, respectively) at 24h post-HTLV-1 transfection, and IRF7 mRNA expression (~11-fold) was maximal at 36h. However, mRNA transcripts for all these genes decreased to near basal levels by 48-72h. At 96h, when HTLV-1 and *SOCS1* genes expression were maximal, no reactivation of antiviral gene transcription was detected (Fig 2E and 4A). We interpret this result as indicating that early after infection, transient stimulation of the antiviral response occurs and restricts *de novo* HTLV-1 RNA expression; at 72-96h after infection induction of SOCS1 results in the shutdown of the type I IFN response, thus promoting high HTLV-1 mRNA expression.

IFN-α signaling is initiated by binding to the heterodimeric IFN-α receptor, followed by activation of JAK1 and TYK2 protein kinases, resulting in the phosphorylation of STAT1 and STAT2 [47]. To investigate whether HTLV-1 expression interfered with the type I IFN response, primary PBMCs expressing the HTLV-1 provirus pX1M-TM were treated with IFN-α for 10-120 min to focus on early IFN-triggered phosphorylation events. In control PBMCs, STAT1 and JAK1 phosphorylation was detected at 10 and 20 min post-IFN-α treatment, as determined by immunoblotting with specific antibodies (Figure 4B). However, in PBMCs expressing the proviral clone pX1M-TM, IFN-α-induced phosphorylation of JAK1 and STAT1 was reduced > 90 and 70%, respectively (Figure 4B), while total protein levels of JAK1 and STAT1 remained unchanged in control and HTLV-1 expressing PBMCs.

To further characterize the effect of HTLV-1 on antiviral response, PBMCs expressing the HTLV-1 provirus were infected with Sendai virus (SeV) - a strong inducer of the antiviral response - and kinetics of expression of IFN genes was assessed by Q-PCR

(Figure 4C, D, E). At 24h post-transfection, PBMCs had significant HTLV-1 proviral load (~700 fold higher than control, Figure 4C); thus at this time, PBMCs were infected with SeV (20 HAU/mL) to compare the levels IFN-$\alpha_2$ and IFN-β mRNA in the presence or absence of HTLV-1 provirus (Figure 4D and E). Induction of IFN-β and IFN-$\alpha_2$ mRNA was detected in all PBMCs as early as 3h post-SeV infection and was sustained up to 12h (Figure 4D and E). However, in HTLV-1 expressing PBMCs, induction of IFN-β and IFN-$\alpha_2$ mRNA was reduced > 60%, relative to the level observed in the absence of HTLV-1 provirus. Decreased levels of IFN-β and IFN-$\alpha_2$ in cells expressing the HTLV-1 provirus were not due to inhibition of SeV replication, as demonstrated by immunoblot for SeV proteins (Figure 4F).

Similarly, knockdown of SOCS1 in MT-2 cells reversed the inhibition of antiviral gene expression imposed by HTLV-1 (Figure 5). Pooled siSOCS1 resulted in increased *IFN-β* (7.5-fold), *ISG56* (2.5-fold), *IFN-γ* (4-fold) and *CXCL10* (~10-fold) gene expression compared to control siRNA. These results demonstrate that SOCS1 contributes to the inhibition of antiviral responses during HTLV-1 infection.

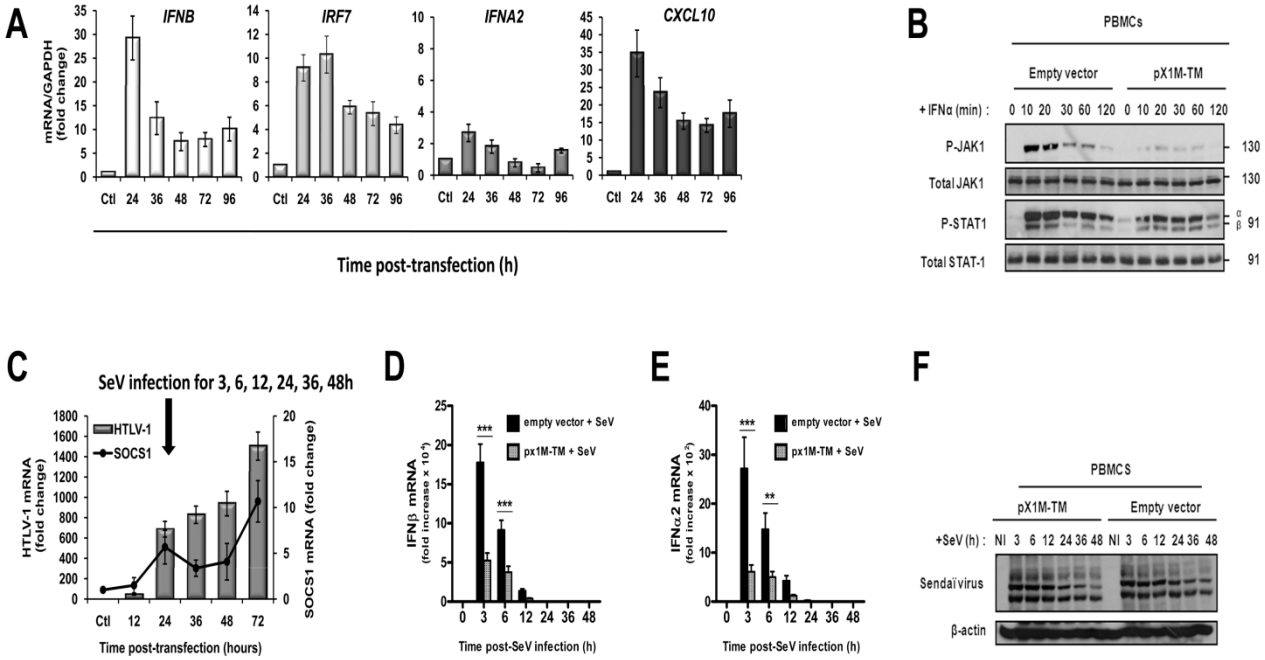

A
IFNB
IRF7
IFNA2
CXCL10
mRNA/GAPDH (fold change)
Ctl 24 36 48 72 96
Time post-transfection (h)
B
PBMCs
Empty vector
pX1M-TM
+ IFNα (min) : 0 10 20 30 60 120 0 10 20 30 60 120
P-JAK1
Total JAK1
P-STAT1
Total STAT-1
130
130
91
91
C
SeV infection for 3, 6, 12, 24, 36, 48h
HTLV-1
SOCS1
HTLV-1 mRNA (fold change)
SOCS1 mRNA (fold change)
Ctl 12 24 36 48 72
Time post-transfection (hours)
D
empty vector + SeV
px1M-TM + SeV
IFNβ mRNA (fold increase x 10⁻⁴)
Time post-SeV infection (h)
E
empty vector + SeV
px1M-TM + SeV
IFNα2 mRNA (fold increase x 10⁻²)
Time post-SeV infection (h)
F
PBMCS
pX1M-TM
Empty vector
+SeV (h) : NI 3 6 12 24 36 48 NI 3 6 12 24 36 48
Sendaï virus
β-actin

**Figure 4. HTLV-1 infection results in the inhibition of type I IFN gene expression.** (A) PBMCs from healthy individuals were electroporated with either HTLV-1 provirus (pX1M-TM) or control empty vector (ctl). At the indicated times, total RNA was extracted and analyzed for, *IFN-β*, *IRF7*, *IFN-α*$_2$, *CXCL10* (A) gene expression. Equivalent mRNA amounts were normalized to *GAPDH* mRNA expression and calculated as fold change from the levels of control cells that were arbitrarily set as 1. (B) HTLV-1 inhibits IFN-α-induced tyrosine phosphorylation of JAK1 and STAT1. PBMCs were electroporated as in (A) and 48h post-transfection cells were left-untreated or treated with 1000U/ml IFN-α. Cells lysates were prepared at indicated times and equal amounts of protein (50 μg) were resolved by SDS-PAGE followed by immunoblotting against Tyr-701-phosphorylated STAT1, total STAT1, Tyr-1022/1023-phosphorylated JAK1, total JAK1, with β-actin shown as a loading control. (C, D, E, F) HTLV-1 inhibits SeV-mediated type I IFN gene expression. PBMCs were electroporated with either HTLV-1 provirus (pX1M-TM) or control empty vector for 24h prior to SeV infection. Total RNA was extracted at the indicated times and analyzed for HTLV-1 (C), IFN-β (D) and IFNα$_2$ (E) mRNA levels by real time PCR. Equivalent mRNA amounts were normalized to GAPDH mRNA expression and calculated as fold change from the expression levels of control cells that were arbitrarily set as 1 (**, $p< 0.05$; *** $p< 0.001$). (F) PBMCs were treated as in C, D, E; cells lysates were prepared at 3-48h post-SeV infection, and equal amounts of protein (20 μg) were resolved by SDS-PAGE followed by immunoblotting with anti-SeV antisera. Immunoblotting against β-actin is shown as a loading control.

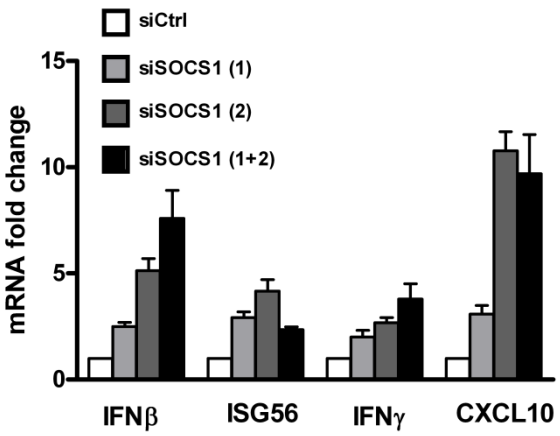

siCtrl
siSOCS1 (1)
siSOCS1 (2)
siSOCS1 (1+2)
mRNA fold change
15
10
5
0
IFNβ
ISG56
IFNγ
CXCL10

**Figure 5. SOCS1 suppresses the antiviral response.** Depletion of SOCS1 restores the type I and II IFN signaling in MT-2 cells. (A) MT-2 cells were electroporated with control or SOCS1 specific-siRNAs (siSOCS1 (1), siSOCS1 (2), or a pool of siSOCS1 (1) and siSOCS1 (2)). At 72h post-transfection, total RNA was extracted and analyzed for IFN-β, ISG56, CXCL10 and IFN-γ mRNA levels.

**HTLV-1-mediated SOCS1 expression induces proteasomal degradation of IRF3.**

Many pathogenic viruses strategically antagonize the early innate antiviral defenses in order to maintain viral replication, often inactivating IFN signaling components as part of their immune evasion strategy (reviewed in [45]. Because IRF3 is essential for IFN gene activation, we assessed IRF3 dimerization (as a measure of activation) in PBMCs expressing the HTLV-1 provirus (Figure 6A). In control PBMCs, SeV infection induced IRF3 dimer formation at 3-12h post-infection, whereas IRF3 dimer formation was not detected in PBMCs expressing the HTLV-1 provirus. Furthermore, IRF3 monomer levels decreased sharply during the course of HTLV-1 replication (Figure 6A). Immunoblot analysis for total IRF3 confirmed that IRF3 levels decreased in a time dependent manner in PBMCs and Jurkat cells expressing the HTLV-1 provirus, a phenomenon not observed in control PBMCs infected with SeV (Figure 6C). IRF-3 was degraded *via* the proteasomal pathway, as the use of the proteasome inhibitor MG132 prevented HTLV-1 mediated reduction of IRF3 protein level (Figure 6B). This observation demonstrates for the first time that HTLV-1 does not activate IRF3 in PBMCs, but rather prevents the initial steps of type I IFN production by targeting IRF3 for proteasomal degradation.

Given that SOCS1 upregulation during HTLV-1 infection inhibits the expression of IFN and ISGs, we sought to investigate the role of SOCS1 in HTLV-1-mediated degradation of IRF3. In HEK293T cells expressing increasing amounts of SOCS1 together with a constant amount of IRF3, SOCS1 expression induced IRF3 degradation in a dose-dependent manner (Figure 7A). RT-PCR analysis with specific IRF3 primers showed that the level of IRF3 mRNA remained unchanged, indicating that SOCS1 had no effect on *IRF3* gene expression (Figure 7A). Interestingly, the addition of the proteasome inhibitors lactacystin (Figure 7B) or MG132 (data not shown) prevented IRF3 degradation in the presence of SOCS1. Furthemore, co-immunoprecipitation experiments demonstrated that SOCS1 physically interacted with IRF3 (Figure 7C), indicating that IRF3 degradation was triggered by physical association with SOCS1.

SOCS1 induces degradation of target proteins by recruiting Elongin B/C to its SOCS-Box domain, leading to the formation of an E3 ubiquitin ligase complex able to modify substrate proteins with K48-linked ubiquitin chains. To confirm that SOCS1-mediated IRF3 degradation required E3 ligase complex activity, increasing amounts of a SOCS1 deletion mutant lacking the SOCS-Box - SOCS1-ΔB/C-Box - was expressed together with a constant amount of IRF3 (Figure 7D); SOCS1-ΔBC did not induce IRF3degradation at any concentration (compare Figures 7D and 7A).

Proteasome-mediated degradation requires the addition of K48-polyubiquitin chain to the target protein; exogenous addition of ubiquitin mutated in its ability to link K48-polyubiquitin chains (ubiquitin-K48R, which contains a single K48R point mutation, or Ubi-KO, which contains no lysines) prevented IRF-3 degradation, while HEK293 cells expressing exogenous SOCS1 readily degraded IRF3 (Figure 7E). In addition, IRF3 turnover was completely reversed in the presence of a 10-fold excess of HA-Ub K48R or HA-Ub KO (Figure 7E), thus confirming that proteosome-mediated IRF3 degradation by SOCS1 requires recruitment of Elongin B/C E3 ligase machinery and is dependent on K48-polyubiquitin chain formation.

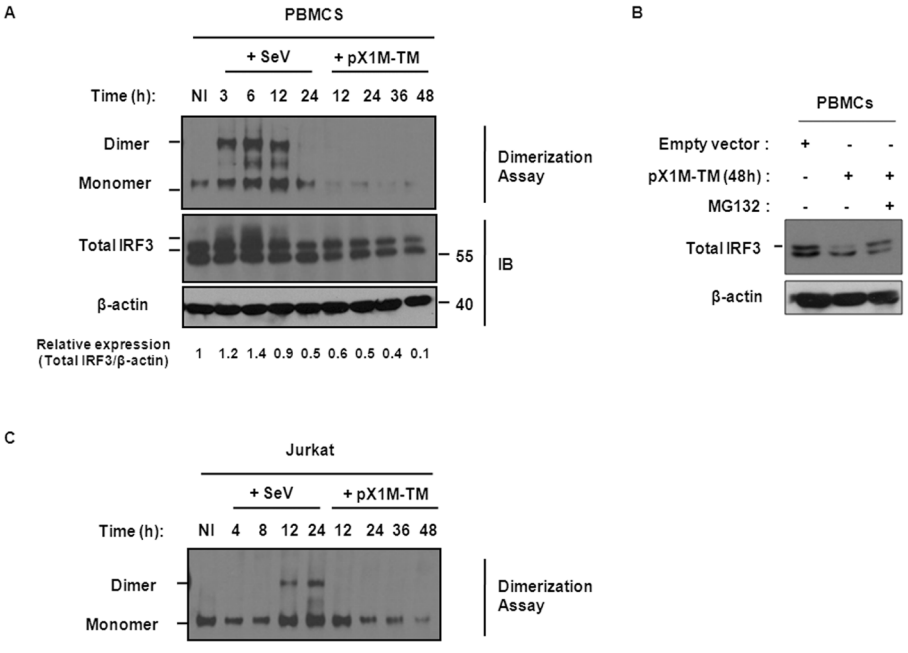

A
PBMCS
+ SeV
+ pX1M-TM
Time (h):
NI 3 6 12 24 12 24 36 48
Dimer
Monomer
Dimerization Assay
Total IRF3
β-actin
55
40
IB
Relative expression (Total IRF3/β-actin)
1 1.2 1.4 0.9 0.5 0.6 0.5 0.4 0.1
B
PBMCs
Empty vector : + - -
pX1M-TM (48h) : - + +
MG132 : - - +
Total IRF3
β-actin
C
Jurkat
+ SeV
+ pX1M-TM
Time (h):
NI 4 8 12 24 12 24 36 48
Dimer
Monomer
Dimerization Assay

**Figure 6. HTLV-1 induces degradation of endogenous IRF3.** (A). PBMCs from healthy individuals were electroporated with either HTLV-1 provirus (pX1M-TM) or empty vector. PBMCs transfected with the control vector were subsequently infected with SeV. At the indicated times, lysates from transfected or infected PBMCs were electrophoretically resolved under non-denaturing or denaturing conditions. Western blot analysis was used to locate monomer and dimer forms of IRF3 in the non-denaturing gel (upper panel), and global IRF3 protein in the denaturing gel (lower panel). Immunoblotting against β-actin was used as a loading control. Total IRF3 protein expression levels (upper band) were quantified and normalized to β-actin levels using the Scion Image 4.0 software program. (B) Degradation of IRF3 is inhibited by the proteasome inhibitor (MG132). PBMCs were treated as indicated in (A). At 48h post-HTLV-1 provirus, cells were incubated with 5 μM of MG132 for 6h. Cells lysates were prepared and equal amounts of protein (20 μg) were resolved by SDS-PAGE followed by immunoblotting against IRF3, with β-actin shown as a loading control. (C) Same experiments and analysis for IRF3 dimerization were performed in the Jurkat leukemic T cell line.

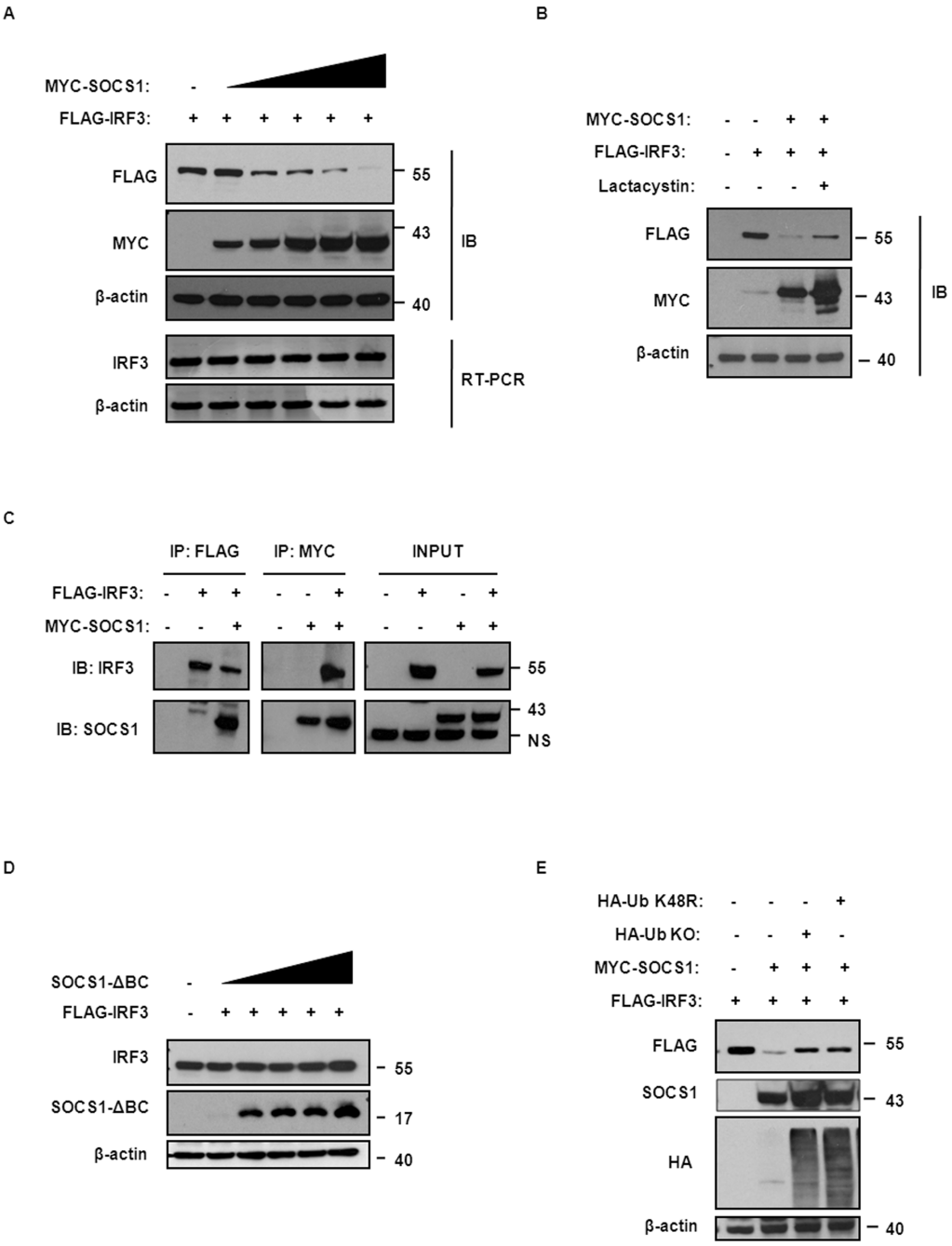

A
MYC-SOCS1: -
FLAG-IRF3: + + + + + +
FLAG
MYC
β-actin
IRF3
β-actin
55
43
40
IB
RT-PCR
B
MYC-SOCS1: - - + +
FLAG-IRF3: - + + +
Lactacystin: - - - +
FLAG
MYC
β-actin
55
43
40
IB
C
IP: FLAG
IP: MYC
INPUT
FLAG-IRF3: - + + - - + - + - +
MYC-SOCS1: - - + - + + - - + +
IB: IRF3
IB: SOCS1
55
43
NS
D
SOCS1-ΔBC -
FLAG-IRF3 - + + + + +
IRF3
SOCS1-ΔBC
β-actin
55
17
40
E
HA-Ub K48R: - - - +
HA-Ub KO: - - + -
MYC-SOCS1: - + + +
FLAG-IRF3: + + + +
FLAG
SOCS1
HA
β-actin
55
43
40

**Figure 7. IRF3 interacts with SOCS1 and undergoes SOCS1-mediated ubiquitination and degradation.** (A) HEK293 T cells co-transfected with increasing amounts of SOCS1 promotes degradation of IRF3 in a concentration-dependent manner. HEK293 T cells were co-transfected with Flag-tagged IRF3 and increasing amount of Myc-tagged SOCS1 expression vectors. Cells were collected 24h post-transfection for either RNA extraction or whole cell extract (WCE) preparation. Immunoblot analysis for SOCS1 and IRF3 protein expression was performed by incubating with antibodies against Myc, Flag, or β-actin, respectively (upper panel). RNA (1 μg) from each sample was subjected to RT-PCR for selective amplification of specific IRF3 mRNA and the constitutively expressed β-actin, as a control. PCR products were separated on a 2% agarose gel and visualized with ethidium bromide staining (lower panel). (B) Degradation of IRF3 is inhibited by the proteasome inhibitor (lactacystin). HEK293 T cells were co-transfected with expression vectors as indicated in (A) and incubated with 10 μM of lactacystin. Twenty four hours post-transfection, equal amounts of proteins (30μg) were resolved by SDS-PAGE followed by immunoblotting with antiserum specific for Flag and Myc. Immunoblotting against β-actin was performed as loading control. (C) Interaction between IRF3 and SOCS1. HEK293 T cells were co-transfected with expresssion vectors for Myc-tagged SOCS1 and Flag-tagged IRF3; at 24h post-transfection, cells lysates were immunoprecipitated with Flag or Myc antibodies, and the precipitates were immunoblotted to assess Flag and Myc protein expression. (D) IRF3 is degraded in a SOCS-Box-dependent manner. HEK293 T cells were co-transfected with Flag-tagged IRF3 and increasing amount of a deletion mutant of SOCS1 (SOCS1-ΔBC-Box) expression vectors. Cells were collected 24h post-transfection for WCE preparation. Immunoblot analysis for SOCS1 and IRF3 protein expression were performed and incubated with antibodies against SOCS1, Flag, or β-actin, respectively. (E) SOCS1 mediated IRF3 ubiquitination. HEK293 T cells were co-transfected with Flag-tagged IRF3, Myc-tagged SOCS1 and either KO or K48R ubiquitin mutant expression vectors. Cells were collected 24h post-transfection for WCE preparation. Immunoblot analysis for

IRF3, SOCS1, Ub protein expression were performed and incubated with antibodies against Myc, Flag, Ha or β-actin, respectively.

## DISCUSSION

The complexity of gene expression dysregulation in ATL or HAM/TSP diseases has been highlighted in a number of gene expression profiling [48,49] and protein profiling studies [50]. The present study however represents the first comparative genome-wide array analysis to establish gene expression profiles for HTLV-1-associated disease states. With a unique cohort of 30 HTLV-1-infected individuals from the Caribbean basin and a custom ImmuneArray [51], we identified ~1039 significant immune-related genes that were differentially regulated in CD4+ T cells from 11 AC, 7 ATL, 12 HAM/TSP patients, compared with CD4+ T cells from 8 NI donors from the same geographical region. Clear clinical discrimination was observed between the ATL, HAM/TSP and NI patients, both by unsupervised hierarchical cluster and principal component analysis. Our analysis revealed that the gene expression profile in ATL cells was clearly distinct from healthy CD4+ T cells, although similarities in gene expression patterns were observed between HAM/TSP samples and NI controls. This difference between HAM/TSP and ATL CD4+ T cells likely reflects numerous alterations in gene expression that occur during ATL transformation [12]. In contrast, evolution to HAM/TSP does not involve cellular transformation, but rather is characterized by a high HTLV-1 proviral load and the establishment of a pro-inflammatory microenvironment due to cytokine/chemokine production of infected and bystander immune cells. It is possible that T-lymphocytes derived from early-stage HAM/TSP patients have a profile similar to healthy cells and that gene expression changes are observed only at later stages of the disease, an interesting hypothesis that needs to be investigated further. Interestingly, AC patients did not cluster as an individual group but rather distributed amongst NI, ATL and HAM/TSP patients, suggesting that extensive analysis of the genes modulated in NI, HAM/TSP and/or ATL groups may help to identify candidate genes important for early diagnosis of HTLV-1 diseases. Here, the major cellular pathways identified involved cell adhesion (CXCR4, CD2, CD63), antimicrobial defense (KLRB1, SPN, SELPLG), innate immune signaling (SOCS1, TRAF3, AIM2, TLR2, IKBKG, STAT3), antigen presentation (TRA

alpha locus), and chemotaxis (CCL14, SPN, CCL13) thus supporting the idea of a global disruption of the immune system during HTLV-1 infection.

Among the many genes modulated during HTLV-1 infection, the suppressor of the interferon signaling - SOCS1 - was upregulated in HAM and AC patients but not in ATL. This observation is in agreement with a previous report published by Nishiura *et al.* demonstrating that SOCS1 mRNA levels were increased in HAM/TSP patients compared to NI [44]. We now demonstrate that CD4 + T cells from HAM/TSP and AC patients express increased levels of SOCS1 which strongly correlates with HTLV-1 mRNA load. Since HAM/TSP patients are characterized by a very high proviral load, we hypothesized that SOCS1 upregulation in HAM/TSP may represent an immune evasion strategy used by HTLV-1 to dampen the early IFN antiviral response. Indeed, in PBMCs expressing a HTLV-1 infectious molecular clone, and in cell lines harboring an intact HTLV-1 provirus, high levels of *SOCS1* gene expression correlated with high levels of HTLV-1 transcription. Increasing HTLV-1 proviral expression blocked expression of type I IFN genes such as IFNB, IRF7, IFN-$\alpha_2$, as well as the IFN-γ stimulated chemokine gene CXCL10, with maximal inhibition observed when HTLV-1 and *SOCS1* gene expression levels were coordinately elevated. Furthermore, depletion of SOCS1 using siRNA decreased HTLV-1 replication and restored the type I IFN response.

IFN-α/β is known to have a negative impact on retrovirus replication. Although few studies have reported its effect on HTLV-1, type I IFN constitutes a potent anti-retroviral mechanism that limits HTLV-1 replication [22,23]. Moreover, clinical studies using IFN-β therapy in HAM/TSP patients have demonstrated benefits in reducing HTLV-1 mRNA load and the number of pathogenic CD8+ T cells, as well as minimizing disease progression during therapy [52]. Accumulating evidence indicates that HTLV-1 possesses evasion mechanisms to counteract type I IFN signaling: for example, HTLV-1 down-regulates JAK-STAT activation by reducing phosphorylation of Tyk2 and STAT2, possibly through a Gag- or Pr-mediated mechanism [53]; and Tax further negatively

modulates IFN-α-induced JAK/STAT signaling by competing with STAT2 for CBP/p300 coactivators [54].

SOCS1 is a cytokine-inducible intracellular negative regulator that inhibits type I and II IFN signaling by triggering the degradation of various components of the JAK-STAT cascade (reviewed in [32,55]. SOCS1 can also be induced during virus infection and plays a positive role in viral replication [56,57,58]. SOCS proteins exert their negative effect by promoting the ubiquitination and proteosomal degradation of key proteins involved in cytokine signaling pathways: MAL in Toll like receptor 4 signaling (TLR4), JAK2 in IFN-γ mediated signaling and NF-κB p65/RelA are all known targets of SOCS1 [38,59,60]. Here, we identified IRF3 as an important target for SOCS1-induced proteasomal degradation that impacts the early type I IFN antiviral response. IRF3 is ubiquitously expressed in the cytoplasm and is activated in response to viral infection, triggering IFN-β and other early ISGs expression, thus initiating the antiviral response. To counter type I IFN, many viruses have evolved strategies to interfere with IRF3 activation as an efficient means to limit IFN-β production [26,61]. Interference of IRF3 activation also dampens the second wave of IFN signaling, including production of IFN-α. The mechanisms of IRF3 antagonism vary, and include inhibition of IRF3 phosphorylation, nuclear translocation, or transcription complex assembly as well as down-regulation of IRF3 by ubiquitin-mediated degradation. In this context, bovine herpes virus 1 infected cell protein 0 (bICP0) has been shown to act as an E3 ligase and promote IRF3 degradation in a proteasome-dependent manner, thus inhibiting the IFN response [62].

The interaction between SOCS1 and IRF3 during HTLV-1 infection promotes proteasome-mediated degradation of IRF3 and thus abrogates early IFN antiviral signaling. SOCS1-dependent IRF3 degradation required the elongin B and C binding sites within SOCS1 and K48-linked polyubiquitination of IRF3. Indeed, the SOCS box-mediated function of SOCS1 is chiefly exerted via its ubiquitin ligase activity [63] and biochemical binding studies have shown that the SOCS box interacts with the elongin

B/C complex, a component of the ubiquitin/proteasome pathway that forms an E3 ligase with Cul2 (or Cul5) and Rbx-1 [40,59]. Thus, SOCS1 serves as an adaptor to bring target proteins to the elongin B/C-Cullin E3 ligase complex for ubiquitination. Although we show from our current experiments that SOCS1 directly mediates K48-linked ubiquitination of IRF3, further studies are required to elucidate the details of SOCS1-mediated IRF3 ubiquitination, as well as the mechanisms of regulation of SOCS1 during HTLV-1 infection.

The present study reveals a novel mechanism of viral evasion of the IFN response in HTLV-1 infected T lymphocytes – the consequence of which can be directly related to the efficiency of HTLV-1 replication in patients suffering from HAM/TSP. Future studies are required to elucidate putative alternate consequences of SOCS1 upregulation in T cells [64], as well as the effect of HTLV-1 induced SOCS1 expression in other relevant viral reservoirs such as dendritic cells and astrocytes [65,66]. Collectively, SOCS1-mediated degradation of IRF3 during HTLV-1 infection has substantial implications in the framework of known HTLV-1 pathobiology and as such opens new avenues of exploration for designing effective therapeutic strategies.

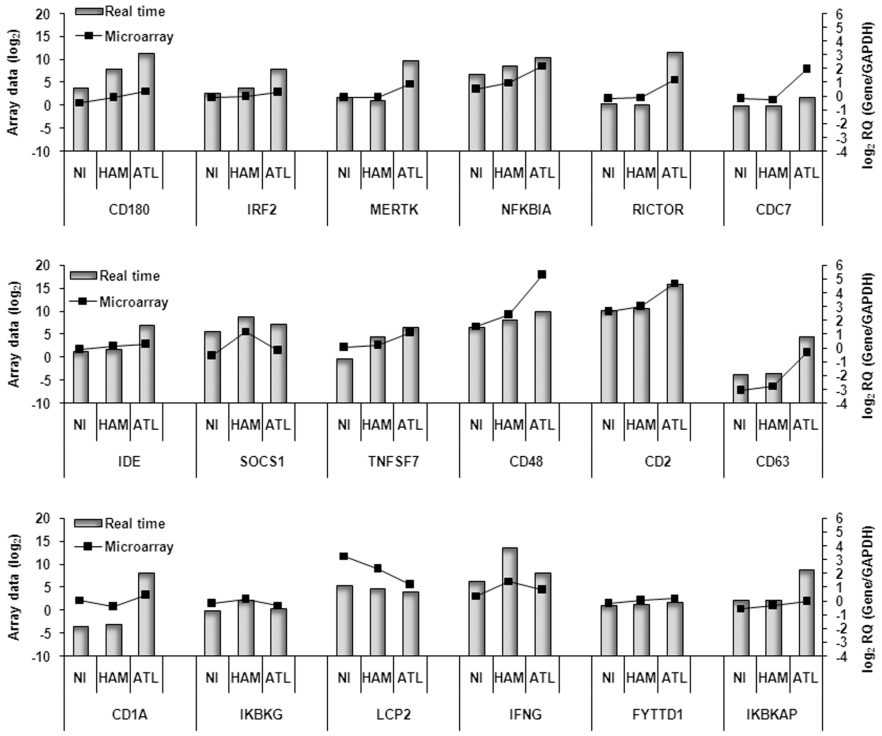
**ACKNOWLEDGMENTS**

The authors wish to thank Dr. Steven Jacobson, NIAID-NIH, for providing RNA samples from patients with HAM/TSP and Dr. Nathalie Grandvaux, Department of Biochemistry, University of Montreal, for helpful discussions. We also thank Dr David Derse, National Cancer Institute-Frederick, Frederick, USA, for providing HTLV-1 provirus (pX1M-TM) and Dr Gerardo Ferbeyre, Department of Biochemistry, University of Montreal, for providing MYC-SOCS1 expression vector.

**AUTHOR CONTRIBUTION**

Conceived and designed the experiments: SO and JH. Performed the experiments: SO, ED, AZ, RND. Analyzed the data: SO, ED, PW, RND, MA, JH. Contributed reagents/materials/analysis tools: PW, SO, MK, GP. Wrote the paper: SO, MA, TLAN, RND, JH.

A




B

| | | Patients | | |
|---|---|---|---|---|
| | Gene | NI | HAM | ATL |
| Array data ($log_2$) | *AIM2* | -0.7 | 0.0 | 0.1 |
| Real time data ($log_2$RQ) | | 5.9 | 6.8 | 8.6 |
| Array data ($log_2$) | *CXCL9* | 0.3 | 0.0 | 1.8 |
| Real time data ($log_2$RQ) | | 11.7 | 12.0 | 13.2 |
| Array data ($log_2$) | *TNFSF11* | 0.0 | -0.1 | 2.3 |
| Real time data ($log_2$RQ) | | 4.6 | 6.7 | 10.7 |
| Array data ($log_2$) | *IL23A* | -0.4 | 0.2 | 0.8 |
| Real time data ($log_2$RQ) | | 9.6 | 9.5 | 14.2 |
| Array data ($log_2$) | *ENG* | -3.6 | -2.8 | -4.0 |
| Real time data ($log_2$RQ) | | 6.2 | 6.2 | 0.0 |
| Array data ($log_2$) | *SELPLG* | -0.1 | 0.4 | 3.0 |
| Real time data ($log_2$RQ) | | 6.2 | 7.2 | 7.2 |
| Array data ($log_2$) | *IL7* | 0.5 | 0.0 | 0.9 |
| Real time data ($log_2$RQ) | | 0.9 | 3.5 | 8.7 |

C

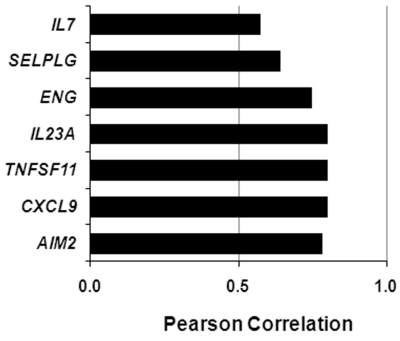


**Figure S1. Confirmation of microarray results by Q-PCR.** (A) Total RNA of 9 donors (3 NI, 3 HAM, 3 ATL) was reverse transcribed and cDNA was amplified using primers specific for indicated genes by real-time PCR. Values were normalized to GAPDH and relative quantification (RQ) was calculated by the comparative CT method. (B) Pearson correlation data represent the strength and direction of the linear relationship between real-time PCR assays and microarray data presented in (A). Genes with a Pearson correlation value between 0.6 and 0.8 are represented in (A).

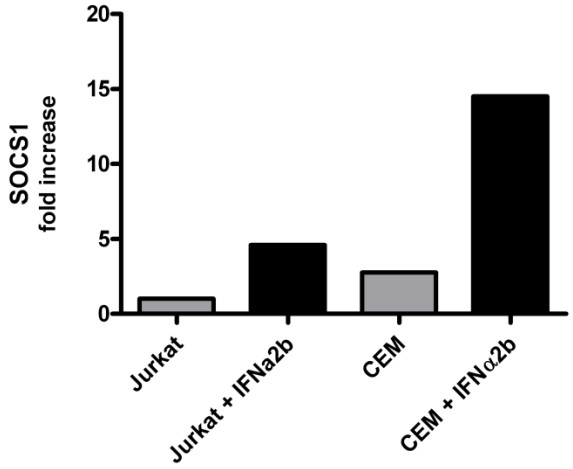

SOCS1
fold increase
20
15
10
5
0
Jurkat
Jurkat + IFNa2b
CEM
CEM + IFNα2b

**Figure S2. CEM and Jurkat CD4+ T cell lines express SOCS1 when treated with IFNα2b.** Expression of SOCS1 was measured in HTLV-1-negative T cell lines (CEM, Jurkat) with and without 1000U/ml of IFNα2b. cDNA was analyzed by quantitative real time PCR to measured SOCS1 expression. Equivalent mRNA amounts were normalized to GAPDH expression and calculated as fold change with the levels of uninfected Jurkat cells set arbitrarily as 1.

**Supplementary Table 1. Cohort #1 of HTLV-1 infected and non-infected donors**. Patients recruited in Martinique and French Guyana for microarray study (Figure 1).

| Hyb ID | Ethnicity | Gender | Diagnosis | Location | |
|---|---|---|---|---|---|
| HISS0042 | Black | M | AC | Martinique | |
| HISS0044 | Black | F | AC | Martinique | |
| HISS0045 | Black | F | AC | Martinique | |
| HISS0046 | Black | F | AC | Martinique | |
| HISS0047 | Black | M | AC | Martinique | |
| HISS0013 | Black | F | AC | Guyana | |
| HISS0014 | Black | F | AC | Guyana | |
| HISS0015 | Black | M | AC | Guyana | |
| HISS0056 | Black | ND | AC | Guyana | |
| HISS0057 | Black | ND | AC | Guyana | |
| HISS0058 | Black | ND | AC | Guyana | |
| | | | | | **ATL cells (%)** |
| HISS0017 | Black | ND | ATL | Martinique | 40% |
| HISS0018 | Black | F | ATL | Martinique | >5% |
| HISS0029 | Black | ND | ATL | Martinique | >5% |
| HISS0024 | Black | F | ATL | Guyana | 39% |
| HISS0010 | Black | F | ATL | Guyana | >5% |
| HISS0022 | Black | M | ATL | Guyana | 88% |
| HISS0023 | Black | M | ATL | Martinique | 3% |
| | | | | | **EDSS** |
| HISS0021 | Black | F | HAM/TSP | Martinique | 5 |
| HISS0031 | Black | F | HAM/TSP | Martinique | 7 |
| HISS0040 | Black | F | HAM/TSP | Martinique | 6 |
| HISS0041 | Black | M | HAM/TSP | Martinique | 7.5 |
| HISS0043 | Black | M | HAM/TSP | Martinique | 6 |
| HISS0048 | Black | M | HAM/TSP | Martinique | 7.5 |
| HISS0049 | Black | M | HAM/TSP | Martinique | 6 |
| HISS0051 | Black | ND | HAM/TSP | Guyana | ND |
| HISS0052 | Black | ND | HAM/TSP | Guyana | ND |
| HISS0053 | Black | ND | HAM/TSP | Guyana | ND |
| HISS0054 | Black | ND | HAM/TSP | Guyana | ND |
| HISS0055 | Black | ND | HAM/TSP | Guyana | ND |
| | | | | | |
| HISS0060 | Black | ND | Healthy | Martinique | |
| HISS0061 | Black | ND | Healthy | Martinique | |
| HISS0062 | Black | ND | Healthy | Martinique | |
| HISS0063 | Black | ND | Healthy | Martinique | |
| HISS0064 | Black | ND | Healthy | Martinique | |
| HISS0065 | Black | ND | Healthy | Martinique | |
| HISS0066 | Black | ND | Healthy | Martinique | |
| HISS0067 | Black | ND | Healthy | Martinique | |

Hyb ID: microarray hybridization identifier; EDSS: Kurtzke Expanded Disability Status Scale; ND: not determined.

**Supplementary Table 2. Cohort #2 of HAM/TSP patients and non-infected donors.** Patient samples used to validate increased SOCS1 expression and correlation with HTLV-1 proviral load for Figure 2C.

| Patient ID | Ethnicity | Gender | Age | EDSS | Disease Duration in years |
|---|---|---|---|---|---|
| P1 | Black/African American | M | 72 | 6.0 | 13 |
| P2 | Black/African American | F | 51 | 6.0 | 6 |
| P3 | Black/African American | F | 54 | 8.0 | 3 |
| P4 | Caucasian | F | 69 | 2.5 | 11 |
| P5 | Black/African American | F | 54 | 6.5 | 2 |
| P6 | Hispanic | F | 43 | 6.0 | 5 |
| P7 | Black/African American | F | 47 | 6.0 | 8 |
| P8 | Black/African American | F | 61 | 6.5 | 14 |
| P9 | Black/African American | F | 68 | 6.5 | 12 |
| P10 | Black/African American | M | 64 | 1.0 | 22 |
| C694 | | | | NA | NA |
| C750 | | | | NA | NA |
| C185 | | | | NA | NA |
| C211 | | | | NA | NA |
| C125 | | | | NA | NA |

EDSS: Kurtzke Expanded Disability Status Scale; NA: not applicable.

**Supplementary Table 3.**

| List of human primers used (SYBR GREEN) | |
|---|---|
| *NFKBIA* | F 5'-GATCACCAACCAGCCAGAAATT-3'<br>R 5'-TCTCGGAGCTCAGGATCACA-3 |
| *RICTOR* | F 5'-CACTTACTACTTACCGGAAGCCTGTT-3'<br>R 5'-GGTAGACGTGAGGACGCTGTAAT-3' |
| *IRF2* | F 5'-TCCTATGCAGAAAGCGAAACG-3'<br>R 5'-CCCCTCGGCACTCTCTTCA-3' |
| *CD180* | F 5'-GAGGAGACCACGTGTGCAAA-3'<br>R 5'-CAGGAAAGCTTGACATCAGATAGC-3' |
| *CD2* | F 5'-GAAATGATGAGGAGCTGGAGACA-3'<br>R 5'-CCGGCCCCTTTCTTCAGT-3' |
| *CD63* | F 5'-GAGAATTACCCGAAAAACAACCA-3'<br>R 5'-CACTTAAAATCTGCCTGCATCCT-3' |
| *MERTK* | F 5'-CGAGCTCGGATCTCTGTTCAA-3'<br>R 5'-GGTGACGGCTGCAATCCT-3' |
| *CD1A* | F 5'-TGACACCTGCCCACGTTTC-3'<br>R 5'-CCGCTGGAGATGTGCCTTT-3' |
| *SELPLG* | F 5'-GCCTGCTGGCCATCCTAA-3'<br>R 3'-ACCGCCAGCACCACAGT-3' |
| *CXCL10* | F 5'-TTCCTGCAAGCCAATTTTGTC-3'<br>R 5'-TCTTCTCACCCTTCTTTTTCATTGT-5' |
| *IFNB* | F 5'-TTGTGCTTCTCCACTACAGC-3'<br>R 3'-CTGTAAGTCTGTTAATGAAG-3' |
| *IFNA2* | F 5'-CCTGATGAAGGAGGACTCCATT-3'<br>R 5'-AAAAAGGTGAGCTGGCATACG-3' |
| *IRF7* | F 5'-GAGCCCTTACCTCCCCTGTTAT-3'<br>R 5'-CCACTGCAGCCCCTCATAG-3' |

| | |
|---|---|
| *IL23A* | F 5'-TTCTGCTTGCAAAGGATCCA-3'<br>R 5'-TCCGATCCTAGCAGCTTCTCA-3' |
| *CXCL9* | F 5'-GTGCAAGGAACCCCAGTAGTGA-3'<br>R 5'-TAGTCCCTTGGTTGGTGCTGAT-3' |
| *GAPDH* | F 5'-ACAGTCCATGCCATCACTGCC-3'<br>R 5'-GCCTGCTTCACCACCTTCTTG-3' |
| *HTLVpx* | F 5'-CAAAGTTAACCATGCTTATTATCAGC-3'<br>R 5'-ACACGTAGACTGGGTATCCGAA-3' |

**List of reference numbers corresponding to the human primers used (Taq Man)**

| | |
|---|---|
| *CD48* | Hs00381156 |
| *LCP2* | Hs00175501 |
| *CDC7* | Hs00177487 |
| *IFNG* | Hs00174143 |
| *SOCS1* | Hs00705164 |
| *IKBKAP* | Hs00175353 |
| *FYTTD1* | Hs00260265 |
| *IKBKG* | Hs00415849 |
| *IDE* | Hs00610438 |
| *AIM2* | Hs00175457 |
| *TNFSF7* | Hs00174297 |
| *TNFSF11* | Hs00243522 |
| *ENG* | Hs00164438 |
| *IL7* | Hs00174202 |
| *GAPDH* | Hs99999905 |

## REFERENCES


1. Grant C, Barmak K, Alefantis T, Yao J, Jacobson S, et al. (2002) Human T cell leukemia virus type I and neurologic disease: events in bone marrow, peripheral blood, and central nervous system during normal immune surveillance and neuroinflammation. J Cell Physiol 190: 133-159.
2. Bangham CR, Meekings K, Toulza F, Nejmeddine M, Majorovits E, et al. (2009) The immune control of HTLV-1 infection: selection forces and dynamics. Front Biosci 14: 2889-2903.
3. Ohshima K (2007) Pathological features of diseases associated with human T-cell leukemia virus type I. Cancer Sci 98: 772-778.
4. Proietti FA, Carneiro-Proietti AB, Catalan-Soares BC, Murphy EL (2005) Global epidemiology of HTLV-I infection and associated diseases. Oncogene 24: 6058-6068.
5. Sabouri AH, Usuku K, Hayashi D, Izumo S, Ohara Y, et al. (2008) Impaired function of human T-lymphotropic virus type 1 (HTLV-1)-specific CD8+ T cells in HTLV-1-associated neurologic disease. Blood 112: 2411-2420.
6. Azran I, Schavinsky-Khrapunsky Y, Priel E, Huleihel M, Aboud M (2004) Implications of the evolution pattern of human T-cell leukemia retroviruses on their pathogenic virulence (Review). Int J Mol Med 14: 909-915.
7. Tsukasaki K, Hermine O, Bazarbachi A, Ratner L, Ramos JC, et al. (2009) Definition, prognostic factors, treatment, and response criteria of adult T-cell leukemia-lymphoma: a proposal from an international consensus meeting. J Clin Oncol 27: 453-459.
8. Grassmann R, Aboud M, Jeang KT (2005) Molecular mechanisms of cellular transformation by HTLV-1 Tax. Oncogene 24: 5976-5985.
9. Matsuoka M, Jeang KT (2007) Human T-cell leukaemia virus type 1 (HTLV-1) infectivity and cellular transformation. Nat Rev Cancer 7: 270-280.
10. Jeang KT, Giam CZ, Majone F, Aboud M (2004) Life, death, and tax: role of HTLV-I oncoprotein in genetic instability and cellular transformation. J Biol Chem 279: 31991-31994.
11. Boxus M, Twizere JC, Legros S, Dewulf JF, Kettmann R, et al. (2008) The HTLV-1 Tax interactome. Retrovirology 5: 76.
12. Peloponese JM, Jr., Kinjo T, Jeang KT (2007) Human T-cell leukemia virus type 1 Tax and cellular transformation. Int J Hematol 86: 101-106.
13. Nagai M, Osame M (2003) Human T-cell lymphotropic virus type I and neurological diseases. J Neurovirol 9: 228-235.
14. Puccioni-Sohler M, Chimelli L, Mercon M, Goncalves RR, Pimenta G, et al. (2003) Pathological and virological assessment of acute HTLV-I-associated myelopathy complicated with encephalopathy and systemic inflammation. J Neurol Sci 207: 87-93.
15. Afonso PV, Ozden S, Prevost MC, Schmitt C, Seilhean D, et al. (2007) Human blood-brain barrier disruption by retroviral-infected lymphocytes: role of myosin light chain kinase in endothelial tight-junction disorganization. J Immunol 179: 2576-2583.

16. Nagai M, Brennan MB, Sakai JA, Mora CA, Jacobson S (2001) CD8(+) T cells are an in vivo reservoir for human T-cell lymphotropic virus type I. Blood 98: 1858-1861.
17. Yamano Y, Nagai M, Brennan M, Mora CA, Soldan SS, et al. (2002) Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP). Blood 99: 88-94.
18. Lepoutre V, Jain P, Quann K, Wigdahl B, Khan ZK (2009) Role of resident CNS cell populations in HTLV-1-associated neuroinflammatory disease. Front Biosci 14: 1152-1168.
19. Grant C, Oh U, Yao K, Yamano Y, Jacobson S (2008) Dysregulation of TGF-beta signaling and regulatory and effector T-cell function in virus-induced neuroinflammatory disease. Blood 111: 5601-5609.
20. Vendrame D, Sourisseau M, Perrin V, Schwartz O, Mammano F (2009) Partial Inhibition of HIV Replication by Type-I Interferons: Impact of Cell-to-Cell Viral Transfer. J Virol.
21. Hong S, Cao J, Tu YT (2009) Evolution of HIV-1 in a patient population failing multiple-drug therapy. Microbiol Immunol 53: 535-539.
22. Kinpara S, Hasegawa A, Utsunomiya A, Nishitsuji H, Furukawa H, et al. (2009) Stromal cell-mediated suppression of human T-cell leukemia virus type 1 expression in vitro and in vivo by type I interferon. J Virol 83: 5101-5108.
23. Mahieux R, Hermine O (2005) In vivo and in vitro treatment of HTLV-1 and HTLV-2 infected cells with arsenic trioxide and interferon-alpha. Leuk Lymphoma 46: 347-355.
24. Hishizawa M, Imada K, Kitawaki T, Ueda M, Kadowaki N, et al. (2004) Depletion and impaired interferon-alpha-producing capacity of blood plasmacytoid dendritic cells in human T-cell leukaemia virus type I-infected individuals. Br J Haematol 125: 568-575.
25. Randall RE, Goodbourn S (2008) Interferons and viruses: an interplay between induction, signaling, antiviral responses and virus countermeasures. J Gen Virol 89: 1-47.
26. Hiscott J (2007) Triggering the innate antiviral response through IRF-3 activation. J Biol Chem 282: 15325-15329.
27. Sharma S, tenOever BR, Grandvaux N, Zhou GP, Lin R, et al. (2003) Triggering the interferon antiviral response through an IKK-related pathway. Science 300: 1148-1151.
28. Fitzgerald KA, McWhirter SM, Faia KL, Rowe DC, Latz E, et al. (2003) IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol 4: 491-496.
29. Genin P, Lin R, Hiscott J, Civas A (2009) Differential regulation of human interferon A gene expression by interferon regulatory factors 3 and 7. Mol Cell Biol 29: 3435-3450.
30. Honda K, Takaoka A, Taniguchi T (2006) Type I interferon gene induction by the interferon regulatory factor family of transcription factors. Immunity 25: 349-360.

31. Levy DE, Marie I, Smith E, Prakash A (2002) Enhancement and diversification of IFN induction by IRF-7-mediated positive feedback. J Interferon Cytokine Res 22: 87-93.
32. O'Shea JJ, Gadina M, Schreiber RD (2002) Cytokine signaling in 2002: new surprises in the Jak/Stat pathway. Cell 109 Suppl: S121-131.
33. Yasukawa H, Sasaki A, Yoshimura A (2000) Negative regulation of cytokine signaling pathways. Annu Rev Immunol 18: 143-164.
34. Kubo M, Hanada T, Yoshimura A (2003) Suppressors of cytokine signaling and immunity. Nat Immunol 4: 1169-1176.
35. Fenner JE, Starr R, Cornish AL, Zhang JG, Metcalf D, et al. (2006) Suppressor of cytokine signaling 1 regulates the immune response to infection by a unique inhibition of type I interferon activity. Nat Immunol 7: 33-39.
36. Mansell A, Smith R, Doyle SL, Gray P, Fenner JE, et al. (2006) Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat Immunol 7: 148-155.
37. De Sepulveda P, Ilangumaran S, Rottapel R (2000) Suppressor of cytokine signaling-1 inhibits VAV function through protein degradation. J Biol Chem 275: 14005-14008.
38. Ryo A, Suizu F, Yoshida Y, Perrem K, Liou YC, et al. (2003) Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. Mol Cell 12: 1413-1426.
39. Babon JJ, Sabo JK, Zhang JG, Nicola NA, Norton RS (2009) The SOCS box encodes a hierarchy of affinities for Cullin5: implications for ubiquitin ligase formation and cytokine signaling suppression. J Mol Biol 387: 162-174.
40. Piessevaux J, Lavens D, Peelman F, Tavernier J (2008) The many faces of the SOCS box. Cytokine Growth Factor Rev 19: 371-381.
41. Dalpke A, Heeg K, Bartz H, Baetz A (2008) Regulation of innate immunity by suppressor of cytokine signaling (SOCS) proteins. Immunobiology 213: 225-235.
42. Mbow ML, Sarisky RT (2004) What is disrupting IFN-alpha's antiviral activity? Trends Biotechnol 22: 395-399.
43. Pothlichet J, Chignard M, Si-Tahar M (2008) Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway. J Immunol 180: 2034-2038.
44. Nishiura Y, Nakamura T, Fukushima N, Moriuchi R, Katamine S, et al. (2004) Increased mRNA expression of Th1-cytokine signaling molecules in patients with HTLV-I-associated myelopathy/tropical spastic paraparesis. Tohoku J Exp Med 204: 289-298.
45. Hiscott J, Nguyen TL, Arguello M, Nakhaei P, Paz S (2006) Manipulation of the nuclear factor-kappaB pathway and the innate immune response by viruses. Oncogene 25: 6844-6867.
46. Nagai M, Yamano Y, Brennan MB, Mora CA, Jacobson S (2001) Increased HTLV-I proviral load and preferential expansion of HTLV-I Tax-specific CD8+ T cells in cerebrospinal fluid from patients with HAM/TSP. Ann Neurol 50: 807-812.
47. Levy DE, Darnell JE, Jr. (2002) Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 3: 651-662.

48. Pise-Masison CA, Radonovich M, Dohoney K, Morris JC, O'Mahony D, et al. (2009) Gene expression profiling of ATL patients: compilation of disease-related genes and evidence for TCF4 involvement in BIRC5 gene expression and cell viability. Blood 113: 4016-4026.
49. Baba M, Okamoto M, Hamasaki T, Horai S, Wang X, et al. (2008) Highly enhanced expression of CD70 on human T-lymphotropic virus type 1-carrying T-cell lines and adult T-cell leukemia cells. J Virol 82: 3843-3852.
50. Wu K, Bottazzi ME, de la Fuente C, Deng L, Gitlin SD, et al. (2004) Protein profile of tax-associated complexes. J Biol Chem 279: 495-508.
51. Solis M, Wilkinson P, Romieu R, Hernandez E, Wainberg MA, et al. (2006) Gene expression profiling of the host response to HIV-1 B, C, or A/E infection in monocyte-derived dendritic cells. Virology 352: 86-99.
52. Oh U, Yamano Y, Mora CA, Ohayon J, Bagnato F, et al. (2005) Interferon-beta1a therapy in human T-lymphotropic virus type I-associated neurologic disease. Ann Neurol 57: 526-534.
53. Feng X, Ratner L (2008) Human T-cell leukemia virus type 1 blunts signaling by interferon alpha. Virology 374: 210-216.
54. Zhang J, Yamada O, Kawagishi K, Araki H, Yamaoka S, et al. (2008) Human T-cell leukemia virus type 1 Tax modulates interferon-alpha signal transduction through competitive usage of the coactivator CBP/p300. Virology 379: 306-313.
55. Kile BT, Alexander WS (2001) The suppressors of cytokine signaling (SOCS). Cell Mol Life Sci 58: 1627-1635.
56. Mahller YY, Sakthivel B, Baird WH, Aronow BJ, Hsu YH, et al. (2008) Molecular analysis of human cancer cells infected by an oncolytic HSV-1 reveals multiple upregulated cellular genes and a role for SOCS1 in virus replication. Cancer Gene Ther 15: 733-741.
57. Kamio M, Yoshida T, Ogata H, Douchi T, Nagata Y, et al. (2004) SOCS1 [corrected] inhibits HPV-E7-mediated transformation by inducing degradation of E7 protein. Oncogene 23: 3107-3115.
58. Ryo A, Tsurutani N, Ohba K, Kimura R, Komano J, et al. (2008) SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag. Proc Natl Acad Sci U S A 105: 294-299.
59. Kamizono S, Hanada T, Yasukawa H, Minoguchi S, Kato R, et al. (2001) The SOCS box of SOCS-1 accelerates ubiquitin-dependent proteolysis of TEL-JAK2. J Biol Chem 276: 12530-12538.
60. Ali S, Nouhi Z, Chughtai N, Ali S (2003) SHP-2 regulates SOCS-1-mediated Janus kinase-2 ubiquitination/degradation downstream of the prolactin receptor. J Biol Chem 278: 52021-52031.
61. Weber F, Kochs G, Haller O (2004) Inverse interference: how viruses fight the interferon system. Viral Immunol 17: 498-515.
62. Saira K, Zhou Y, Jones C (2007) The infected cell protein 0 encoded by bovine herpesvirus 1 (bICP0) induces degradation of interferon response factor 3 and, consequently, inhibits beta interferon promoter activity. J Virol 81: 3077-3086.
63. Alexander WS (2002) Suppressors of cytokine signaling (SOCS) in the immune system. Nat Rev Immunol 2: 410-416.

64. Palmer DC, Restifo NP (2009) Suppressors of cytokine signaling (SOCS) in T cell differentiation, maturation, and function. Trends Immunol 30: 592-602.
65. Baker BJ, Akhtar LN, Benveniste EN (2009) SOCS1 and SOCS3 in the control of CNS immunity. Trends Immunol 30: 392-400.
66. Kobayashi T, Yoshimura A (2005) Keeping DCs awake by putting SOCS1 to sleep. Trends Immunol 26: 177-179.
67. Osame M, editor (1990) Review of WHO Kagoshima meeting and diagnostic guidelines for HAM/TSP: New York, NY: Raven Press. 191-197 p.
68. Hinuma Y, Nagata K, Hanaoka M, Nakai M, Matsumoto T, et al. (1981) Adult T-cell leukemia: antigen in an ATL cell line and detection of antibodies to the antigen in human sera. Proc Natl Acad Sci U S A 78: 6476-6480.
69. Kobayashi N, Yamamoto N, Koyanagi Y, Schneider J, Hunsmann G, et al. (1984) Translation of HTLV (human T-cell leukemia virus) RNA in a nuclease-treated rabbit reticulocyte system. EMBO J 3: 321-325.
70. Iwamura T, Yoneyama M, Yamaguchi K, Suhara W, Mori W, et al. (2001) Induction of IRF-3/-7 kinase and NF-kappaB in response to double-stranded RNA and virus infection: common and unique pathways. Genes Cells 6: 375-388.
71. Huang FJ, Steeg PS, Price JE, Chiu WT, Chou PC, et al. (2008) Molecular basis for the critical role of suppressor of cytokine signaling-1 in melanoma brain metastasis. Cancer Res 68: 9634-9642.
72. Zitzmann K, Brand S, De Toni EN, Baehs S, Goke B, et al. (2007) SOCS1 silencing enhances antitumor activity of type I IFNs by regulating apoptosis in neuroendocrine tumor cells. Cancer Res 67: 5025-5032.

## Preamble for Manuscript II

In the previous chapter, we identified SOCS1 as being highly expressed in HAM/TSP and AC but not in ATL patients. We further demonstrated that HTLV-1-induced SOCS1 had a positive role in viral replication through inhibition of the IFN response, suggesting that targeting SOCS1 could represent a potential new approach to enhance the therapeutic potency of type I IFNs in HAM/TSP disease. Although treatments of hematological malignancies have improved considerably over the past decade, the growing therapeutic arsenal has not benefited adult-T-cell Leukemia (ATL) patients who are completely refractory to conventional chemotherapeutic regimens. The development of optional therapies such as nucleosides analog in combination with IFN-α has been shown to improve the response rate and survival; however, most patients relapse and succumb to disease, thus underlying the need for novel approaches to purge CD4+/CD25+ leukemic T-cells. Oncolytic viruses (OVs) constitute a novel biotherapeutic approach to treat malignancies refractory to standard therapy. Replication-competent OVs selectively infect and replicate in tumor cells that harbor defects in key antiviral, survival and growth signaling pathways – effectively leading to their destruction. Normal cells and tissues are spared because intact antiviral responses naturally limit OVs replication. OVs represent a highly targeted anti-cancer therapy with limited toxicity compared with conventional treatments. Thus, the aggressive phenotype of ATL, coupled with its resistance to standard chemotherapeutic regimens, make this leukemia an appropriate target for OVs therapy. In this chapter, we evaluated the ability of VSV to lyse primary HTLV-1-infected T-lymphocytes from individuals with ATL.

# - MANUSCRIPT II -

## Oncolytic Activity of Vesicular Stomatitis Virus in primary Adult T-Cell Leukemia

*Published in: Oncogene. 2006 Jan 19;25(3):349-58.*

**S Olière[2,3,8], R Césaire[1,8], E Sharif-Askari[2,3,8], M Loignon[2,3], A Lézin[1], S Olindo[4], G Panelatti [5], M Kazanji[6], R Aloyz[2,3], L Panasci[2,3], JC Bell[7] and J Hiscott[2,3]**

*[1]Laboratoire de Virologie-Immunologie and UMR433 INSERM, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; [2]Molecular Oncology Group, Lady Davis Institute for Medical Research, Jewish General Hospital, Montreal, Quebec, Canada; [3]Departments of Microbiology and Medicine, McGill University, Montreal, Canada; [4]Service de Neurologie, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; [5]Service de Médecine Interne, Centre Hospitalier Universitaire de Fort-de-France, Martinique, France; [6]Laboratoire Retrovirus, Institut Pasteur de Cayenne, French Guyana, France and [7]Ottawa Regional Cancer Centre, University of Ottawa, Ottawa, Canada*

[8]Authors contributed equally to the work.

#Corresponding Author: Dr. John Hiscott
Lady Davis Institute for Medical Research
3755 Cote Ste. Catherine
Montreal, Quebec, Canada H3T 1E2
Telephone: (514) 340-8222 Ext. 5265
Fax: (514) 340-7576
E-mail: john.hiscott@mcgill.ca

Running Title: Oncolytic activity of VSV in ATL
Key words: ATL, HTLV-1, HAM/TSP, VSV, apoptosis

This research was supported by grants from the National Cancer Institute with funds from the Canadian Cancer Society to JH and JB, the Canadian Institutes of Health Research and CANVAC, the Canadian Network for Vaccines and Immunotherapeutics. ESA is supported by a Fellowship from the FRSQ and JH is a recipient of a CIHR Senior Investigator award.

**ABSTRACT**

Treatments for hematological malignancies have improved considerably over the past decade, but the growing therapeutic arsenal has not benefited adult T-cell leukemia (ATL) patients. Oncolytic viruses such as vesicular stomatitis virus (VSV) have recently emerged as a potential treatment of solid tumors and leukemias *in vitro* and *in vivo*. In the current study, we investigated the ability of VSV to lyse primary human T-lymphotropic virus type 1 (HTLV-1)-infected T-lymphocytes from patients with ATL. *Ex vivo* primary ATL cells were permissive for VSV and underwent rapid oncolysis in a time-dependent manner. Importantly, VSV infection showed neither viral replication nor oncolysis in HTLV-1-infected, non-leukemic cells from patients with HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), and in naive CD4+ T-lymphocytes from normal individuals or in *ex vivo* cell samples from patients with chronic lymphocytic leukemia (CLL). Interestingly, activation of primary CD4+ T-lymphocytes with anti-CD3/CD28 monoclonal antibody, and specifically with anti-CD3, was sufficient to induce limited viral replication and oncolysis. However, at a similar level of T-cell activation, VSV replication was increased four-fold in ATL cells compared to activated CD4+ T-lymphocytes, emphasizing the concept that VSV targets genetic defects unique to tumor cells to facilitate its replication. In conclusion, our findings provide the first essential information for the development of a VSV-based treatment for ATL.

## INTRODUCTION

Adult T-cell leukemia (ATL) is an aggressive malignancy of mature activated, CD4/CD25+ T- lymphocytes that occurs worldwide in populations where human T-lymphotropic virus type 1 (HTLV-1) infection is endemic – southwestern Japan, the Caribbean Basin, inter-tropical Africa, and the southern United States. HTLV-1 has also been etiologically linked to a neurological disorder, HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Uchiyama *et al.*, 1977; Hinuma *et al.*, 1982; Gessain *et al.*, 1985; Osame *et al.*, 1986). Mechanistically, the HTLV-1 Tax oncoprotein plays a central role in the initiation of transformation by transactivating several cellular pathways (Yoshida, 2001; Franchini *et al*., 2003), and by promoting the oligo/polyclonal expansion of HTLV-1-infected T cells (Mortreux et al., 2003). Subsequently, Tax-mediated accumulation of host genome alterations leads to Tax-independent proliferation, escape from the CD8+ T-lymphocyte anti-Tax response, and emergence of the malignant CD4/CD25+ T-lymphocyte clone (Yasunaga and Matsuoka, 2003). Despite extensive progress in the understanding of the molecular virology and immunology of HTLV-I-associated diseases, there has been only minimal improvement in the treatment of ATL. Conventional as well as high-dose chemotherapy has shown only limited benefit, and the aggressive subtypes of ATL carry a very poor prognosis, with a median survival of less than 1 year (Shimoyama, 1991). The combination of antiviral nucleosides and interferon (IFN)-α has been shown to improve the response rate and survival; however, most patients relapse and succumb to disease, thus underlining the need for new therapeutic approaches (Bazarbachi *et al*., 2004). One promising and novel cancer therapeutic approach against tumor cells is virotherapy. Several naturally occurring or genetically engineered viruses are effective in killing tumor cells in many experimental models (Steele, 2000; Wu *et al*., 2001; Chiocca, 2002). Recently, vesicular stomatitis virus (VSV), an enveloped negative-strand RNA virus belonging to the Rhabdoviridae family, has been shown to possess intrinsic oncolytic properties (Balachandran and Barber, 2000; Stojdl *et al*., 2000). VSV can infect a wide variety of mammalian cells and host defense to VSV infection is based on the antiviral activity of

IFN before the generation of neutralizing antibody response (Grigera *et al*., 1996). The ability of VSV to replicate in and lyse tumor cells occurs in part due to defects in the IFN antiviral response in tumor cells (Stojdl *et al*., 2000, 2003; Fernandez *et al*., 2002). Recently, Stojdl *et al*. (2003) demonstrated that 80% of VSV-permissive cell lines had impaired responses to either IFN-α or IFN-β. Complete destruction of cell line-derived xenograft tumors in animal models following intratumoral injection of wild-type or genetically modified VSV have been documented (Balachandran and Barber, 2000; Stojdl *et al*., 2000, 2003; Balachandran *et al*., 2001; Fernandez *et al*., 2002; Ebert *et al*., 2003; Huang *et al*., 2003; Obuchi *et al*., 2003). Furthermore, systemic intravenous administration of VSV in mouse models has also shown potency in treating primary and metastasic tumors (Balachandran *et al*., 2001). The importance of IFN in the host defense against VSV was highlighted by demonstrating that mice harboring defects in IFN signaling are highly sensitive to normally sublethal exposure to VSV (Muller *et al*., 1994; Durbin *et al*., 1996), a situation that is dramatically reversed by prophylactic IFN treatment (Stojdl *et al*., 2003). Recent analysis has demonstrated that defective control of mRNA translation initiation plays a crucial role in cell permissiveness to VSV (Balachandran and Barber, 2004), and that translation control downstream of PKR activation, frequently deregulated in many transformed cells, can cooperate with attenuated IFN antiviral activity to facilitate VSV oncolysis. While most studies of VSV oncolysis in vitro and/or in animal models have been performed with cell lines, preclinical data in primary cancer cells are still lacking. In this study, we investigated the oncolytic activity of VSV in primary CD4+ T-lymphocytes from ATL patients. *Ex vivo* VSV infection induced significant oncolytic activity in primary ATL cell in a time- and dose-dependent manner. These studies provide the first essential information for the development of a VSV based treatment for ATL.

## MATERIALS AND METHODS

### *Cells and reageants*

HTLV-1 T-cell lines MT-2 and MT-4 cells were purchased from American Type Culture Collection (ATCC) and maintained in RPMI 1640 medium supplemented with 10% FCS (Wisent) and 100 U/ml penicillin/streptomycin (referred to as complete RPMI 1640). The human Epstein–Barr virus transformed B-cell chronic lymphocytic leukemia (B-CLL) I-83 cell line and Epstein–Barr virus-negative B-CLL WSU cell line were cultured in complete RPMI 1640. Peripheral blood mononuclear cells (PBMCs) from four ATL patients (samples ATL-1, ATL-2, ATL-3, and ATL-4), two patients with HAM/TSP (HAM-1 and HAM-2), three patients with B-CLL (CLL-1, CLL-2, and CLL-3), one patient with T-CLL (CLL-4), and three healthy individuals were obtained after informed consent. PBMCs were isolated by centrifugation (400 g at $20^0$C for 25 min) of blood on a Ficoll–Hypaque gradient (Amersham,Pharmacia Biotech). PBMCs were resuspended in RPMI 1640 medium supplemented with 15% heat-inactivated FBS,
100 U/ml penicillin–streptomycin, and 70 U/ml of human recombinant IL-2 (rIL-2, Roche Diagnostics, Mannheim, Germany).

### *Isolation of T cells*

Freshly isolated PBMCs were incubated with CD4 enrichment cocktail (Miltenyi Biotec) and CD4+ T-lymphocytes were negatively selected with the high-speed autoMACS system (Miltenyi Biotec) according to the manufacturer's instructions. In all cases, the purity of CD4+ T-lymphocytes was between 90 and 95% as determined by flow cytometry. Isolated CD4+ T-cells were cultured in complete RPMI 1640.

### *T-cell activation*

Purified CD4+ T-lymphocytes ($10^6$/ml) were mock activated or activated *in vitro* for 24 h with 5 mg/ml of immobilized anti-CD3 monoclonal antibody, and 1 mg/ml of immobilized anti-CD28 monoclonal antibody (BD Biosciences), in the presence of 70 U/ml of rIL-2. Cells were then harvested, washed, and cultured in complete RPMI 1640.

***Virus production, quantification, and infection***

Wild-type VSV (Indiana serotype) and rVSV-GFP were propagated in BHK-21 cells as described previously (Stojdl *et al*., 2003). Viruses were obtained from cell-free supernatants and titrated on Vero cells by standard plaque assay. Cells were mock infected or infected with VSV at an MOI of 1 PFU/cell for 1 h in RPMI 1640 at $37^0$C. In some experiments, caspase-3 inhibitors zVAD-FMK or D-VAD-FMK were added to a final concentration of 100 mM at 20 min prior to VSV infection. The cells were then washed with PBS and incubated with complete RPMI 1640 at $37^0$C for the indicated times. Following incubation, cells were harvested in PBS, pelleted, and processed for protein analysis. For indicated experiments, cells were mock infected or infected with rVSV-GFP under similar conditions and GFP expression was analyzed by FACS caliburt flow cytometry.

***Immunoblot analysis***

Cells destined for immunoblotting were washed with PBS and lysed in lysis buffer (0.05% NP40, 1% glycerol, 30mM NaF, 40mM β-glycerophosphate, 10mM $Na_3VO_4$, 10ng/ml of protease inhibitors (leopeptine, aprotinine, and pepstatine)). Protein concentration was determined with Bio-Rad protein assay reagent (BioRad), and 20 mg of protein was then resolved using 12% SDS–PAGE and transferred to nitrocellulose membrane (Hybond C Super; Amersham, Oakville, Canada). Blots were blocked for 1 h at 25˚C in 5% non-fat dried milk in PBS-T (PBSþ0.5% Tween-20). Membranes were then incubated overnight with rabbit anti-VSV, anti-Rho GD Pdissociation inhibitor (Rho-GDI) (BD Pharmingen, 1:5000), anti-procaspase-3 (Upstate, 1:1000), anti-cleaved caspase-3 (Cell Signaling, 1:2000), anti-DNA fragmentation factor-45 (DFF-45) (Sharif-Askari et al., 2001) (1:3500), or mouse anti β-actin (Chemichon, 1:10 000) at 25˚C. The blots were then washed three times in PBST and reincubated in the presence of horseradish peroxidase-conjugated goat anti-rabbit or anti mouse antibodies (Amersham, 1:3000) for 1 h at $25^0$C. Following three washes with PBST, the proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham, Oakville, Canada).

***Immunofluorescence staining and analysis***

After washing twice with PBS, cells were stained with monoclonal allophycocyanin-labeled anti-CD4, phycoerytrin (PE)-labeled anti-CD25 or PE-labeled anti-CD69 (BD Pharmingen), for 30 min in PBS/1% FCS. After a final wash with ice-cold PBS, cells were resuspended in 400ml FACS buffer (PBS/CytoFix (BD Pharmingen)). Flow cytometric analyses ($1x10^4$ cells/measurement) were performed by FACS caliburt flow cytometer with CELLQuestt software (Becton Dickinson).

***Measurement of apoptosis***

Apoptotic cells were analysed by annexin V/7-amino-actinomycin D (7-AAD) double staining for detection of the apoptotic plasma membrane (phosphatidylserine translocation). Mock- or VSV-infected cells were washed and resuspended in 300ml of ice-cold annexin V binding buffer (HEPESNaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$ (BD Pharmingen)), incubated on ice for 15 min with 1 ml of each of fluorescein isothiocyanate (FITC)-conjugated annexin V and 7-AAD (BD Pharmingen), and followed by flow cytometric analysis. For DNA content staining, cells were collected, washed in ice-cold PBS/5mm EDTA, and fixed in ethanol (70% in PBS) overnight at $-20^0$C. After fixation, cells were then washed in PBS, and stained with a PI staining solution (PBS, 50 mg/ml PI, 100 U/ml RNase A and 1 mg/ml glucose) for 2 h at room temperature before FACSs analysis. For each sample, the forward versus right-angle scatter cytogram was used to exclude debris and aggregates. Apoptosis was determined by quantification of the sub-$G_0$ population ($1x10^4$ cells/measurement).

## RESULTS

### VSV replicates and induces rapid cell death in HTLV-1-transformed cells

Wild-type VSV has been shown to induce potent cytolytic effects against a wide range of tumor cells, including 80% of the NCI human tumor cell line panel (Weinstein *et al*., 1997). Thus, we first sought to determine the ability of VSV to replicate and induce cell death in the HTLV-1-infected T-cell lines, MT-2 and MT-4. VSV infection was accompanied by cell death; quantification by acridine orange–ethidium bromide fluorescent-dye staining showed that cell death was time-dependent (Figure 1a), and by 48 h post-VSV infection, no significant viable cells were detected. Cell death was correlated with VSV replication; as shown in Figure 1b, after infection with a VSV strain expressing GFP (rVSVGFP), a significant percentage of cells were positive for GFP expression in both MT-2 and MT-4, illustrating the permissiveness of HTLV-1-transformed T cells for VSV infection. At 12 h post-infection, 45% of infected cells were GFP positive in MT-4 cells as compared to 10% in MT-2 cells, suggesting higher VSV replication in MT-4 than MT-2 cells.

### VSV induces caspase-dependent cell death in HTLV-1-transformed cells

VSV-induced cytolytic activity was further confirmed by increased binding of annexin V-FITC to externalized phosphatidylserine of infected but not of control cells. Double staining for annexin V and 7-AAD revealed that phosphatidylserine exposure was induced in a time-dependent manner in both MT-2 and MT-4 cell lines following VSV infection (Figure 1C), and by FACS, the percentage of apoptotic cells at 12 h post-infection increased to 88 and 46% for MT-4 and MT-2, respectively. The number of apoptotic cells in the infected population is higher than the number of VSV positive cells, suggesting that apoptosis may occur through both direct virus-mediated cytolysis and indirect mechanisms involving the release of proapoptotic cytokines. To address the mechanism of VSV killing in HTLV-1-transformed cells, caspase activation and substrate cleavage was assessed in MT-4 cells (Woo *et al*.,, 1998;Wolf and Green, 1999). As shown in Figure 2a, immunoblot analysis using anti-VSV antiserum revealed significant

VSV replication, with VSV G and N protein expression observed as early as 2 h post-infection (Figure 2a, lane 4). Concomitant with viral replication, VSV infection for 4 h was sufficient to cleave procaspase-3 to its enzymatically active p20 and p17 subunits (Figure 2a, lane 5). The enzymatic activity of caspase-3 in VSV-infected cells was further monitored by immunoblot analysis of two caspase-3 substrates, the Rho-GDI, and the DFF45 (Na *et al*., 1996; Wolf *et al*., 1999). Both substrates were processed in a time-dependent manner beginning at 4 h post-infection (Figure 2a, lanes 5–9). Furthermore, the use of two broad-spectrum caspase-3 inhibitors – zVAD and DEVD – significantly delayed the onset of VSV-induced caspase-3 activation and Rho-GDI processing in MT-4 cells (Figure 2b); the 19 and 17 kd forms of cleaved caspase-3 were weakly detectable at 10 h post-infection in the presence of DEVD (Figure 2b, lanes 8 and 9), while in the presence of zVAD, cleaved caspase-3 was barely detected at 6–12 h (Figure 2b, lanes 13–16). In the presence of both inhibitors, the majority of the 32 kd procaspase-3 remained uncleaved (Figure 2b). Rho-GDI cleavage in the presence of both inhibitors was also significantly delayed (Figure 2b, lanes 5–8 and 13–16). There was in contrast only a slight delay in the kinetics of VSV replication in the presence of DEVD or zVAD, with VSV G and N protein expression observed at 4 h post-infection (Figure 2b, lanes 4, 11, and 12). Collectively, these data demonstrate that HTLV-1-transformed T-cells are permissive for VSV replication, which in turn induces rapid caspase-dependent oncolysis of infected cells.

| *Patient* | *Sex* | *Age (years)* | *Type of leukemia* | *Survival* | *Chemotherapy at sampling* | *Circulating leukemic cells (%)* | *Increase in % annexin V-positive cells at 48 h in VSV- versus mock-infected PBMCs* |
|---|---|---|---|---|---|---|---|
| ATL-1 | M | 39 | ATL (acute form) | 40 days | Untreated | 79 | 55 |
| ATL-2 | F | 51 | ATL (acute form) | 30 days | Untreated | 39 | 50 |
| ATL-3 | F | 60 | ATL (acute form) | >9 months | Untreated | 77 | 56 |
| ATL-4 | M | 41 | ATL (acute form) | 14 months | Untreated | 40 | 62 |
| CLL-1 | M | 60 | B-CLL | >10 years | Chlorambucil | 90 | 0 |
| CLL-2 | M | 83 | B-CLL | >10 years | Chlorambucil | 45 | 6 |
| CLL-3 | F | 87 | B-CLL | >5 years | Untreated | 72 | 3 |
| CLL-4 | M | 85 | T-CLL | >5 years | Untreated | 75 | 7 |
| HAM/TSP-1 | M | 48 | — | — | — | — | 13 |
| HAM/TSP-2 | F | 69 | — | — | — | — | 3 |
| Healthy control | M | 42 | — | — | — | — | 6 |

**Table 1. Clinical and biological features of ATL patients and PBMCs following *ex vivo* infection with VSV.** At 48 h post-infection, mock- and VSV-infected cells were analyzed for annexin V staining by flow cytometry. VSV-induced cell death was estimated by the difference between the percentages of annexin V-positive cells in the VSV- and mock-infected samples. Data from CLL, HAM/TSP and healthy controls are indicated.

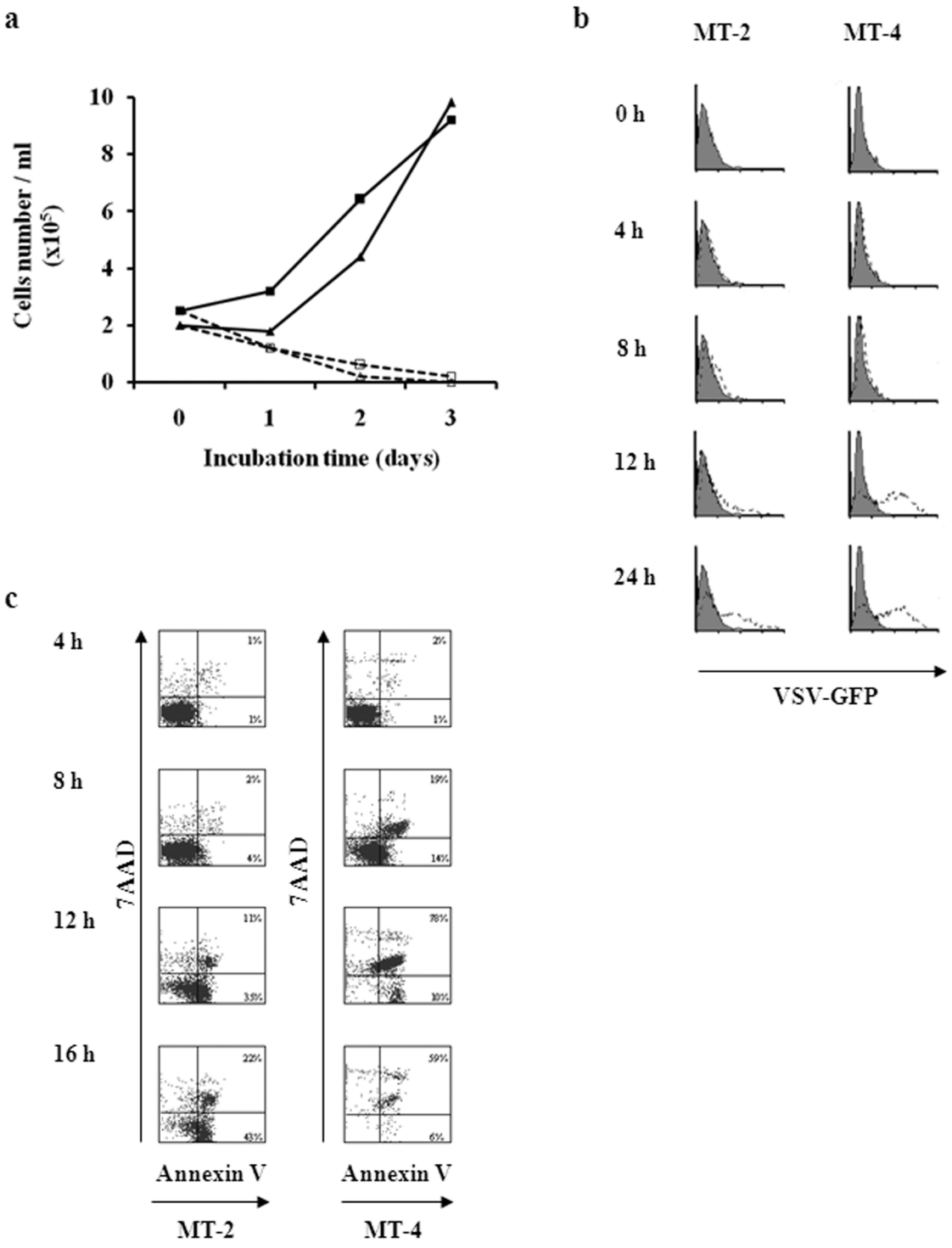

a
Cells number / ml (x10^5)
Incubation time (days)
b
MT-2
MT-4
0 h
4 h
8 h
12 h
24 h
VSV-GFP
c
4 h
8 h
12 h
16 h
7AAD
Annexin V
MT-2
MT-4

**Figure 1. VSV replication in HTLV-1-transformed cells.** (a) Growth of VSV-infected MT2 and MT4 cell lines. Cells were mock infected or infected (MOI of 1 PFU/cell) with VSV-HR strain for the indicated times. Cells were stained with acridine orange and ethidium bromide, and viable cells were counted by fluorescence microscopy. (■) MT2; (□) MT2+VSV; (▲) MT4; and (Δ) MT4+VSV. (b) Kinetics of VSV-GFP replication in MT-2- and MT-4-transformed cell lines. At the indicated times post-infection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (c) Kinetics of VSV-induced cell death in MT-2 and MT-4 cell lines. At the indicated times, apoptosis was measured using annexin V/7-AAD staining by flow cytometry.

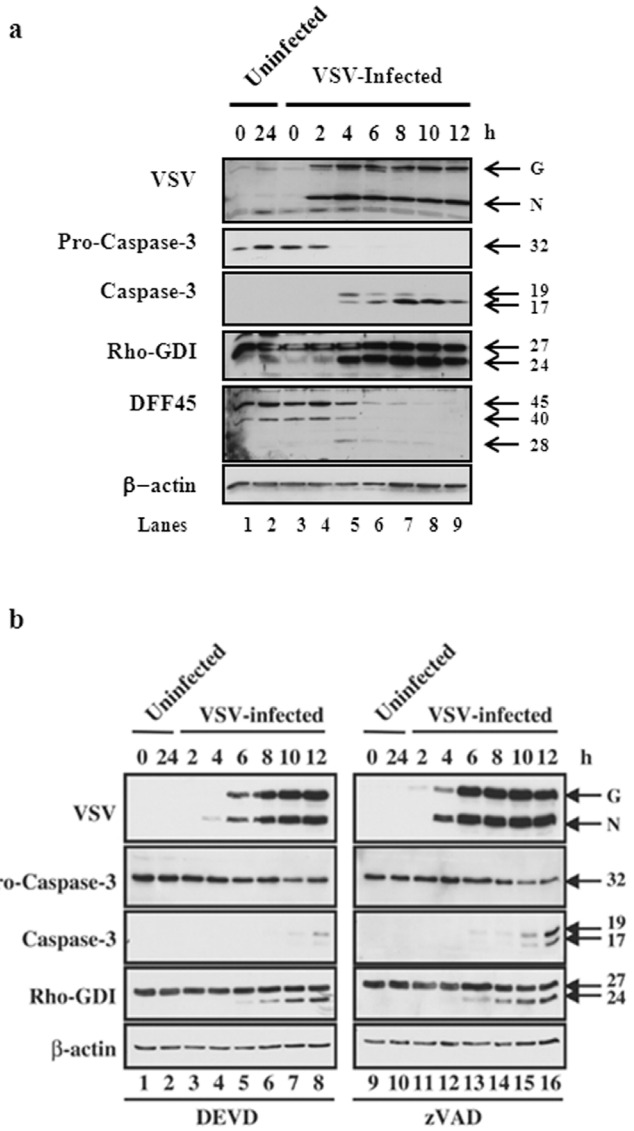

a
Uninfected
VSV-Infected
0 24 0 2 4 6 8 10 12 h
VSV
G
N
Pro-Caspase-3
32
Caspase-3
19
17
Rho-GDI
27
24
DFF45
45
40
28
β−actin
Lanes 1 2 3 4 5 6 7 8 9
b
Uninfected
VSV-infected
Uninfected
VSV-infected
0 24 2 4 6 8 10 12 0 24 2 4 6 8 10 12 h
VSV
G
N
Pro-Caspase-3
32
Caspase-3
19
17
Rho-GDI
27
24
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
DEVD
zVAD

**Figure 2. Caspase-dependent cell death in VSV-infected HTLV-1-transformed cells.** (a) At the indicated times post-VSV infection (MOI of 1 PFU/cell), the kinetics of caspase-3 activation, Rho-GDI and DFF45 substrate cleavage, and VSV G and N protein expression were assessed in 20mg of whole-cell lysates using SDSPAGE and immunoblot analysis with β-actin as a loading control. Similar results were gathered from three independent experiments. (b) Caspase inhibitors zVAD and DEVD (100 mM final) were added to MT-4 cells 20 min prior to VSV infection and at different times post-infection cell lysates were analyzed for caspase-3 activation, Rho-GDI and DFF45 substrate cleavage, and VSV G and N protein expression, as above.

**VSV replicates and induces rapid cell death in primary ATL cells**

To investigate the *ex vivo* responses of primary ATL cells to VSV infection, PBMCs were obtained from four patients with acute ATL disease; samples were collected at the time of diagnosis before chemotherapy. The survival time of ATL-1, -2, -3, and -4 patients were 40 days, 30 days, >9 months, and 14 months, respectively. Hematological analysis revealed that PBMCs from ATL-1, -2, -3, and -4 patients contained substantial leukemic cells: 79, 39, 77, and 40% atypical lymphocytes, respectively (Table 1). Following Ficoll purification and culture, the percentage of CD4+/CD8-/CD25+ T leukemic cells ranged between 76 and 89%. In parallel, PBMCs were collected from two patients with HAM/TSP, and a healthy volunteer (naive PBMC) (Table 1). Cells (ATL, HAM/TSP, and normal PBMC) were mock infected or infected with the rVSV-GFP (MOI of 1.0PFU/cell) for various times and the level of GFP expression (as a measure of virus replication) was examined by flow cytometry. GFP-expressing cells (10–15%) were detected in all ATL samples beginning at 24 h and the percentage of GFP-positive cells increased in a time-dependent manner (30–40% at 72 h) (Figure 3a, and data not shown). On the other hand, no increase in GFP expression was detected in HAM/TSP or naive PBMC even after 72 h of VSV infection (Figure 3a), illustrating the permissiveness of VSV in ATL cells. VSV induced dramatic cell death (>50% at 24 h and >70% at 72 h) only in ATL cells but not in HAM/TSP or naive PBMC (Figure 3b). In parallel, immunoblot analysis from ATL-infected cells clearly revealed time-dependent processing of procaspase-3 and Rho-GDI at 24 h post-infection, concomitant with viral replication (Figure 3c). High levels of apoptotic ATL cells at 24 and 72 h again indicates that cell death may be occurring through both direct virus mediated and indirect apoptotic mechanisms.

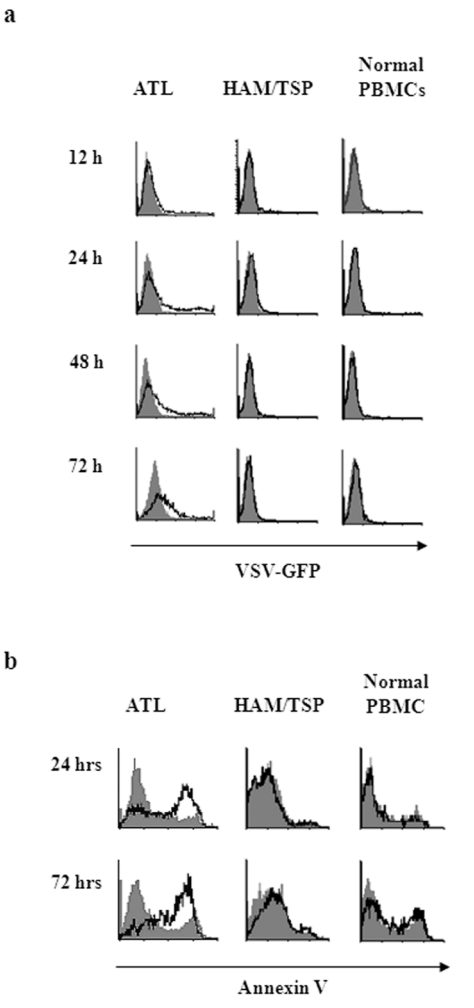

a
ATL
HAM/TSP
Normal PBMCs
12 h
24 h
48 h
72 h
VSV-GFP
b
ATL
HAM/TSP
Normal PBMC
24 hrs
72 hrs
Annexin V
c
0 96 24 48 72 96 h
- - + + + + VSV
VSV
G
N
M
Pro-Caspase-3
32
Cleaved Caspase-3
20
17
Rho-GDI
27
β−actin
24

**Figure 3. VSV oncolysis of primary ATL cells.** (a) Kinetics of VSV replication in ATL cells. ATL, HAM/TSP, and normal PBMC were isolated as described in Materials and methods. Cells were mock infected or infected (MOI of 1 PFU/cell) with rVSV-GFP recombinant virus. At the indicated times, cells were analyzed for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (b) Induction of cell death in ATL cells. At 24 and 72 h post-infection, mock- and VSV-GFP-infected cells were analyzed for annexin V staining by flow cytometry. The full histogram represents mock-infected cells. The empty histogram represents VSV-GFP-infected cells. (c) Kinetics of caspase-3 activation and VSV replication in ATL cells. At the indicated times post-infection, caspase-3 activation, Rho- GDI processing, and VSV replication were detected in 20 mg of whole-cell lysates by SDS–PAGE and immunoblot analysis, with β-actin as a loading control. Similar results were gathered from four patient samples.

**VSV replicates and induces cell death in CLL cell lines but not in primary CLL lymphocytes**

To investigate the specificity of VSV oncolysis, the ability of VSV to replicate and lyse two other leukemic lines, I-83 (Epstein–Barr virus-transformed B-CLL) and WSU (Epstein–Barr virus-negative B-CLL) cell lines, was evaluated. Similar to HTLV-1-infected cell lines, almost no viable I-83 or WSU cells were detected 48 h post-VSV infection (Figure 4a). As shown in Figure 4b, both I-83 and WSU were GFP positive (>70 and 40%, respectively) at 24 h after rVSV-GFP infection. The time-dependent induction of cell death was confirmed by annexin V and 7-AAD staining in infected but not control cells, and revealed extensive cell death (60 and 76%, respectively) as early as 16 h post-infection (Figure 4c). VSV replication was then assessed in primary cells from three B-CLL and one T-CLL patients, with the samples containing more than 60% leukemic cells. In contrast to I-83 and WSU cell lines, primary CLL cells infected *ex vivo* under similar conditions were not permissive to VSV replication (Figure 4d). Furthermore, VSV infection of primary CLL cells showed no increase in cell death as measured by annexin V staining (Table 1), although these CLL samples were efficiently killed by chlorambucil, etoposide or campthotecin (data not shown). Similar results were observed for primary CLL cells at an MOI as high as 100 PFU/cell. These data emphasize the *ex vivo* specificity of VSV oncolytic activity in primary ATL but not primary CLL cells.

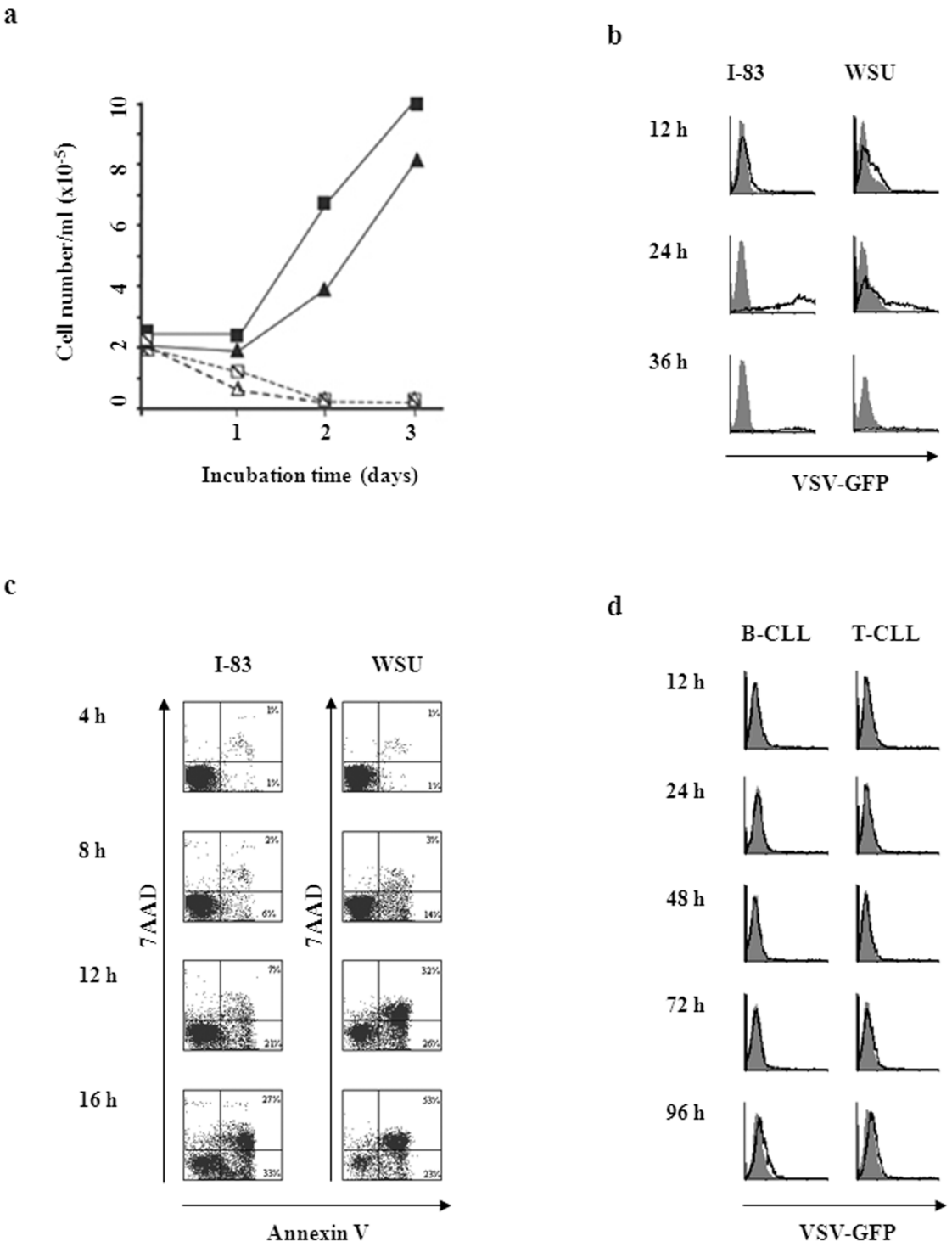
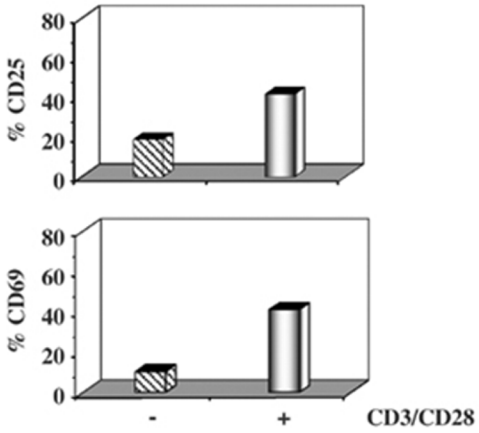

a
Cell number/ml (x10-5)
0
2
4
6
8
10
1
2
3
Incubation time (days)
b
I-83
WSU
12 h
24 h
36 h
VSV-GFP
c
I-83
WSU
4 h
8 h
12 h
16 h
7AAD
7AAD
Annexin V
d
B-CLL
T-CLL
12 h
24 h
48 h
72 h
96 h
VSV-GFP

**Figure 4. VSV replication in CLL cells.** (a) Kinetics of VSV-induced cell death in I-83 and WSU CLL cell lines. Cells were mock infected or infected (MOI of 1 PFU/cell) with VSV-HR strain for the indicated times. Cells were stained with acridine orange and ethidium bromide, and viable cells were counted by fluorescence microscopy. (■) I-83; (□) I-83+VSV; (▲) WSU; and (Δ) WSU+VSV. (b) VSV replication in I-83 and WSU B-CLL cell lines. (b) At the indicated times post-infection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells. The empty histogram represents GFP expression in VSV-infected cells. (c) Kinetics of VSV-induced cell death in I-83 and WSU cell lines. At the indicated times, apoptosis was measured using annexin V/7-AAD staining by flow cytometry. (d) VSV infection in primary CLL lymphocytes. B-CLL and T-CLL PBMC were isolated as described in Materials and methods. At the indicated times post-infection, cultures were evaluated for VSV replication by flow cytometry. The full histogram represents GFP expression in mock-infected cells and the empty histogram represents GFP expression in VSV-infected cells.

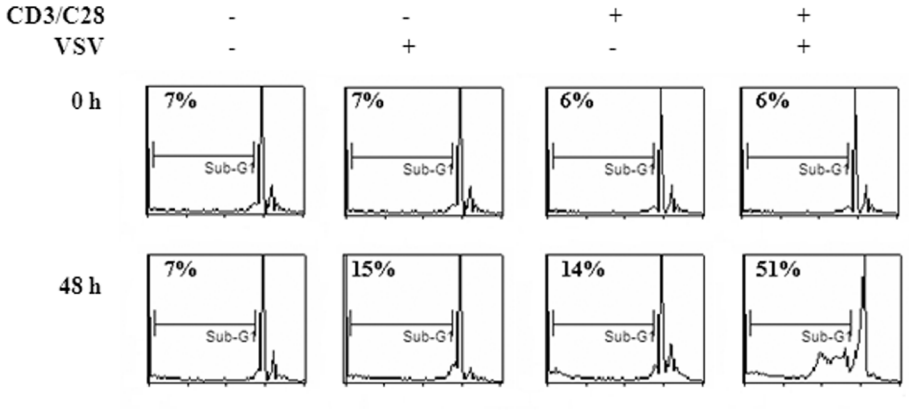
**Activation of CD4+ T-lymphocytes renders them susceptible to VSV replication and cell death**

Previous studies have demonstrated that activation of the IL-2/IL-2R loop during the acute phase of HTLV-1 infection contributes to the activation and proliferation of HTLV-1-infected CD4+CD25+ subset of ATL cells (Maruyama *et al*., 1987; Satoh *et al*., 2002). Thus, we next examined the effect of cell activation on VSV permissiveness and cytolytic activity. CD4+ T-lymphocytes were mock activated or activated with anti-CD3/CD28 (5 mg/ml) and IL-2 (70 U/ml) for 24 h, followed by infection with VSV (MOI of 1.0 PFU/cell) for different time intervals. The activation status of CD4+ T-cells was first analyzed by flow cytometry against CD25 and CD69 activation markers at the time of VSV infection (Figure 5a). VSV infection of anti-CD3/CD28-activated primary CD4+ T-cells resulted in VSV replication (Figure 5c, right panel) compared to non-stimulated cells (Figure 5c, left panel), indicating a relationship between T-cell activation status and VSV replication. Furthermore, VSV replication in anti-CD3/CD28-activated, CD4+ lymphocytes resulted in significant cell death by 48 h post-infection with more than 50% of the cells in the sub-G fraction, whereas in unstimulated primary CD4+ T cells, the sub-G fraction was only 7%. Treatment with anti-CD3/CD28 or VSV alone modestly increased the sub-G proportion to 15% (Figure 5b). In another experiment, PMA treatment of CD4+ T- cells induced significantly higher levels of cell activation compared to anti-CD3/CD28 stimulation (CD69 positivity ~99 versus ~33%, respectively) and was accompanied by extensive VSV replication and cell death (data not shown).

a


% CD25
80
60
40
20
0
% CD69
80
60
40
20
0
-
+
CD3/CD28

b


CD3/C28 - - + +
VSV - + - +
0 h
7%
Sub-G1
7%
Sub-G1
6%
Sub-G1
6%
Sub-G1
48 h
7%
Sub-G1
15%
Sub-G1
14%
Sub-G1
51%
Sub-G1

c

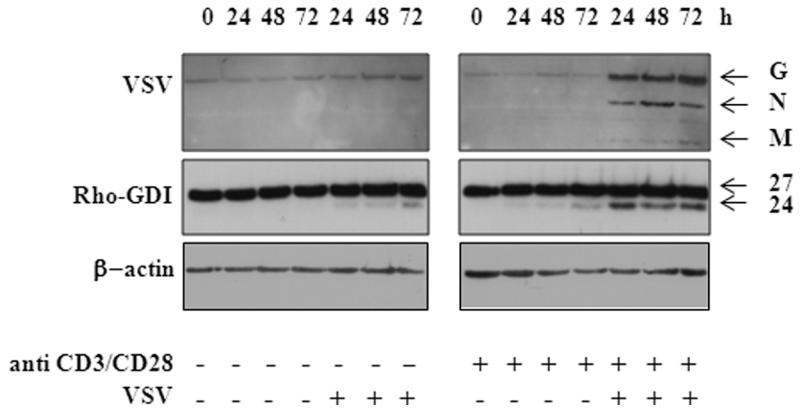
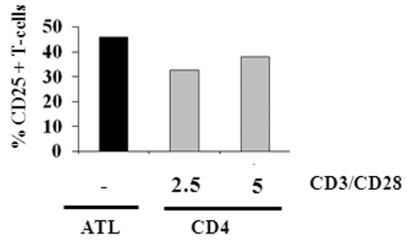

0 24 48 72 24 48 72 0 24 48 72 24 48 72 h
VSV
G
N
M
Rho-GDI
27
24
β−actin
anti CD3/CD28 - - - - - - - + + + + + + +
VSV - - - - + + + - - - - + + +

**Figure 5. VSV-induced oncolysis in activated CD4 T-lymphocytes.** Isolated CD4+ T-lymphocytes were incubated with anti-CD3/anti-CD28/IL-2 or PMA for 24 h. (a) Expression of CD25 and CD69 activation markers were assessed by flow cytometry at the time of VSV infection to determine the CD4+ T-lymphocyte activation status. (b) Activated and non-activated CD4+ lymphocytes were mock infected or infected (MOI of 1 PFU/cell) with VSV-HR strain for the indicated times post-infection. Apoptosis was determined by quantification of the sub-$G_0$ population ($1x10^4$ cells/measurement). (c) Kinetics of VSV replication and Rho-GDI processing in activated and non-activated CD4+ T-lymphocytes. VSV replication and Rho-GDI processing were detected in 20 mg of whole-cell lysates by SDS–PAGE and immunoblot analysis, with β-actin as a loading control.

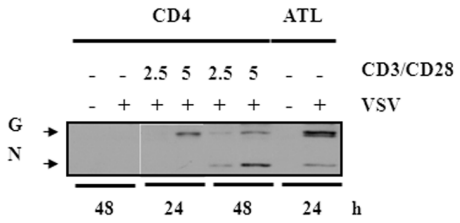
**ATL cells are more permissive to VSV replication than activated CD4+ T-lymphocytes**

Next, the replicative capacity of VSV was examined in ATL cells versus CD4 T-lymphocytes activated to similar levels. For this purpose, anti-CD3/CD28 was used to optimize the intensity of T-cell activation, using an ATL sample that was 86% CD4+, and a primary T-lymphocyte sample that was 96% CD4+. Activation of CD4+ T-cells with anti-CD3/CD28 increased the CD25 expression level (~32% with 2.5 mg/ml of anti-CD3 and ~38% with 5 mg/ml of anti-CD3) comparable to the CD25 level detected for ATL cells, 46% (Figure 6a). Following VSV infection for 24 and 48 h, immunoblot analysis demonstrated that VSV replication in activated CD4+ T-lymphocytes was time dependent and directly related to the intensity of cell activation (Figure 6b). Nevertheless, ATL cells showed fourfold higher levels of viral G and N proteins at 24 h after infection compared to activated CD4+ T-cells under similar conditions, indicating that ATL leukemic cells were more permissive to VSV than activated, primary T-lymphocytes. To further evaluate T-cell activation and VSV permissiveness, anti-CD3 and anti-CD28 were used separately to stimulate the CD4+ T-cell population (Figure 6c). Clearly, CD3 stimulation of the CD3 pathway was sufficient to render T cells permissive to VSV replication (Figure 6c, lanes 3 and 4), whereas stimulation of the CD28 pathway had little effect on the capacity of the T-cell population to support VSV replication (Figure 6c, lanes 5–7). As expected, the combination of CD3/CD28 rendered CD4+ T-cells permissiveness for VSV replication, but with minimal additive effect compared to CD3 stimulation alone (Figure 6c, compare lanes 3 and 4 with lanes 9 and 10). In related experiments, no change in the expression of translational components eIF2α, B or Bε (data not shown) was observed, as described previously (Balachandran and Barber, 2004). Thus, a critical component of the ability of VSV to replicate in CD4+ T-lymphocytes is the stimulation of the TCR/CD3 activation pathway.

a


% CD25+ T-cells
50
40
30
20
10
0
-
2.5
5
CD3/CD28
ATL
CD4

b


CD4
ATL
- - 2.5 5 2.5 5 - -
CD3/CD28
- + + + + + - +
VSV
G
N
48
24
48
24
h

c

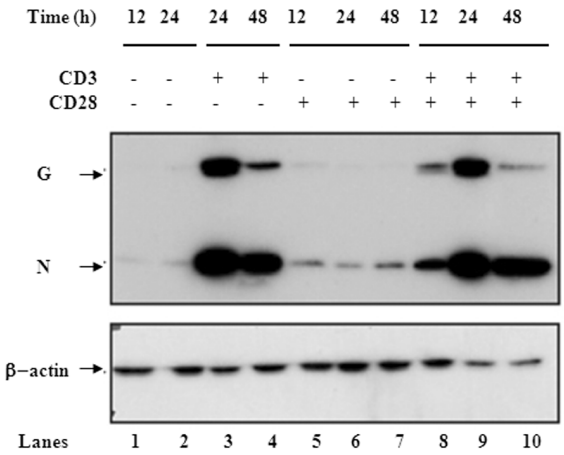

Time (h)
12 24 24 48 12 24 48 12 24 48
CD3
- - + + - - - + + +
CD28
- - - - + + + + + +
G
N
β−actin
Lanes
1 2 3 4 5 6 7 8 9 10

**Figure 6. Enhanced VSV replication in ATL versus activated CD4 T-lymphocytes.** (a) Activation of CD4 T-lymphocytes. Purified isolated CD4 T-lymphocytes were incubated with 2.5 or 5.0 mg/ml of anti-CD3 plus anti-CD28/IL-2 for 24 h and CD25 expression levels were assessed by flow cytometry at 24 h after treatment. (b) Kinetics of VSV replication in CD4 and ATL lymphocytes. Activated CD4 lymphocytes and ATL cells were mock infected or infected (VSV-HR, MOI of 1 PFU/cell), and at the indicated times post-infection, total cell lysates were prepared and VSV G and N protein expression was analyzed in 20 mg of total cell lysates by SDS–PAGE and immunoblot analysis. (c) CD3 activation of CD4+ T-lymphocytes. Purified isolated CD4+ T-lymphocytes were incubated with 5.0 mg/ml of anti-CD3 monoclonal antibody or anti-CD28 monoclonal antibody for 24 h and then infected with VSV at an MOI of 1 PFU/cell. At the indicated times post-infection, total cell lysates were prepared and VSV G and N protein expression was analyzed in 20 mg of total cell lysates by SDS–PAGE and immunoblot analysis with β -actin as a loading control.

## DISCUSSION

Oncolytic viruses provide an attractive potential cancer therapeutic because of their ability to replicate selectively within tumor but not normal cells. Several oncolytic viruses have demonstrated promising results in preclinical and early clinical studies (Kirn *et al*., 2001; Chiocca, 2002; Hawkins *et al*., 2002; Lichty *et al*., 2004), including naturally attenuated replication competent viruses such as reovirus (non-enveloped, double-stranded (ds)RNA), parvoviruses (nonenveloped, single-stranded DNA), Newcastle disease virus, measles virus, and VSV (Gromeier and Wimmer, 2001; Kirn *et al*., 2001; Norman *et al*., 2001; Bell *et al*., 2002; Hawkins *et al*., 2002). The present study provides the first conclusive evidence that VSV can replicate selectively in and cause extensive caspase-dependent oncolysis of primary *ex vivo* ATL cells. Importantly, VSV infection did not induce cell death in naive primary CD4+ T-lymphocytes from healthy volunteers or in PBMCs from patients with HAM/TSP. VSV specifically replicates in tumor cells due to the attenuation of the antiviral IFN response, a phenotype that frequently arises during tumor evolution (Linge *et al*., 1995; Sun *et al*., 1998; Matin *et al*., 2001). The induction of type I IFN-α/β is a potent host defense mechanism against viral infection, and many viruses have evolved strategies to overcome the antiviral effects of this cytokine. However, VSV is extremely sensitive to the antiviral actions of IFN (Belkowski and Sen, 1987); hence, VSV infection in humans is asymptomatic in most cases or results in a mild febrile illness. In contrast, mice harboring a defective IFN system are highly sensitive to normally harmless exposure of VSV (Muller *et al*., 1994; Durbin *et al*., 1996). Stojdl *et al*., (2003) demonstrated recently that 80% of the NCI 60 cell line collection (Weinstein *et al*., 1997) is permissive for VSV infection. Indeed, most of these cells were found to have impaired responses to either IFN-α or IFN-β (Stojdl *et al*., 2003). In the context of the present study, we observed that IFN production in ATL cells is defective compared to primary T cells (Oliere *et al*., unpublished) and experiments are underway to examine the relationship between IFN induction and VSV replication. Subsequent biochemical analysis provided evidence that translation control downstream of PKR activation, frequently deregulated in many transformed cells, can cooperate with

the attenuated IFN antiviral activity to facilitate VSV oncolysis (Balachandran and Barber, 2004). Elevated levels of eIF2Bε are required for increased permissiveness of transformed cells to VSV replication. Cells transfected with siRNA against eIF2B were almost completely protected against VSV-induced cytolysis and produced 10-fold lower yield of virus than control cells (Balachandran and Barber, 2004). In the present study, no evidence of changes in the expression of eIF2B was obtained in resting or activated CD4+ T-lymphocytes, suggesting that alternative mechanisms may be operative in the T-cell context. In this regard, triggering the TCR/CD3 activation pathway was sufficient to render normal CD4+ T-lymphocytes permissive for VSV replication. This preliminary observation suggests that dissection of the CD3 pathway may identify critical downstream regulators of VSV replication and/or susceptibility to oncolysis. Since activation of RAS/RAF signaling is downstream of CD3 activation, the possibility exists that activated RAS may be required for VSV oncolysis, as previously demonstrated for Reovirus-induced oncolysis (reviewed in Norman *et al*., 2001). Interestingly, non-leukemic cells from patients with HAM/TSP were resistant to VSV infection. Since HAM/TSP cells are known to spontaneously proliferate *in vitro* (Cavrois *et al.,* 1996; Mortreux *et al.,* 2003), an important caveat to consider in the interpretation of the present results is that the HTLV-1-infected sub-population (1–5%) within the HAM/TSP PBMC samples may in fact be susceptible to VSV oncolysis. It is well documented that HTLV-1 infection, and specifically the viral Tax oncoprotein, leads to T-cell activation via an IL-2/IL-2 receptor autocrine loop, as well as induction of the NF-κB and CREB pathways (Yoshida, 2001; Franchini *et al.,* 2003); activation of these Tax-mediated pathways may also influence the susceptibility of the HTLV-1-infected subpopulation in HAM/TSP to VSV infection. It will be of interest to determine the differential sensitivities of the HTLV-1-infected and -uninfected compartments in HAM/TSP.

Programmed cell death represents the convergence of multiple apoptotic pathways from numerous distinct initiating events and insults (Eastman and Rigas, 1999; Nguyen and Wells, 2003). While many of the details of direct and indirect mechanisms of VSV-induced apoptosis remain to be determined, the role of viral replication in VSV oncolysis

is also controversial. It has been reported that VSV-induced cell death occurs at early stages after infection, and requires viral particle internalization and uncoating, but not viral replication or *de novo* viral protein synthesis (Gadaleta *et al*., 2002). In contrast, other studies demonstrated that VSV-induced apoptosis correlates with viral protein expression, and that activation of caspase-3-like proteases was required for VSV-induced apoptosis but not viral replication (Hobbs *et al*., 2001, 2003). In addition, VSV matrix M protein has been shown to inhibit host cell gene expression and nuclear export of host mRNA via an association between M protein and Nup98, a host nuclear pore protein, with subsequent induction of apoptosis (Kopecky *et al*., 2001; Desforges *et al*., 2002; Kopecky and Lyles, 2003). The present results in HTLV-1-transformed MT-4 cells and in primary ATL demonstrate that VSV-induced apoptosis correlated with VSV replication, and that caspase-3 activation and subsequent apoptotic events involving Rho-GDI cleavage and DFF45 processing occurred only following VSV replication. Nonetheless, both direct virus-mediated apoptotic mechanisms and indirect mechanisms involving pro-apoptotic cytokine release may be involved in ATL killing by VSV.

Based on the discrepancies between VSV killing of established hematopoietic cells and primary leukemic cells documented in the present study, the oncolytic activity of VSV in immortalized cell lines may not systematically reflect VSV killing in their primary cancer counterparts, thus underscoring the need to assess the sensitivity of primary cancer cells to virotherapy. While CLL cell lines were permissive for viral replication, primary CLL cells were resistant to VSV replication and subsequent cell death. This discrepancy in VSV oncolysis may be due to the fact that CLL cells do not proliferate but remain in $G_0$ *ex vivo* (Meinhardt *et al*., 1999; Caligaris-Cappio, 2003). That VSV oncolysis may be linked to the state of cell growth or activation was addressed in primary T cells by demonstrating that TCR/CD3 activation enhanced permissiveness to VSV replication. It should be noted, however, that even in activated T cells, viral replication was about fourfold higher in ATL cells than in activated CD4 cells, further supporting the idea that VSV replication may be influenced by genetic defects unique to tumor cells, including deficiencies in antiviral IFN-α/β and p53 signaling pathways (Stojdl *et al*., 2000, 2003;

Takaoka *et al*., 2003). Thus, p53 dysfunction, myc overexpression, or p16INK4a inactivation in ATL cells (Yoshida, 2001; Matsuoka, 2003) could increase the susceptibility of ATL cells to VSV and other oncolytic viruses (Balachandran *et al*., 2001; Chiocca, 2002). Although we provide information on the *ex vivo* oncolytic potential of VSV in primary ATL cells, we cannot predict the oncolytic efficiency of VSV *in vivo* before the onset of potentially neutralizing antibody response. Interestingly, naturally occurring VSV variants with a mutated matrix protein unable to block the IFN production in infected cells have been shown to retain oncolytic activity in a variety of *in vivo* models (Stojdl *et al*., 2003). The possibility to enhance the oncolytic activity of VSV using genetic engineering to insert immunomodulatory or suicide cassettes has also been demonstrated both *in vitro* and *in vivo* models (Fernandez *et al.,* 2002). These findings further highlight the potential of VSV in cancer virotherapy and emphasize the importance of future investigations in primary *ex vivo* tumor cells.

## ACKNOWLEDGMENTS

We thank the members of the Molecular Oncology Group, Lady Davis Institute, and McGill University for helpful discussions. We also thank Lama Fawaz for helpful discussions. This research was supported by grants from the National Cancer Institute with funds from the Canadian Cancer Society to JH and JB, the Canadian Institutes of Health Research and CANVAC, and the Canadian Network for Vaccines and Immunotherapeutics (to JH). ESA is supported by a Fellowship from the FRSQ and JH is a recipient of a CIHR Senior Investigator award.

## REFERENCES


Balachandran S, Barber GN. (2000). *IUBMB Life* **50**: 135–138.

Balachandran S, Barber GN. (2004). *Cancer Cell* **5**: 51–65.

Balachandran S, Porosnicu M, Barber GN. (2001). *J Virol* **75**:3474–3479.

Bazarbachi A, Ghez D, Lepelletier Y, Nasr R, De The H, El Sabban ME *et al.* (2004). *Lancet Oncol* **5**: 664–672.

Belkowski LS, Sen GC. (1987*). J Virol* **61**: 653–660.

Bell JC, Garson KA, Lichty BD, Stojdl DF. (2002). *Curr Gene Ther* **2**: 243–254.

Caligaris-Cappio F. (2003). *Br J Haematol* **123**: 380–388.

Cavrois M, Gessain A, Wain-Hobson S, Wattel E. (1996). *Oncogene **12***: 2419–2423.

Chiocca EA. (2002). *Nat Rev Cancer* **2**: 938–950.

Desforges M, Despars G, Berard S, Gosselin M, McKenzie MO, Lyles DS *et al.* (2002). *Virology* **295**: 63–73.

Durbin JE, Hackenmiller R, Simon MC, Levy DE. (1996). *Cell* **84**: 443–450.

Eastman A, Rigas JR. (1999). Semin Oncol 26: 7–16.

Ebert O, Shinozaki K, Huang TG, Savontaus MJ, Garcia-Sastre A, Woo SL. (2003). *Cancer Res* **63***: 3605–3611.*

Fernandez M, Porosnicu M, Markovic D, Barber GN. (2002). *J Virol* **76**: 895–904.

Franchini G, Nicot C, Johnson JM. (2003). *Adv Cancer Res* **89**: 69–132.

Gadaleta P, Vacotto M, Coulombie F. (2002). *Virus Res* **86**:87–92.

Gessain A, Barin F, Vernant JC, Gout O, Maurs L, Calender A *et al.* (1985). *Lancet* **2**: 407–410.

Grigera PR, Garcia-Briones M, Periolo O, la Torre JL, Wagner RR. (1996). *J Virol* **70**: 8492–8501.

Gromeier M, Wimmer E. (2001). *Curr Opin Mol Ther* **3**: 503–508.

Hawkins LK, Lemoine NR, Kirn D. (2002). *Lancet Oncol* **3**: 17–26.

Hinuma Y, Komoda H, Chosa T, Kondo T, Kohakura M, Takenaka T *et al.*. (1982). *Int J Cancer* **29**: 631–635.

Hobbs JA, Hommel-Berrey G, Brahmi Z. (2003). *Hum Immunol* **64**: 82–92.

Hobbs JA, Schloemer RH, Hommel-Berrey G, Brahmi Z. (2001). *Virus Res* **80**: 53–65.

Huang TG, Ebert O, Shinozaki K, Garcia-Sastre A, Woo SL. (2003). *Mol Ther* **8**: 434–440.

Kirn D, Martuza RL, Zwiebel J. (2001). *Nat Med* **7**: 781–787.

Kopecky SA, Lyles DS. (2003). *J Virol* **77**: 5524–5528.

Kopecky SA, Willingham MC, Lyles DS. (2001). *J Virol* **75**: 12169–12181.

Lichty BD, Stojdl DF, Taylor RA, Miller L, Frenkel I, Atkins H *et al.* (2004). *Hum Gene Ther* **15**: 821–831.

Linge C, Gewert D, Rossmann C, Bishop JA, Crowe JS. (1995). *Cancer Res* **55**: 4099–4104.

Maruyama M, Shibuya H, Harada H, Hatakeyama M, Seiki M, Fujita T *et al.* (1987). *Cell* **48**: 343–350.

Matin SF, Rackley RR, Sadhukhan PC, Kim MS, Novick AC, Bandyopadhyay SK. (2001). *Cancer Res* **61**: 2261–2266.

Matsuoka M. (2003). *Oncogene* **22**: 5131–5140.

Meinhardt G, Wendtner CM, Hallek M. (1999). *J Mol Med* **77**: 282–293.

Mortreux F, Gabet AS, Wattel E. (2003). *Leukemia* **17**: 26–38.

Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM *et al.* (1994). *Science* **264**: 1918–1921.

Na S, Chuang TH, Cunningham A, Turi TG, Hanke JH, Bokoch GM *et al.* (1996). *J Biol Chem* **271**: 11209–11213.

Nguyen JT, Wells JA. (2003). *Proc Natl Acad Sci USA* **100**: 7533–7538.

Norman KL, Farassati F, Lee PW. (2001). *Cytokine Growth Factor Rev* **12**: 271–282.

Obuchi M, Fernandez M, Barber GN. (2003). *J Virol* **77**: 8843–8856.

Osame M, Usuku K, Izumo S, Ijichi N, Amitani H, Igata A *et al.* (1986). *Lancet* **1**: 1031–1032.

Satoh M, Toma H, Sugahara K, Etoh K, Shiroma Y, Kiyuna S *et al.* (2002). *Oncogene* **21**: 2466–2475.

Sharif-Askari E, Alam A, Rheaume E, Beresford PJ, Scotto C, Sharma K *et al.* (2001). *EMBO J* **20**: 3101–3113.

Shimoyama M. (1991). *Br J Haematol* **79**: 428–437.

Steele TA. (2000). *Proc Soc Exp Biol Med* **223**: 118–127.

Stojdl DF, Lichty B, Knowles S, Marius R, Atkins H, Sonenberg N *et al.* (2000). *Nat Med* **6**: 821–825.

Stojdl DF, Lichty BD, tenOever BR, Paterson JM, Power AT, Knowles S *et al.* (2003). *Cancer Cell* **4**: 263–275.

Sun WH, Pabon C, Alsayed Y, Huang PP, Jandeska S, Uddin S *et al.* (1998). *Blood* **91**: 570–576.

Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H, Kikuchi H *et al.* (2003). *Nature* **424**: 516–523.

Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. (1977). *Blood* **50**: 481–492.

Weinstein JN, Myers TG, O'Connor PM, Friend SH, Fornace Jr AJ, Kohn KW *et al.* (1997). *Science* **275**: 343–349.

Wolf BB, Green DR. (1999). *J Biol Chem* **274**: 20049–20052.

Wolf BB, Schuler M, Echeverri F, Green DR. (1999). *J Biol Chem* **274**: 30651–30656.

Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D *et al.* (1998). *Genes Dev* **12**: 806–819.

Wu JT, Byrne HM, Kirn DH, Wein LM. (2001). *Bull Math Biol* **63**: 731–768.

Yasunaga J, Matsuoka M. (2003). *Int J Hematol* **78**: 312–320.

Yoshida M. (2001). *Annu Rev Immunol* **19**: 475–496.

## Preamble for Manuscript III

Oncolytic viruses constitute a promising novel anti-cancer therapeutic approach, as recent studies have demonstrated that several non-pathogenic viruses are able to exploit tumour-specific genetic defects to selectively replicate in and kill cancer cells, while sparing normal cells. Previously, we investigated the ability of VSV to lyse primary T lymphocytes from patients with Adult T-cell leukemia (ATL) and Chronic Lymphocytic Leukemia (CLL). ATL is an aggressive malignancy of activated CD4+ T-lymphocytes caused by HTLV-1. CLL is characterized by the accumulation of mature B or T-cells, most of which are non-proliferating. We demonstrated that primary ATL cells are permissive to VSV infection and undergo rapid oncolysis *ex vivo* whereas CLL cells are resistant to VSV. As activation of the IL-2/IL-2R loop during the acute phase of HTLV-1 contributes to the activation and proliferation of HTLV-1-infected CD4+/CD25+ subset of ATL cells, we examined the effect of T-cell activation on VSV permissiveness and cytolytic activity. Interestingly, activation of primary CD4+ T-lymphocytes with anti-CD3 and CD28 monoclonal antibodies was sufficient to induce VSV replication and killing. Altogether, these results suggest that cellular signaling pathways that promote VSV replication are engaged during T-cell activation. Accordingly, the purpose of this chapter is to identify the mechanisms governing the permissiveness to VSV replication in primary leukemic and normal activated T cells.

# - MANUSCRIPT III -

## VSV oncolysis of T lymphocytes requires cell cycle entry and translation initiation

*Published in: J Virol. 2008 Jun;82(12):5735-49.*

**Stephanie Oliere [1,2], Meztli Arguello [1,2], Thibault Mesplede [1,2], Vanessa Tumilasci [1,2], Peyman Nakhaei [1,2], David Stojdl [3], Nahum Sonenberg [4], John Bell [5] and John Hiscott [1, 2, *].**

[1]Molecular Oncology Group, Lady Davis Institute - Jewish General Hospital and Depts. of [2]Microbiology and Medicine, McGill University, Montreal Canada H3T 1E2. [3]Apoptosis Research Centre, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada, KIH 8L1. [4]Department of Biochemistry and McGill Cancer Centre, McGill University, Montreal, Quebec, Canada H3G 1Y6. [5]Ottawa Regional Cancer Centre, University of Ottawa, Ottawa Canada, K1Y 4E9.

[*] **Corresponding author:**

**Dr. John Hiscott, Lady Davis Institute for Medical Research, 3755 Cote Ste. Catherine, Montreal, Quebec, Canada H3T 1E2. Phone: (514) 340-8222, ext. 5265. Fax: (514) 340-7576. E-mail address: john.hiscott@mcgill.ca.**

**ABSTRACT**

Vesicular stomatitis virus (VSV) is a candidate oncolytic virus that replicates and induces cell death in cancer cells while sparing normal cells. Although defects in the interferon (IFN) antiviral response facilitate VSV oncolysis, other host factors including translational and growth regulatory mechanisms also appear to influence oncolytic virus activity. We previously demonstrated that VSV infection induces apoptosis in proliferating CD4+ T-lymphocytes from adult T-cell leukemia samples but not in resting T lymphocytes or primary chronic lymphocytic leukemia cells that remain arrested in $G_0$. Activation of primary CD4+ T-lymphocytes with anti-CD3/CD28 is sufficient to induce VSV replication and cell death in a manner dependent on activation of MEK1/2, JNK, or PI3K pathways, but not p38. VSV replication is specifically impaired by the cell cycle inhibitors oloumicine or rapamycin, which induce early $G_1$ arrest, but not by aphidicolin or taxol, which block at $G_1$-S and $G_2$-M phase respectively; this result suggests a requirement for cell cycle entry for efficient VSV replication. The relationship between increased protein translation following $G_0$/G1 transition and VSV permissiveness is highlighted by the absence of mTOR and/or eIF4E phosphorylation whenever VSV replication is impaired. Furthermore, VSV protein production in activated T cells is diminished by siRNA-mediated eIF4E knockdown. These results demonstrate that VSV replication in primary T lymphocytes relies on cell cycle transition from the $G_0$ to $G_1$ phase – which is characterized by a sharp increase in ribogenesis and protein synthesis.

## INTRODUCTION

Oncolytic viruses constitute a promising novel cancer therapeutic approach (review in (9, 10, 47)). Vesicular stomatitis virus (VSV), a RNA virus belonging to the Rhabdoviridae family, possesses intrinsic oncolytic properties that permit cancer cell destruction, while sparing normal cells (8, 53). VSV is exquisitely sensitive to the antiviral effects of the interferon (IFN) pathway and therefore fails to replicate efficiently in primary cells that contain a functional IFN system (6, 73, 74). However, VSV replicates to high titers in transformed cells in which aspects of IFN signaling or downstream effectors including translational control are compromised (4, 21, 27, 72). The oncolytic capacity of VSV has been established *in vitro* and *in vivo*; VSV infection selectively killed a large panel of human tumor cell lines including 80% of the NCI 60 tumor cell bank, cleared bone marrow of leukemic AML cells and effectively arrested metastatic spread of CT26 lung metastases in immunocompetent animals (5, 25, 27, 48). However, 20% of tumor cells tested were partially or completely refractory to VSV oncolysis, suggesting that in the clinical setting many primary cancers may not respond to VSV treatment. For example, although VSV efficiently induced oncolysis of Chronic Lymphocytic Leukemia (CLL) cell lines, primary *ex-vivo* CLL samples were not permissive to VSV replication (17). To date, few studies have addressed the issue of VSV resistance from a mechanistic perspective. While defects in the host antiviral response provide one explanation for VSV-mediated oncolysis, additional regulatory alterations in tumors also facilitate VSV oncolysis; for example, defective control of mRNA translation initiation plays an important role in cell permissiveness to VSV (4, 6, 7, 21, 24).

Ligation of the T cell receptor (TCR) and CD28 in a naive T lymphocyte rapidly leads to activation of distinct but interactive signaling cascades (review in (52, 79)). The Ras pathway activates the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun $NH_2$-terminal kinases (JNK), and p38, whereas the calcium pathway activates phosphatidyl inositol-3 kinase (PI3K)-leading to Akt phosphorylation (18, 68). Such signals culminate in the activation of the transcription

factor families NF-AT, AP-1, and NF-κB, leading to the upregulation of genes involved in protein translation and cell cycle progression.

To exit quiescence, D-type cyclins (CycD) are synthesized *de novo* (reviewed in (69)). CycD-Cdk4/6 complexes accumulate in early G1 phase and promote cell division by phosphorylating Rb protein and sequestering cdk inhibitory proteins (CKIs: Cip/Kip family). The cdk inhibitor $p27^{Kip1}$ is an important regulator of T cell cycle progression: high levels of $p27^{kip1}$ protein are present in resting T cells, preventing $G_1$ to S phase transition by inhibiting the cyclin E/cdk2 complex (41). The activity of $p27^{Kip1}$ is regulated at two levels, acting in early $G_1$ and in $G_1$/S transition: $p27^{Kip1}$ is sequestered by CycD/cdk complexes, and free $p27^{Kip1}$ is degraded *via* the proteasome pathway by Cyclin E/cdk2 dependent and independent mechanisms that require MEK and PI3K activation (reviewed in (20, 32, 50, 62). Once free from $p27^{Kip1}$, newly synthesized Cyclin E and Cyclin A along with cdk2 orchestrate the $G_1$/S phase transition. The mitogen stimulated kinase Akt controls the stability of cyclin E as well as the subcellular localization of Cip/Kip proteins (46, 70). It is now evident that activation of the MAPK and PI3K cascades following TCR/CD28 stimulation regulates the cell cycle by acting at both at the transcriptional and post-translational levels on CKI and cyclin molecules (2, 14).

Cell cycle progression is tightly linked to protein synthesis and ribogenesis, as optimal cell size is required to enter and successfully complete the cell division process. Mammalian target of Rapamycin (mTOR) is a central coordinator of protein synthesis and cell cycle progression (34, 61), although the mechanisms by which mTOR mediates these events are not fully understood. It has been proposed that mTOR controls protein translation through two distinct mechanisms that in turn are crucial for cell cycle progression (28, 29). One branch is regulated by phosphorylation of eIF4E binding protein (4E-BP) by mTOR, leading to its dissociation from eIF4E (37, 63). Free eIF4E, can then be phosphorylated by MAP kinase-interacting protein 1 (Mnk1), resulting in activation of eIF4E and enhanced ribosome recruitment to the translational start site. In T cells, ERK is an upstream regulator of Mnk1 and therefore directly involved in the

activation of eIF4E throughout the cell cycle. The second branch relies on phosphorylation of the ribosomal S6 kinase 1 (S6K1) by mTOR: translation initiation, elongation and/or ribosome biogenesis is stimulated by yet poorly defined mechanisms involving S6K1-mediated phosphorylation of the 40S ribosomal protein S6 and the translation initiation factor 3 (eIF3) (3, 65). The relationship between protein translation and cell cycle control is highlighted by the fact that rapamycin, the best characterized mTOR inhibitor, causes cell cycle arrest in early G1 and reportedly inactivates cdk2 kinase by favouring formation of the cyclin E/cdk2-p27 complex (15, 35).

We previously demonstrated that *ex vivo* primary Adult T-cell leukemia (ATL) cells - rapidly proliferating CD4+/CD25+ T-lymphocytes - are permissive to VSV infection and undergo rapid oncolysis, whereas neither viral replication nor oncolysis were observed in resting T cells or in *ex vivo* cells from patients with chronic lymphocytic leukemia (CLL), corresponding to B-lymphocytes arrested in the $G_0$ phase (17, 23, 42). In this report, we demonstrate that T-cell activation with anti-CD3 and anti-CD28 antibodies renders primary CD4+ T-lymphocytes permissive to VSV replication with concomitant induction of apoptosis. Similarly, activation of primary CLL with PMA/ionomycin to exit from $G_0$ and enter the cell cycle renders them susceptible to VSV infection and oncolysis. As demonstrated in CD4+ T cells, activation of ERK, JNK or AKT pathways, leading to $G_0$ to $G_1$ phase transition, is crucial for VSV replication in primary lymphocytes, due to a global increase in protein translation mediated by the activation of mTOR and eIF4E.

## MATERIALS AND METHODS

### CD4+ T and B-CLL cell isolation.

PBMCs from CLL patients were obtained at the Jewish General Hospital, Montreal, Quebec following informed consent, in agreement with the Jewish General Hospital and McGill University Research Ethics Committee (REC). Leukophoresis from healthy donors were obtained at the Royal Victoria Hospital, Montreal, Quebec following informed consent, in agreement with the Royal Victoria Hospital, the Jewish General Hospital and McGill University Research Ethics Committee (REC). PBMCs were isolated by centrifugation (400g at 20°C for 25 min) of blood on a Ficoll-Hypaque gradient (GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada). B cells or CD4+ T-lymphocytes were isolated using the CD4 or CD19 enrichment cocktail by negative selection with the high-speed autoMACS system (kit # 130-050-301 and 130-091-894, respectively, Miltenyi Biotec) according to the manufacturer's instructions. In all cases, the purity of B cells or CD4+ T-lymphocytes was between 90 and 95% as determined by flow cytometry. Cells were cultured in RPMI 1640 medium (Wisent INC, San Diego, CA, USA) supplemented with 15% heat-inactivated FBS, 100 U/ml penicillin–streptomycin.

### Flow cytometry for surface markers.

After washing twice with PBS, cells were stained with monoclonal allophycocyanin-labeled anti-CD4 (T-cell marker), phycoerytrin (PE)-labeled anti-CD19 (B-cell marker), FITC-labeled anti-CD5 (B-cell marker), PE-labeled anti-CD25 or PE-labeled anti-CD69 (T-cell activation markers), for 30 min in PBS/1% FCS. After a final wash with ice-cold PBS, cells were resuspended in 400µl FACS® buffer (PBS/Cytofix (BD Pharmigen)). Flow cytometric analyses ($1x10^4$ cells/measurement) were performed by FACScalibur™ flow cytometer with CELLQuest™ software (Becton Dickinson). All antibodies were purchased from BD Biosciences (Mississauga, ON, Canada).

**CD4+ T cell and B-CLL activation.**

Freshly isolated CD4+ T-lymphocytes ($10^6$/ml) were mock activated or activated for 12-48h with 5 µg/ml of immobilized anti-CD3 monoclonal antibody, and 1 µg/ml of immobilized anti-CD28 monoclonal antibody (BD Biosciences - Mississauga, ON, Canada). Resting T cells were mock treated or treated with pharmacological inhibitors 1h prior to their activation, as indicated. The inhibitors used in this study were LY294002 (PI3K inhibitor, 20 µM), SB203580 (p38 MAPK inhibitor, 12 µM), SP600125 (JNK inhibitor, 20 µM), UO126 (MEK1/2 inhibitor, 10µM), Rapamycin (25 nM), Oloumicine (100 µM), Taxol (200 nM), Aphidicolin (3 µg/ml), Nocodazole (1 µg/ml). All purchased from Calbiochem (San Diego, CA, USA) except UO126, purchased from Cell Signaling Technologies (Danvers, MA, USA). B cells were stimulated with PMA (25 ng/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ionomycin (1 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h.

**Cell cycle analysis by flow cytometry.**

Enriched human primary T cells untreated or treated for 1h with kinase inhibitors were activated for 48 h with anti-CD3/CD28. Cells were washed with cold PBS/5 mM EDTA, and fixed ethanol (70% in PBS) overnight at -20°C. Fixed cells were washed and incubated in PBS containing 2.5 µg/ml propidium iodide (PI; sigma-Aldrich) and 50 µg/ml Rnase A for 30 min at 37°C. Samples were subjected to FACS (FACScalibur™) analysis using CELLQuest™ software (Becton Dickinson) to determine percentage of cells in $G_0/G_1$, S and $G_2$/M phases of the cell cycle based on DNA content.

**VSV infection of primary CD4+ T lymphocytes and CLL samples.**

Wild-type VSV (VSV-HR, Indiana serotype) and VSV-AV1 were propagated in Vero cells as described previously in (74). Viruses were obtained from cell-free supernatants and titrated on Vero cells by standard plaque assay. T or B cells were mock infected or infected with VSV at 1 MOI for 12-48 h. Virus infection was performed in RPMI in the absence of serum for 1 h, after which it was replaced with fully supplemented growth

medium. Cells were incubated at 37°C for the specified times, washed twice in PBS and stored at –80°C for further use. Viral quantification in supernatants was performed in duplicate with log dilutions on 100% confluent Vero cells plated in six-well dishes.

**Plaque assay.**

Confluent monolayers of Vero cells in 6-well plates were infected with 0.1 ml of serially diluted samples; after 1 hour of infection, at 37 °C, medium was removed and replaced with complete medium containing 0.5% methyl cellulose (Sigma Aldrich – Oakville, ON, Canada) for 48h. Vero cells were fixed in 4% formaldehyde and stained with crystal violet. Plaques were counted and titers were calculated as PFU per milliliter.

**siRNAs and primary human CD4+-T lymphocyte transfection.**

Control and eIF4E - specific RNAi sequences were described previously (75). Transfection of purified activated T cells was carried out by electroporation using the Nucleofection® system (Amaxa, Köln, Germany), according to the protocols proposed by the furnisher. Briefly, CD4+ T cells were activated with an anti-CD3/28 monoclonal antibodies combination for 48h. $10 \times 10^6$ T cells were resuspended in 100 µl of T cell nucleofector solution (Human T Cell Nucleofector kit) containing 300 pmol of double-stranded siRNAs. After electroporation, 400 µl of prewarmed cultured medium were added to the cuvette, and the cells were transferred into cultures plates containing prewarmed culture medium. At the optimal time of gene silencing (usually 48h post-transfection), T cells were mock-infected or infected with VSV 1 MOI for 24h.

**Immunoblot analysis.**

Cells destined for immunoblotting were washed with PBS and lysed in lysis buffer (0.05% NP40, 1% glycerol, 30 mM NaF, 40 mM β-glycerophosphate, 10mM $Na_3VO_4$, 10 ng/ml of protease inhibitors cocktail (Sigma Aldrich – Oakville, ON, Canada). Protein concentration was determined by Bradford assay (BioRad). Whole cell extracts (30 µg) were resolved by SDS-PAGE in a 10% acrylamide gel and transferred to a nitrocellulose membrane (Hybond C Super; GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada).

Membranes were blocked in 5% nonfat dried milk in Tris-buffered saline (TBS) plus 0.1% Tween-20 (TBST) for 1h at room temperature. Membranes were then probed overnight with antibodies against VSV (1:5000; a gift from John Bell, Ottawa Cancer Centre), cleaved-caspase 3, cyclin D3, cyclin A, Phospho-Akt, phospho-p38 MAPK, phospho-JNK, phospho-eIF4E (Ser 209), eIF4E, mTOR, p27*kip1,* β-actin (1:1000; Cell Signaling - Danvers, MA, USA), CDK4, phospho-ERK1/2 and ERK2 (1µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Rb (1:5000; BD PharMingen) in 5% BSA and PBS at 4°C. Incubation mixtures were washed in Tris buffered saline (TBS)/0.05% Tween-20 five times for a total of 25 min. Following washes, the membrane was incubated with peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Amersham) at a dilution of 1:5,000 for 1h at room temperature. Following the incubation with the secondary antibody, membranes were washed again for 5 times 5 min and then visualized with the enhanced chemiluminescence (ECL) detection system as recommended by the manufacturer (ECL, GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada).

**RNA extraction and semi-quantitative RT-PCR.**

Whole RNA from treated cells was extracted using RNase extraction Kit (Qiagen – Mississauga, ON, Canada) according to manufacturer's instructions. RT-PCR was performed using 1 µg of RNA resuspend in RNase-free ddH2O and Oligo $dT_{12-18}$ primer (Invitrogen Canada Inc., Burlington, ON, Canada) according to manufacturer's conditions. Reverse transcription was performed using Superscript II (Invitrogen Canada Inc., Burlington, ON, Canada) at 42°C for 1h. Following the reverse transcription reactions, cDNA samples were brought to 100-µl final volumes of which 5 µl was used as template for each PCR reaction with Taq polymerase (Invitrogen Canada Inc., Burlington, ON, Canada). The primer sequences used in this study for PCR were: L protein, Forward 5'-AAGTTATCAAACGGCCCAGTG-3' and Reverse 5'-ACA AAC TCG TTG GGA GGT TG-3'; M protein, Forward 5'-GCG AAG GCA GGG CTT ATT TG3' and Reverse 5'-CTT TTT CTC GAC AAT CAG GCC-3'. PCR fragments were amplified at an annealing temperature of 55°C for 35 cycles. Products were run on a 2% agarose gel and

revealed through use of a Typhoon 9400 phosphoimager (GE Healthcare Bio-Sciences Inc - Oakville, ON, Canada).

**Statistical analysis.** The data are presented as the mean ± standard error of the mean. Statistical significance for comparison was assessed by an unpaired Student's *t* test. Statistical significance was evaluated using the following p values: $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

## RESULTS

### Activation renders CD4+ T-lymphocytes susceptible to VSV replication.

VSV replicates and induces cell death in primary CD4+ T-lymphocytes from adult T-cell leukemia (ATL) samples (17). To determine whether VSV susceptibility requires T cell activation, VSV replication was monitored in resting and CD3/CD28 activated CD4+ T-lymphocytes. Immunoblot analysis using VSV antiserum revealed that viral protein synthesis was not detectable in resting CD4+ T-lymphocytes, whereas production of VSV proteins - L, G, N, P, M - was detected at 24h post-infection in T lymphocytes pre-incubated for 12h with anti-CD3/CD28 (Fig. 1A). Apoptosis occurred concomittantly with viral replication as indicated by cleavage of caspase 3 (Fig. 1A). Quantification of virus titers by plaque assay revealed that T cell activation led to a 4-log increase in virus titer at 24h post-infection, compared to resting T cells (Fig. 1B). To determine if VSV replication was directly proportional to the level of CD4+ T-cell activation, lymphocytes were stimulated with CD3/CD28 for 12, 24, 48h and subsequently infected with VSV for 12 and 24h (Fig. 1C). VSV protein production was detected as early as 12h post-activation and increased as activation time was prolonged (Fig. 1C, lanes 4-9). In cells activated with CD3/CD28 for 48h, VSV G protein levels were ~ 3.6 fold higher than in cells activated for 12h (Fig. 1C, compare lanes 5 and 9). In agreement with viral protein levels, cells activated for 48h produced ~2 fold more virus than non-stimulated cells at 24h (Fig. 1D). Furthermore, VSV replication in activated primary CD4+ T-lymphocytes

led to induction of apoptosis, as demonstrated by immunoblot for cleaved caspase 3 (Fig. 1C, lanes 6-9).

Since VSV is exquisitely sensitive to interferon (IFN) mediated inhibition and therefore fails to replicate efficiently in primary cells that contain a functional IFN system, we tested the possibility that anti-CD3/CD28 treatment interfered with type 1 (IFN$\alpha/\beta$) production PCR analysis of type I IFN mRNA demonstrated that treatment of CD4+ T-lymphocytes with anti-CD3/CD28 did not inhibit the IFN response, thus ruling out this possibility as a cause for the observed increase in viral replication (Supplementary Fig. 1). These results indicate that triggering the CD3/CD28 activation pathways is sufficient to render normal CD4+ T-lymphocytes permissive to VSV replication, suggesting that downstream effectors of CD3/CD28 are critical for VSV replication and induction of apoptosis.

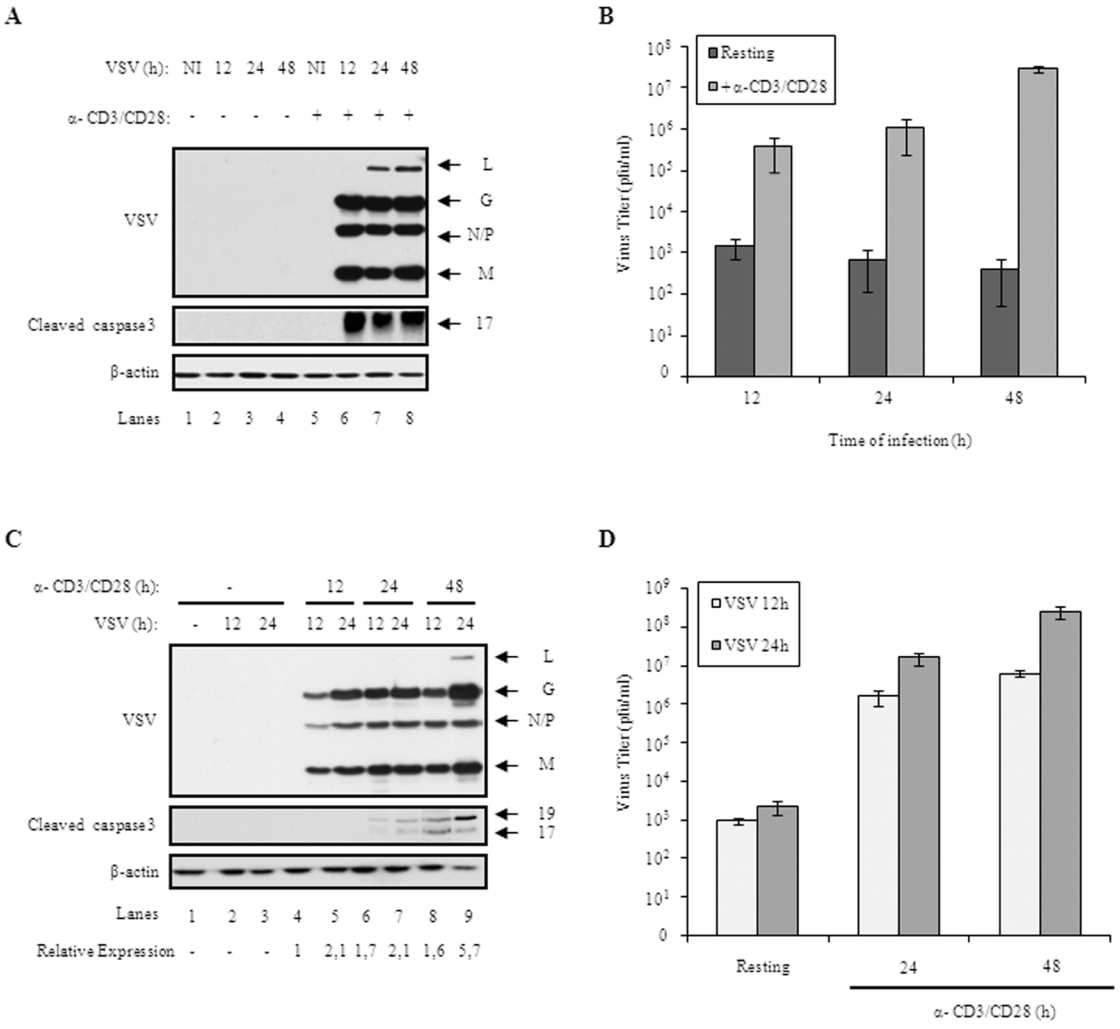

A
VSV (h): NI 12 24 48 NI 12 24 48
α- CD3/CD28: - - - - + + + +
VSV
L
G
N/P
M
Cleaved caspase3
17
β-actin
Lanes 1 2 3 4 5 6 7 8
B
Resting
+α-CD3/CD28
Virus Titer (pfu/ml)
Time of infection (h)
C
α- CD3/CD28 (h): - 12 24 48
VSV (h): - 12 24 12 24 12 24 12 24
VSV
L
G
N/P
M
Cleaved caspase3
19
17
β-actin
Lanes 1 2 3 4 5 6 7 8 9
Relative Expression - - - 1 2,1 1,7 2,1 1,6 5,7
D
VSV 12h
VSV 24h
Virus Titer (pfu/ml)
Resting
24
48
α- CD3/CD28 (h)

**Figure 1. VSV replicates and induces apoptosis in activated CD4+ T-lymphocytes.** (A) Kinetics of VSV replication and caspase 3 cleavage in resting and activated CD4+ T-lymphocytes. Resting CD4+ T-lymphocytes were activated with anti-CD3/CD28 for 24h. Activated and resting CD4+ T-lymphocytes were mock infected or infected with VSV (1 MOI). At the indicated times postinfection VSV replication and caspase 3 cleavage were analysed in 20 μg of total cell lysates by SDS-PAGE followed by immunoblot with anti-cleaved caspase-3, anti-VSV and anti-β-actin as a loading control. (B) Infectious VSV was measured at 12, 24 and 48h postinfection by plaque assay in Vero cells. Virus titers were performed in triplicate; each bar indicates mean +/- standard deviation. (C) VSV protein levels are proportional to T-cell activation time. Purified primary T-lymphocytes were incubated with anti-CD3/CD28 mAbs for 12 to 48h, then infected with VSV (1 MOI). At the indicated times post-infection, VSV replication and caspase 3 cleavage were assessed in 20 μg of whole-cell lysates by immunoblot analysis. VSV N/P protein expression level was quantified and normalized to β-actin level using the Scion Image 4.0. software. (D) Supernatants of cells activated for 24 and 48h were collected 12, 24h postinfection and virus titers determined by standard plaque assay. Assays were performed in triplicate; each bar indicates mean +/- standard error of the mean.

**VSV requires ERK, JNK and AKT but not p38 activation for replication in T lymphocytes.**

Since MAP kinases and PI3 kinase signaling pathways are downstream of CD3/CD28, we postulated that activation of these pathways is required for VSV replication and oncolysis. CD4+ T-cell stimulation with CD3/CD28 antibodies resulted in enhanced ERK, JNK, Akt and p38 phosphorylation as determined by immunoblot with phosphospecific antibodies, while total protein levels remained unchanged (Fig. 2A lanes 1-6; 2B lanes 1-7; 2C lanes 1-6 and 2D lanes 1-5). To examine the essential signaling pathways downstream of CD3/CD28 required for VSV replication in T cells, small molecule inhibitors specific for PI3K (LY294002), MEK1/2 (U0126), SAPK/JNK (SP600125) or p38 (SB203580) were used to block distinct pathways. The concentrations for each of these inhibitors (20 μM, 10 μM, 20 μM, 12 μM, respectively) were chosen based on values reported to be specific and sufficient to inhibit each kinase (2, 26, 55, 76). CD4+ T-lymphocytes were pretreated for 1h with each individual inhibitor, activated with the combination of anti-CD3/CD28 monoclonal antibodies for 12h and subsequently infected with VSV for 12 and 24h. Effectiveness of the drug treatment was confirmed by the dissapearance of phosphorylated substrates by immunoblot (Fig. 2A-D). Thus, U0126 inhibited phosphorylation of ERK by MEK1/2 (Fig. 2A, compare lanes 1-6 to lanes 7-11), SP600125 inhibited phosphorylation of JNK, (Fig. 2B, compare lanes 1-7 to lanes 8-14), LY294002 inhibited phosphorylation of Akt by PI3K (Fig. 2C, compare lanes 1-6 to lanes 7-11) and SB203580 inhibited phosphorylation of p38 (Fig. 2D, compare lanes 1-5 to lanes 6-9). VSV replication was monitored by viral protein immunoblotting (Fig. 2E). Specific inhibition of MEK1/2, JNK or PI3K pathways impaired VSV replication, whereas VSV protein synthesis was not blocked by SB203580 at concentrations that effectively inhibited p38 phosphorylation. Interestingly, MEK1/2, PI3K and JNK inhibitors had no effect on VSV replication when added subsequent to CD4+ T-cell activation (data not shown). To confirm the suppressive effect of MEK1/2, JNK and PI3K inhibitors on VSV replication, virus titers were assessed by plaque assay (Fig. 2F); CD4+

T-lymphocyte activation led to a 4-log increase in virus titer compared to resting T cells, while inhibition of MEK1/2, JNK or PI3K signaling effectively prevented VSV virion production ($p < 0.001$). In agreement with the VSV protein synthesis results, inhibition of the p38 pathway had no effect on viral titer in activated CD4+ T-lymphocytes. These results demonstrate that VSV replication in primary T-lymphocytes depends on the activation of the ERK, JNK and PI3K signaling pathways.

Previous studies have demonstrated that VSV can infect both permissive and nonpermissive cells to produce viral mRNA, yet VSV protein synthesis occurs only in permissive cells (64), suggesting that cell permissiveness depends on post-transcriptional regulation of viral protein synthesis. To determine if VSV permissiveness in primary T-cells is governed by a similar mechanism, the level of individual viral mRNAs in CD4+ T cells following VSV infection was investigated (Fig. 2G). Total RNA was extracted from resting or activated CD4+ T cells challenged with VSV for 24h. RT-PCR analysis of VSV mRNAs demonstrated that both resting and activated CD4+ T-lymphocytes supported VSV M and L mRNA production (Fig. 2G, lanes 2 and 4). The level of viral mRNA production remained unchanged in activated CD4+ T-lymphocytes pretreated with MEK1/2, SAPK/JNK, PI3K and p38 inhibitors (Fig. 2G, compare lanes 2 and 4 to lanes 5-8). Thus, the blockade in VSV replication imposed by MEK1/2, JNK or PI3K pharmacological inhibitors occurs at the level of viral protein synthesis and not at the level of virus entry or mRNA generation.

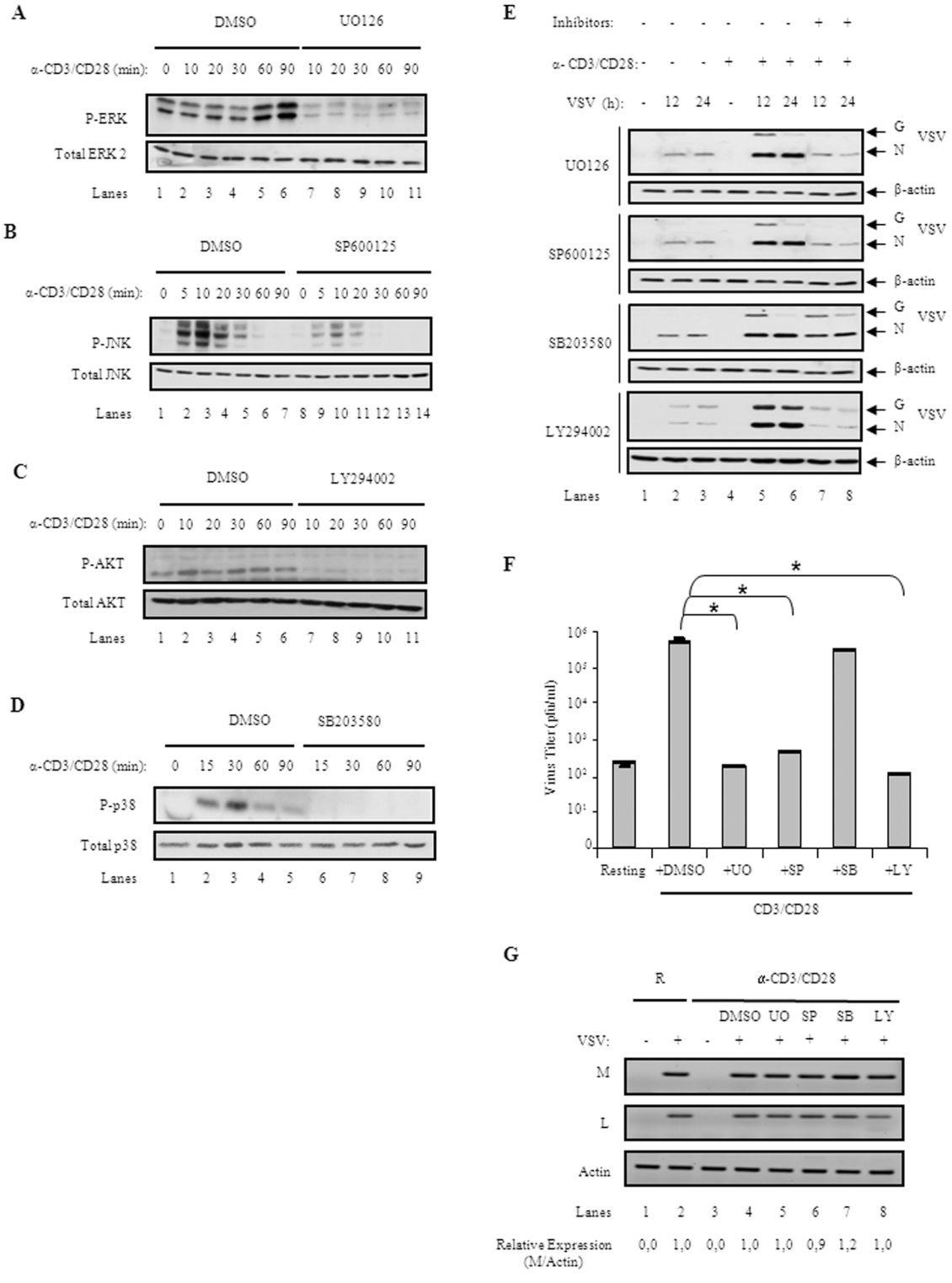

A
DMSO
UO126
α-CD3/CD28 (min): 0 10 20 30 60 90 10 20 30 60 90
P-ERK
Total ERK 2
Lanes 1 2 3 4 5 6 7 8 9 10 11
B
DMSO
SP600125
α-CD3/CD28 (min): 0 5 10 20 30 60 90 0 5 10 20 30 60 90
P-JNK
Total JNK
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14
C
DMSO
LY294002
α-CD3/CD28 (min): 0 10 20 30 60 90 10 20 30 60 90
P-AKT
Total AKT
Lanes 1 2 3 4 5 6 7 8 9 10 11
D
DMSO
SB203580
α-CD3/CD28 (min): 0 15 30 60 90 15 30 60 90
P-p38
Total p38
Lanes 1 2 3 4 5 6 7 8 9
E
Inhibitors: - - - - - - + +
α- CD3/CD28: - - - + + + + +
VSV (h): - 12 24 - 12 24 12 24
UO126
SP600125
SB203580
LY294002
G
N
VSV
β-actin
Lanes 1 2 3 4 5 6 7 8
F
Virus Titer (pfu/ml)
Resting
+DMSO
+UO
+SP
+SB
+LY
CD3/CD28
G
R
α-CD3/CD28
DMSO UO SP SB LY
VSV: - + - + + + + +
M
L
Actin
Lanes 1 2 3 4 5 6 7 8
Relative Expression (M/Actin) 0,0 1,0 0,0 1,0 1,0 0,9 1,2 1,0

**Figure 2. Activation of the MAPK and PI3K pathways is required for VSV replication.** (A-D) CD3/CD28 stimulation induces PI3K and MAPK signaling in CD4+ T-lymphocytes. Resting primary CD4+ T lymphocytes were mock treated (DMSO) or treated with the MEK1/2 inhibitor UO126 (A), the JNK inhibitor SP600125 (B), the PI3-kinase inhibitor LY294002 (C), or the p38 kinase inhibitor SB203580 (D) before activation with anti-CD3/CD28. At the indicated times, cells were lysed and analysed by immunoblot with antibodies specific for total or phosphorylated forms of p38 MAPK, ERK1/2, Akt, JNK. (E) Effect of MAP kinase and PI3K pathway inhibitors on VSV replication in T-lymphocytes. Mock- and inhibitor-treated activated T-lymphocytes were infected with VSV (1 MOI) for 12h and 24h lysed and subject to immunoblot analysis with an anti-VSV antibody. Data are representative of 3 differents experiments. (F) Effect of various MAP kinase and PI3K inhibitors on virus yield. CD4+ T-lymphocytes pretreated with UO126, SB203580, SP600125, LY294002 and activated as above before to be infected with VSV (1 MOI) for 24h. Supernatants were collected and viral titers determined by standard plaque assay. (G) Effect of various MAP kinase and PI3K inhibitors on VSV mRNA transcripts. Cells were treated as described in (E) and collected 24h after infection for total RNA extraction; resting T-cells are designated as R. 1 μg of RNA from each samples were subjected to RT-PCR for selective amplification of specific VSV mRNAs (M, L) and the constitutively expressed actin, as a control. PCR products were separated on a 2 % agarose gel and visualized with ethidium bromide staining. The VSV M mRNA expression level were quantified and normalized to actin level using the Scion Image 4.0 software.

**Inhibition of ERK, JNK or PI3K leads to cell cycle arrest in primary T lymphocytes.**

Because anti-CD3/CD28 stimulation of T lymphocytes induces cell proliferation through the activation of MEK1/2, JNK and PI3K pathways, we sought to examine the relationship between VSV replication and induction of the cell cycle by MAPK. Treatment of T-lymphocytes with anti-CD3/CD28 for 48h resulted in exit from $G_0$ phase and cell cycle entry as demonstrated by FACS analysis of PI-labeled cells; T cell activation led to a 30% increase in cells in $S+G_2/M$ phases of the cell cycle (Fig. 3A compare panels a and b; Fig. 3B). As expected, addition of MEK1/2 or PI3K inhibitors for 1h prior to CD3/CD28 stimulation decreased the percentage of cells in $S+G_2/M$ phases to background levels (3-5%) (Fig. 3*A*, compare panel b to panels c and f; Fig. 3B). Surprisingly, inhibition of the JNK pathway by SP600125 prevented T-lymphocyte cell cycle entry/progression, resulting in $G_0/G_1$ arrest (2%) (Fig. 3A, panel d; Fig. 3B). Simultaneous assessment of apoptosis by DNA content analysis showed that these inhibitors did not affect cell survival (data not shown). To our knowledge, this constitutes the first report that JNK activation is specifically required for T lymphocyte $G_0/G_1$ phase transition. Inhibition of p38 had no effect on T cell proliferation (Fig. 3A, panel e; Fig. 3B).

To confirm that CD3/CD28 stimulated T lymphocytes underwent $G_0/G_1$ transition, the induction or degradation of proteins controlling cell cycle progression was assessed by immunoblot. High levels of the cdk inhibitor $p27^{kip1}$ were present in resting cells whereas cyclin D3 (cycD3) and cyclin-dependent kinase 4 (cdk4) were undetectable, thus confirming $G_0$ arrest (Fig. 3*C*, lane 1). CD3/CD28 stimulation resulted in rapid degradation of $p27^{kip1}$, concomitant with induction of cyclin D3 and cdk4 expression (Fig. 3C, lanes 2-5). $p27^{kip1}$ downregulation and cycD3/cdk4 induction was inhibited by pretreatment with LY294002, SP600125, or UO126 but not by SB203580 (Fig. *3*C). Entry into S phase was monitored by measuring hyperphosphorylation of Retinoblastoma protein (Rb) - a cycD/cdk4 substrate - and cyclin A protein expression. As demonstrated on Figure 3D, CD3/D28 stimulation increased Rb phosphorylation as well as cyclin A

expression, whereas pretreatment with LY294002, SP600125 and UO126 - but not SB203580 - abrograted both Rb hyperphosphorylation and cyclin A expression following T cell activation. These results confirm that inhibition of MEK1/2, SAPK/JNK or PI3K activity arrests CD4+ T-lymphocytes in $G_0/G_1$ phase by preventing the expression of positive regulators of cell cycle entry, such as cyclin D3 and cdk4, or preventing the downregulation of cell cycle inhibitors such as $p27^{Kip1}$.

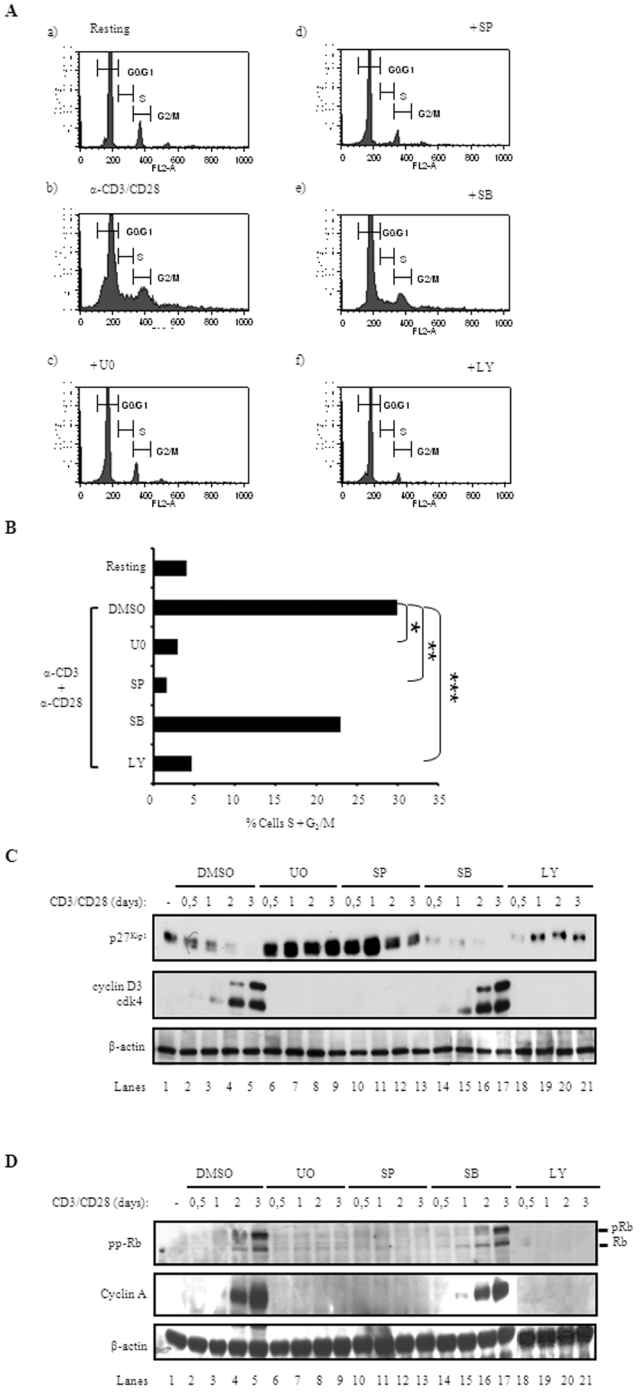

A
a) Resting
b) α-CD3/CD28
c) +U0
d) +SP
e) +SB
f) +LY
G0/G1
S
G2/M
FL2-A
B
Resting
DMSO
U0
SP
SB
LY
α-CD3 + α-CD28
*
**
***
% Cells S + G2/M
C
DMSO
UO
SP
SB
LY
CD3/CD28 (days):
p27Kip1
cyclin D3
cdk4
β-actin
Lanes
D
DMSO
UO
SP
SB
LY
CD3/CD28 (days):
pp-Rb
pRb
Rb
Cyclin A
β-actin
Lanes

**Figure 3. Proliferation and cell cycle progression of primary T cells is dependent on MEK1/2, JNK and PI3K activation.** (A) and (B) Resting CD4+ T cells were left untreated (a), treated with anti-CD3/CD28 (b), or pretreated with MEK1/2 inhibitor UO126 (c), JNK inhibitor SP600125 (d), P38 inhibitor SB203580 (e), or PI3K inhibitor LY294002 (f) before stimulation with anti-CD3/CD28. Cell cycle analysis was performed by flow cytometry on PI-labeled cells (A) and percentage of cells in S, $G_2$ and M phase was determined as the mean of triplicate measurements (B). Results are representative of more than 3 independent experiments using separate donors. (C) CD3/CD28 stimulation leads to $G_1$ progression, as demonstrated by immunoblot showing down-regulation of $P27^{Kip1}$ and synthesis of cyclin D3 and Cdk4. Cell lysates were prepared at different times, and equal amounts of protein (30 μg) were resolved by SDS-page followed by immunoblotting with antiserum specific for $p27^{kip1}$, cyclin D3, and Cdk4. Immunoblot against β-actin was performed as loading control. *D*, CD3/CD28 stimulation leads to $G_1$/S phase transition: immunoblot for Rb and cyclin A proteins. Cells were treated and cultured as in (A). *Top panel*, arrow indicates hyper-phosphorylated Rb. Immunoblot against anti-β-actin was used as loading control.

**VSV replication is dependent on cell cycle entry and increased protein translation.**

Activation of the ERK, JNK or PI3K pathways appears to confer VSV permissiveness to activated CD4+ T-cells by mediating cell cycle entry, which is characterized by a sharp increase in protein translation. To test this hypothesis, activated CD4+ T-lymphocytes were arrested at different stages of the cell cycle using the pharmacological inhibitors olomoucine, rapamycin, aphidicolin, nocodazole and taxol before challenge with VSV. FACS analysis of PI-labeled cells confirmed that CD3/CD28 stimulation induced cell cycle progression (Fig. 4A, panel b, Fig. 4B). Olomoucine - a purine-derivative drug that inhibits different CDKs (36, 38) - and rapamycin - an mTOR targeting, immunosuppressive drug that blocks the elimination of p27Kip1 and inactivates the kinase activity of the $G_1$cyclin/cdk complex (31, 40) - arrested T-lymphocytes in $G_0/G_1$ (97.5% and 95.9%, respectively). Aphidicolin - an inhibitor of DNA polymerase α (44, 54, 66) - blocked the cell cycle between the $G_1$ and S phases (92.3%). Taxol and nocodazole block the cell cycle in $G_2$/M (30.3% and 15%, respectively) by modulating microtubule polymerization (16, 39) (Fig. 4A, panels c-g, Fig. 4B). Simultaneous assessment of apoptosis by DNA content analysis showed that these inhibitors did not affect cell survival (data not shown). To investigate if cell cycle arrest in $G_0/G_1$ specifically impaired VSV replication, CD4+ T-lymphocytes were pre-treated or not with inhibitors and activated with anti-CD3/CD28 for 12h prior to VSV infection. VSV protein synthesis was decreased by olomoucine or rapamycin (Fig. 4C, compare lanes 4-5 to lanes 6-7; Fig. 4D compare lanes 2-3 to lanes 4-5) but not with aphidicolin, nocodazole or taxol (Fig. 4C, compare lanes 4-5 to lanes 8-13), demonstrating that VSV replication in primary T-lymphocytes is exclusively dependent on $G_0/G_1$ phase transition.

The effect of rapamycin on the cell cycle is largely dependent on its ability to inhibit mTOR, a regulator of 5'-cap dependent translation and ribogenesis (reviewed in (49, 61)). mTOR regulates cell growth by phosphorylating S6K and 4EPB1 and its activity is controlled through phosphorylation by the Serine/Threonine kinase Akt. Phosphorylation of 4EBP1 leads to its dissociation from the translation initiation factor eIF4E which can

then be phosphorylated by MAP kinase-interacting protein 1 (Mnk1) (reviewed in (49)), resulting in activation of eIF4E and enhanced ribosome recruitment to the translational start site. Thus, eIF4E activity is regulated through expression, phosphorylation and interaction with eIF4BP1 (37, 58). We therefore investigated the phosphorylation status of mTOR (3, 78), as well as the levels of total and phosphorylated eIF4E (11, 59) as readouts for 5'-cap dependent translation and ribogenesis. CD3/CD28 stimulation of CD4+ T-lymphocytes induced mTOR phosphorylation in a time-dependent manner (Fig. 5A, lanes 1-2 and 5B, lanes 1-5); eIF4E expression and phosphorylation was also induced following T cell activation. Addition of the PI3K inhibitor LY294002 1h prior to CD3/CD28 stimulation abolished mTOR and eIF4E phosphorylation (Fig. 5A, lane 6 and 5B, lanes 18-21), whereas the ERK inhibitor UO126 abrogated eIF4E phosphorylation only (Fig. 5A, lanes 3 and 5B, lanes 6-9). A modest decrease in the total level of eIF4E was also observed in activated T cells pretreated with the ERK (UO126) or PI3K (LY294002) inhibitors, respectively. This result is in agreement with previous reports on the role of PI3K and ERK1/2 signaling in the control of protein translation (51). Surprisingly, inhibition of the JNK pathway by SP600125 inhibited mTOR phosphorylation activated T-lymphocytes, as well as eIF4E phosphorylation and expression (Fig. 5A, lanes 4 and 5B, lanes 10-13). To our knowledge, this represents the first report that JNK activation following TCR stimulation is necessary for translational control via mTOR and eIF4E. As expected, SB203850 had no effect on mTOR and eIF4E phosphorylation (Fig. 5A, lanes 5 and 5B, lanes 14-17). These results suggest that the dependence of VSV on $G_0/G_1$ phase transition in primary lymphocytes is related to a global enhancement in protein translation that depends on mTOR and eIF4E activity.

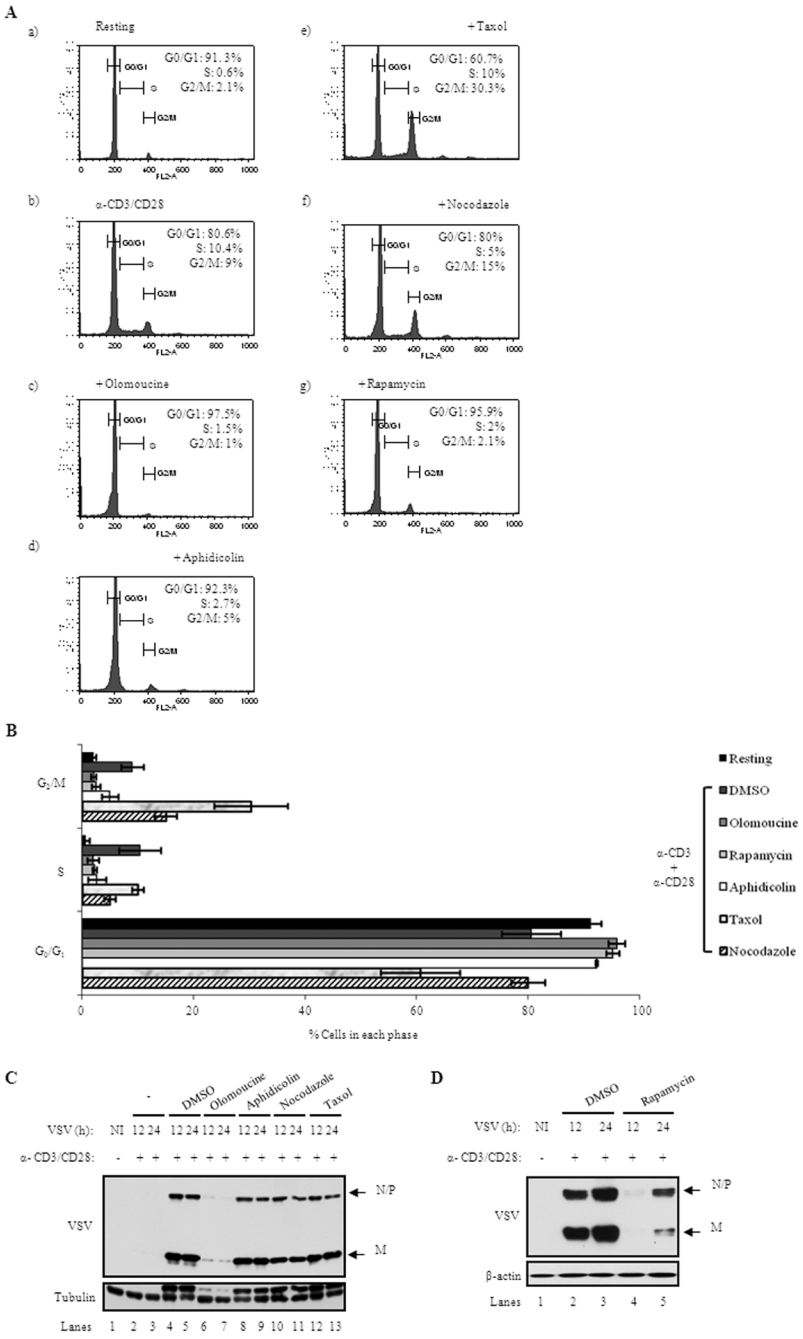
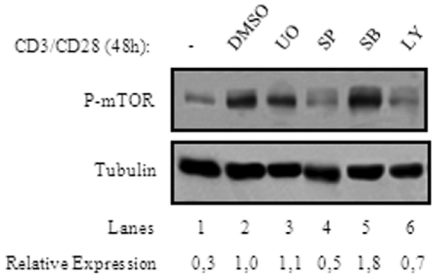

A
a) Resting
G0/G1: 91.3%
S: 0.6%
G2/M: 2.1%
b) α-CD3/CD28
G0/G1: 80.6%
S: 10.4%
G2/M: 9%
c) +Olomoucine
G0/G1: 97.5%
S: 1.5%
G2/M: 1%
d) +Aphidicolin
G0/G1: 92.3%
S: 2.7%
G2/M: 5%
e) +Taxol
G0/G1: 60.7%
S: 10%
G2/M: 30.3%
f) +Nocodazole
G0/G1: 80%
S: 5%
G2/M: 15%
g) +Rapamycin
G0/G1: 95.9%
S: 2%
G2/M: 2.1%
FL2-A
B
G2/M
S
G0/G1
Resting
DMSO
Olomoucine
Rapamycin
Aphidicolin
Taxol
Nocodazole
α-CD3 + α-CD28
% Cells in each phase
C
DMSO
Olomoucine
Aphidicolin
Nocodazole
Taxol
VSV (h):
α- CD3/CD28:
VSV
Tubulin
N/P
M
Lanes
D
DMSO
Rapamycin
β-actin

**Figure 4. VSV protein synthesis requires cell cycle entry.** (A) Effect of pharmacological inhibitors of the cell cycle on CD3/CD28-stimulated primary T cells. Resting CD4+ T- cells were left untreated (a) or pretreated with Oloumicine (c), Aphidicolin (d), Taxol (e), Nocodazole (f) or Rapamycin (g) for 1h before stimulation with anti-CD3/CD28 for 48h. Activated T cells are shown in (b). Cell cycle analysis was performed by flow cytometry on PI-labeled cells. (A) and (B) Percentage (%) of cells in various phases of the cell cycle is indicated. (C) and (D) Effect of Rapamycin, Oloumicine, Aphidicolin, Taxol and Nocodazole on VSV protein synthesis. Human CD4+ T-cells were treated as in (A), then infected with VSV (1 MOI). Cells were collected 12h and 24h post-infection, lysed and subject to immunoblot analysis with anti-VSV antisera. Results are representative of more than 3 independent experiments using separate donors.

**A**


CD3/CD28 (48h):
-
DMSO
UO
SP
SB
LY
P-mTOR
Tubulin
Lanes 1 2 3 4 5 6
Relative Expression 0,3 1,0 1,1 0,5 1,8 0,7

**B**

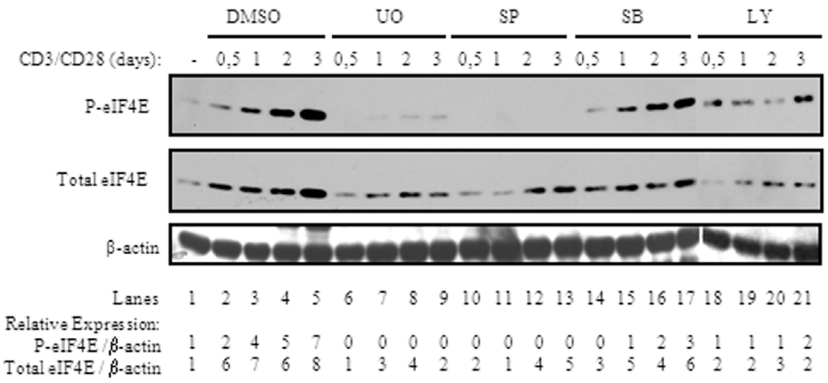
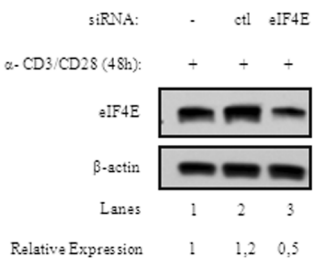

DMSO
UO
SP
SB
LY
CD3/CD28 (days): - 0,5 1 2 3 0,5 1 2 3 0,5 1 2 3 0,5 1 2 3 0,5 1 2 3
P-eIF4E
Total eIF4E
β-actin
Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
Relative Expression:
P-eIF4E / β-actin 1 2 4 5 7 0 0 0 0 0 0 0 0 0 1 2 3 1 1 1 2
Total eIF4E / β-actin 1 6 7 6 8 1 3 4 2 2 1 4 5 3 5 4 6 2 2 3 2

**Figure 5. mTOR and eIF4E phosphorylation requires ERK1/2, JNK and Akt activation in T lymphocytes.** (A) and (B) Isolated CD4+ T-lymphocytes were mock treated (DMSO) or treated with the MEK1/2 inhibitor UO126, the JNK inhibitor SP600125, the p38 kinase inhibitor SB203580 or the PI3-kinase inhibitor LY294002, 1 hour prior to activation with anti-CD3/CD28 for 0 to 2 days. Cell lysates (30 μg) were resolved by SDS-page followed by immunoblotting with antibody specific for mTOR phosphorylation (A), eIF4E phosphorylation as well total eIF4E protein (B). mTOR phosphorylation level in (A) was quantified and normalized to β-actin levels using the Scion Image 4.0 software.

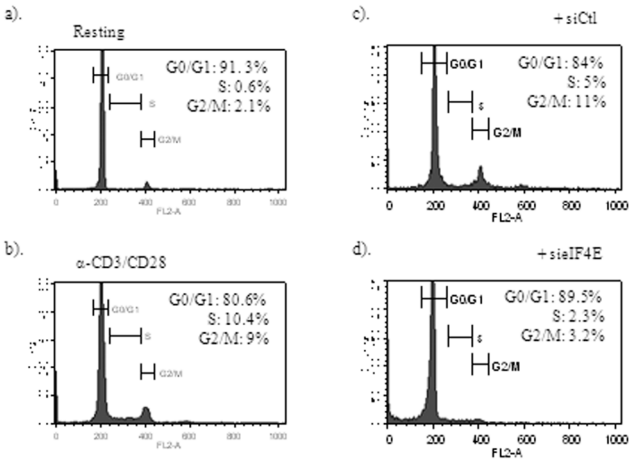
**siRNA against eIF4E inhibits VSV replication in activated T lymphocytes.**

Because it is generally accepted that VSV protein synthesis requires the cap-dependent translational machinery (8), RNA interference (RNAi) was next used to examine whether the high level of eIF4E found in activated T-lymphocytes was required for VSV protein synthesis. Cells were stimulated with anti-CD3/CD28 treated for 48h with siRNA directed against eIF4E mRNA (Fig. 6A). A 50 % decrease in the level of eIF4E protein compared to the control siRNA was observed (Fig. 6A, compare lane 1 to lanes 2-3); simultaneously, siRNA directed against eIF4E induced cell cycle arrest in $G_0/G_1$ (89.5%), as demonstrated by PI staining (Fig. 6B, compare panels c and d; Fig. 6C). VSV infection was performed on siRNA-treated and preactivated T-lymphocytes (Fig. 6D), and by 12h post-infection, VSV protein production was detected in control siRNA cells but a 50% decrease in VSV N/P protein level was observed in eIF4E siRNA expressing cells. These results demonstrate that eIF4E plays an important role in regulating VSV replication in CD4+ T lymphocytes, either through a global regulation of cellular protein synthesis that leads to $G_0/G_1$ transition, or specifically through translation initiation of viral 5'-capped mRNAs.

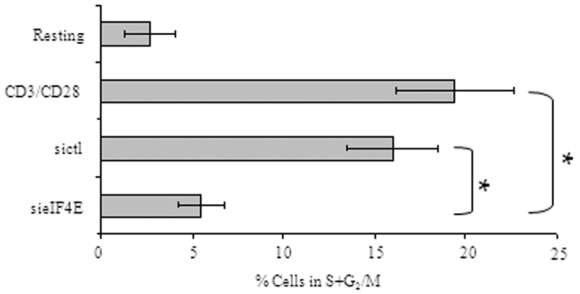
**A**


siRNA: - ctl eIF4E
α- CD3/CD28 (48h): + + +
eIF4E
β-actin
Lanes 1 2 3
Relative Expression 1 1,2 0,5

**B**


a).
Resting
G0/G1: 91.3%
S: 0.6%
G2/M: 2.1%
c).
+ siCtl
G0/G1: 84%
S: 5%
G2/M: 11%
b).
α-CD3/CD28
G0/G1: 80.6%
S: 10.4%
G2/M: 9%
d).
+ sieIF4E
G0/G1: 89.5%
S: 2.3%
G2/M: 3.2%
FL2-A

**C**


Resting
CD3/CD28
sictl
sieIF4E
0
5
10
15
20
25
% Cells in S+G2/M
*
*

**D**

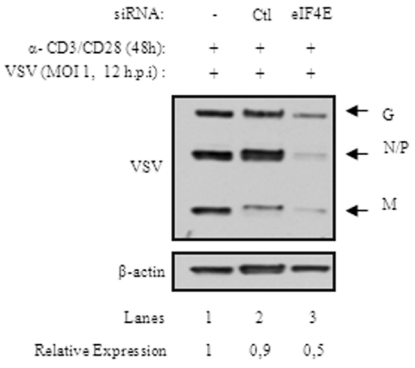

siRNA: - Ctl eIF4E
α- CD3/CD28 (48h): + + +
VSV (MOI 1, 12 h.p.i) : + + +
VSV
G
N/P
M
β-actin
Lanes 1 2 3
Relative Expression 1 0,9 0,5

**Figure 6. siRNA directed against eIF4E inhibits VSV replication in primary activated CD4+ T-lymphocytes.** (A) Primary CD4+ T-lymphocytes were stimulated with anti-CD3/CD28 for 48h before electroporation with eIF4E-specific siRNA or scrambled siRNA. After an additional 48h incubation period, cells were lysed and analysed by immunoblot with an antibody against total eIF4E. Mock denotes cells electroporated without siRNA. (B) Cell cycle analysis was performed on cells described in (A) by PI-labelling and flow cytometry analysis. (B) and (C) The percentage (%) of cells in various phases of the cell cycle is indicated. (D) eIF4E knockdown and control cells were infected with VSV (1 MOI) for 12h. Cells were lysed and subject to immunoblot with anti-VSV antisera. The eIF4E and VSV N/P protein expression levels in (A) and (B) were quantified and normalized to β-actin levels using the Scion Image 4.0 software. Levels of eIF4E were expressed as a percentage of mock cells.

**Cell cycle entry and translation initiation renders CLL cells susceptible to VSV replication.**

Cell cycle transition from $G_0$ to $G_1$ phase - concomitant with an increase in protein translation - is essential for VSV replication in lymphocytes. Primary *ex-vivo* CLL cells are resistant to VSV oncolysis due to a Bcl-2 mediated arrest in $G_0$ phase (30); (45); (43); (56), and thus represent an interesting model to investigate whether cell cycle induction is sufficient to confer VSV susceptibility to primary CLL cells. *Ex-vivo* CLL samples were stimulated with PMA/ionomycin for 12 to 72h prior to VSV infection; FACS analysis on PI-labeled cells demonstrated that treatment with PMA/ionomycin of B-CLL cells induced their progression through the cell cycle in a time-dependent manner, leading to an increase of cells in S+G2M phases from 5 % to 18 % (Fig. 7A). Cell cycle entry was further confirmed by immunoblot for $p27^{Kip1}$ protein, which was degraded in a time-dependent manner (Fig. 7B, compare lanes 1-4 to lanes 5-8). Interestingly, treatment of CLL cells with PMA/ionomycin induced eIF4E phosphorylation in a time-dependent manner (Fig. 7C, compare lanes 1-4 to lanes 5-8). VSV protein levels and caspase 3 cleavage were also monitored at 12 and 72h post-infection (Fig. 7D); VSV protein decreased in unstimulated primary CLL (Fig. 7D, lanes 2-5) and no caspase 3 cleavage was observed 24h post-infection (Fig. 7D, lanes 2-5). In agreement with this result, activated CLL cells produced ~3 fold more virus than unstimulated CLL at 48h post-infection (Fig. 7E). Furthermore, PMA/ionomycin treatment of CLL cells stimulated VSV replication, concomitant with a time-dependent increase in the cleaved forms of caspase 3 (Fig. 7D, lanes 7-10). Thus, using the example of $G_0/G_1$ arrested primary CLL, resistance to VSV oncolysis can be overcome by pretreatment with pharmacological compounds that induce cell cycle entry and translation initiation.

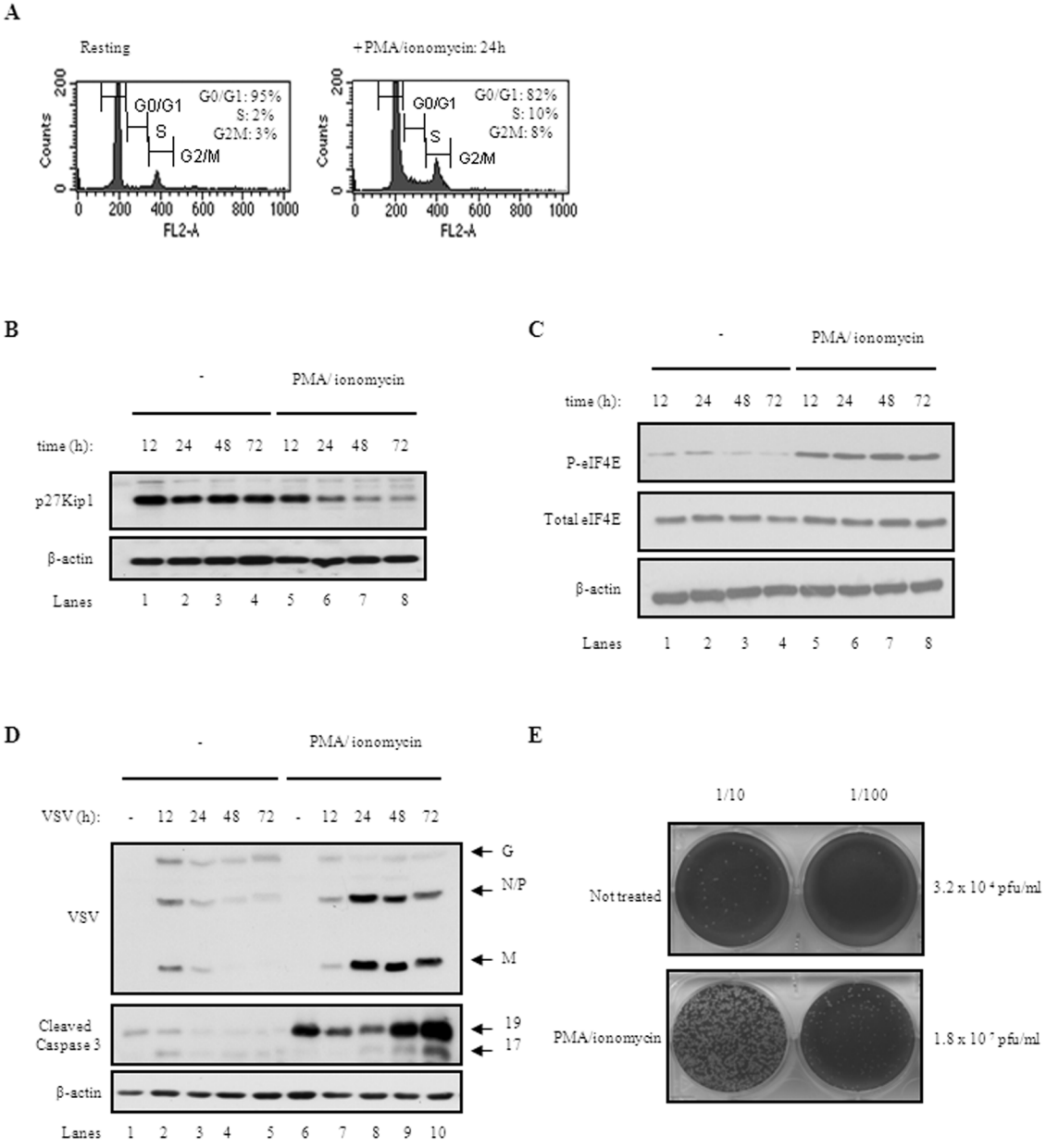

A
Resting
+PMA/ionomycin: 24h
G0/G1
S
G2/M
G0/G1: 95%
S: 2%
G2M: 3%
G0/G1: 82%
S: 10%
G2M: 8%
Counts
FL2-A
B
-
PMA/ionomycin
time (h):
12 24 48 72 12 24 48 72
p27Kip1
β-actin
Lanes
1 2 3 4 5 6 7 8
C
-
PMA/ionomycin
time (h):
12 24 48 72 12 24 48 72
P-eIF4E
Total eIF4E
β-actin
Lanes
1 2 3 4 5 6 7 8
D
-
PMA/ionomycin
VSV (h):
- 12 24 48 72 - 12 24 48 72
VSV
G
N/P
M
Cleaved Caspase 3
19
17
β-actin
Lanes
1 2 3 4 5 6 7 8 9 10
E
1/10
1/100
Not treated
3.2 x 10 4 pfu/ml
PMA/ionomycin
1.8 x 10 7 pfu/ml

**Figure 7. Pharmacological induction of cell cycle entry of primary CLL cells is sufficient to overcome VSV resistance.** (A) PMA and ionomycin treatment induces cell cycle entry of CLL cells. Isolated CLL were mock treated (DMSO) or treated with 25 ng PMA and 1 μg ionomycin. After 48h, cell cycle analysis was performed by flow cytometry on PI-labeled cells. The percentage of cells in various phases of the cell cycle is indicated. Cell lysates from CLL samples were prepared at 12, 24, 48 and 72h after PMA/ionomycin stimulation and equal amount of protein (20 μg) were resolved by SDS-page followed by immunoblotting with antibody specific for $P27^{kip1}$ (B), eIF4E phosphorylation and total eIF4E protein (C). (D) Effect of PMA/ionomycin on VSV replication. CLL cells were treated as in (A) for 12h prior to infection with VSV (1 MOI). (D) Cells lysates were prepared at 12, 24, 48, 72h postinfection and equal amount of protein (20 μg) were resolved by SDS-page followed by immunoblotting with anti-VSV antisera. Immunoblot against β-actin is shown as loading control. (E) Supernatants were collected at 48h postinfection and virus titers determined by standard plaque assay. Images of the assay plate are also shown. Assays were performed in triplicate.

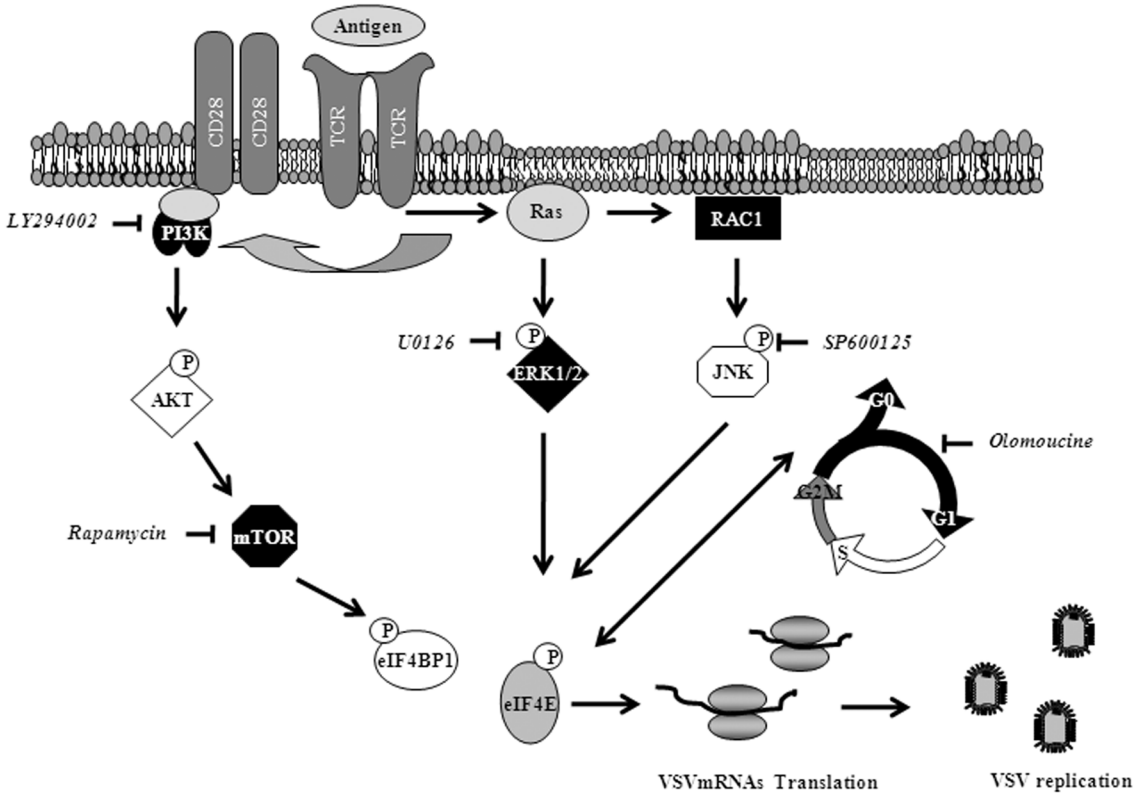
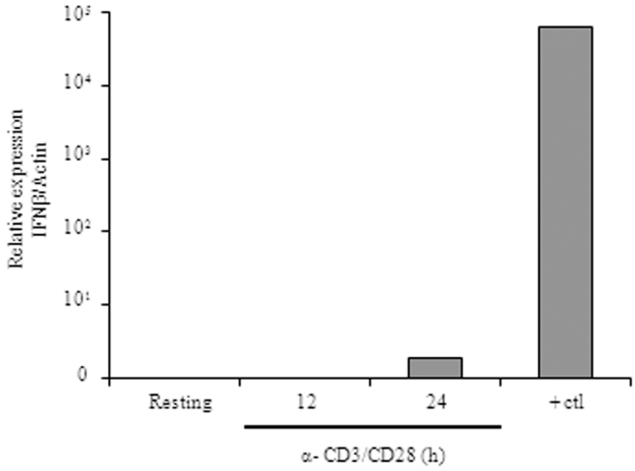
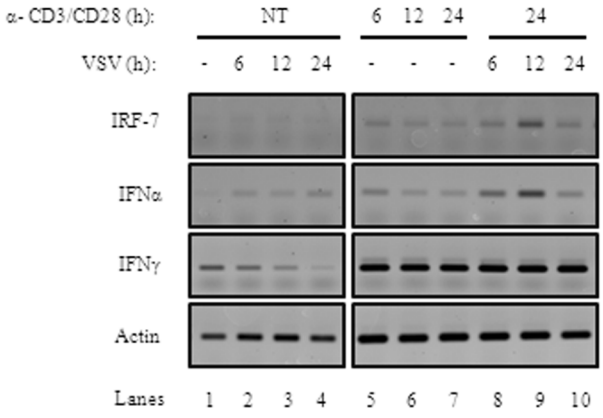

Antigen
CD28
CD28
TCR
TCR
LY294002
PI3K
Ras
RAC1
U0126
P
ERK1/2
P
JNK
SP600125
P
AKT
G0
Olomoucine
G2M
G1
S
Rapamycin
mTOR
P
eIF4BP1
P
eIF4E
VSVmRNAs Translation
VSV replication

**Figure 8. Schematic diagram illustrating the signaling pathways that impact VSV replication in activated T cells.** The point of action of pharmacological inhibitors is indicated. Following CD3/CD28 stimulation, activation of ERK, JNK or AKT pathways - leading to $G_0$ to $G_1$ phase transition - is crucial for VSV replication in primary lymphocytes, due to a global increase in protein translation mediated by the activation of mTOR and eIF4E.

**A**


Relative expression
IFNβ/Actin
10⁵
10⁴
10³
10²
10¹
0
Resting
12
24
+ctl
α- CD3/CD28 (h)

**B**


α- CD3/CD28 (h):
NT
6 12 24
24
VSV (h):
- 6 12 24 - - - 6 12 24
IRF-7
IFNα
IFNγ
Actin
Lanes
1 2 3 4 5 6 7 8 9 10

**Supplemental Figure 1. VSV replication in primary activated CD4+ T-lymphocytes is not due to a CD3/CD28 induced defect in the IFN pathway.** (A) Resting CD4+ T-lymphocytes were stimulated with anti-CD3/CD28 and DNAse-treated total RNA prepared at the indicated times. The expression of *IFNB* mRNA was investigated by real-time PCR 12 and 24 h post-stimulation. THP1 cells infected with Sendaï virus for 6h were used as positive control for IFNβ mRNA production. Results are presented as a quantification based on the relative expression levels of target gene mRNA *versus B-Actin* mRNA, as a reference gene. (B) Resting and stimulated CD4+ T-lymphocytes were mock infected or infected with VSV (1 MOI) and total RNA prepared at the indicated times. The expression of *IRF7, IFNA2*, *IFNG* mRNA and the constitutively expressed actin, as a control was investigated by RT-PCR. PCR products were separated on a 2 % agarose gel and visualized with ethidium bromide staining.

## DISCUSSION

The emergence of VSV as potential oncolytic virus has made dissection of the molecular determinants of host-cell permissiveness to VSV an important objective. Although promising results have been obtained in cell lines and murine cancer models (25, 27, 48), accumulating evidence indicates that certain types of cancer are partially or completely resistant to VSV oncolysis. In a previous study, we demonstrated that CD4+ T-lymphocytes from proliferating primary ATL samples were susceptible to VSV oncolysis, while resting primary T cells were restricted for VSV replication (17). In this study, we demonstrate that stimulation of primary human CD4+ T-lymphocytes with anti-CD3/CD28 allows VSV replication and VSV-induced cell death by triggering the MAPK, PI3K and mTOR/eIF4E pathways and cell cycle entry.

Stimulation of T cells by TCR plus co-receptor engagement leads to the activation of two main signaling pathways: Ras and PI3K. The Ras pathway signals through the MAP kinases ERK, JNK and p38, whereas PI3K activates Akt. It is generally accepted that ERK, JNK and Akt play a role in cell cycle control, but not p38 (1). Similarly, by using the pharmacological inhibitors U0126, SP600125, LY-294002 or SB203580 we demonstrate that VSV replication in primary T lymphocytes requires activation of MEK1/2, JNK and PI3K but not p38. These inhibitors had no effect on production of VSV mRNA but led to a decrease in viral protein levels and virus titer indicating that these pathways affect VSV mRNA translation but not virus entry or mRNA production. Since inhibition of any one of the kinases blocked VSV replication, it appears that the activities of MEK1/2, JNK and PI3K are non-redundant in regards to VSV permissiveness.

VSV viral protein synthesis appears to depend on cell cycle entry because inhibitors that blocked VSV protein synthesis also resulted in $G_0/G_1$ arrest. Indeed, pre-treatment of naive T cells with U0126, SP600125 or LY-294002 but not SB203580 prior to activation led to arrest in $G_0/G_1$, preventing degradation of $p27^{kip1}$ and induction of cyclin D3/cdk4.

Remarkably, the inhibitors had no effect on VSV replication if added to preactivated T lymphocytes, suggesting that VSV infection is dependent on global activation events and not on the direct action of these kinases or their targets in viral replication. The use of pharmacological inhibitors to specifically block cell cycle progression independently of MAPK or PI3K activity confirmed that entry into the $G_1$ phase but not progression through S or $G_2$/M allowed VSV replication. $G_0/G_1$ cell cycle arrest induced by rapamycin or oloumicine treatment abrogated VSV replication as did the inhibitors of CD3/CD28 signaling. In contrast, robust VSV protein expression was observed in the presence of aphidicolin or taxol, which induced cell cycle arrest in $G_1$/S and $G_2$/M phase, respectively. Therefore, entry in $G_1$ phase of the cell cycle is sufficient to render normal primary human CD4+ T-lymphocytes susceptible to VSV replication.

T cells arrested in $G_0$ phase of the cell cycle have low rates of mRNA and protein synthesis. Phosphorylation of the mammalian target of rapamycin (mTOR) and the translation initiation factor eIF4E is observed in T cells following CD3/CD28 stimulation, which leads to a surge in protein synthesis that allows cell cycle progression (reviewed in (49, 61)). Our data demonstrates that the Ras pathway through ERK, and the PI3K pathway through Akt, regulate translation initiation and/or ribosome biogenesis, as their inhibition by U0126 and LY-294002 prevented eIF4E and mTOR phosphorylation (49, 60, 63). Interestingly, JNK is also required for mTOR and eIF4E activation in T lymphocytes, as the presence of the inhibitor SP600125 prevented mTOR phosphorylation as well as eIF4E protein induction and phosphorylation following CD3/CD28 stimulation. This is to our knowledge the first report indicating that, in primary T cells, JNK activation leads to mTOR and eIF4E phosphorylation. As expected, p38 inhibition had no effect on mTOR and eIF4E phosphorylation following CD3 and CD28 engagement.

Rapamycin induces $G_1$ cell cycle arrest and blocks mRNA translation by binding to and inhibiting mTOR. This prevents phosphorylation of eIF4-BP1 and therefore blocks the release of the translation initiation factor eIF4E. Rapamycin also inhibits the

phosphorylation of the ribosomal S6 kinase 1 (S6K1), a downstream target of mTOR, further blocking translation initiation of 5'-capped and/or 5'TOP mRNAs (57); (13, 77). In this study, we demonstrate that rapamycin blocks VSV protein production in primary activated CD4+ T lymphocytes, suggesting that mTOR-mediated translation initiation and ribosome biogenesis during $G_1$ phase is crucial for VSV replication in primary human CD4+ T-lymphocytes. In contrast, Connor *et al.* (22) concluded that rapamycin had no effect on VSV protein synthesis in Hela cells, although this study did not address the cell cycle status of HeLa cells following rapamycin treatment. The discrepancy might be explained by the fact that the antiproliferative effect of rapamycin is maximal in quiescent cells that express high levels of $p27^{kip1}$ but not in exponentially growing cells (12, 71). In primary T-cells rapamycin induced a strong $G_1$ phase arrest that was concomitant with inhibition of VSV replication.

eIF4E is a downstream target of mTOR that regulates 5'-cap dependent translation (33). Considering that VSV mRNA is capped and polyadenylated, the possibility that eIF4E is directly involved in VSV replication in primary activated T cells was explored using siRNA directed against eIF4E. VSV protein synthesis was indeed inhibited in activated T lymphocytes expressing eIF4E siRNA, suggesting that this host translational factor is required for VSV replication. However, treatment of activated CD4+ T-lymphocytes with eIF4E siRNA led to cell cycle arrest in $G_0/G_1$. Therefore, it was not possible to ascribe a direct role of eIF4E in VSV replication, since it is not clear whether the observed effect is a direct consequence of eIF4E inhibition, or whether it is an indirect consequence of a delay in cell cycle progression.

The results obtained in primary T cells demonstrate that VSV replication in lymphocytes depends on $G_0$/G1 phase transition. We postulated that resistance to VSV-induced oncolysis observed in *ex-vivo* CLL samples is due to the low proliferative index of CLL cells, which are arrested in $G_0$ phase (67). Indeed, treatment of *ex-vivo* primary CLL samples with PMA/ionomycin to induce $G_0/G_1$ transition was sufficient to restore VSV sensitivity: PMA/ionomycin stimulation led to cell cycle entry, as demonstrated by a 13%

increase in cells in S+$G_2$/M phase, and this was sufficient to restore VSV replication. Induction of eIF4E phosphorylation was also observed in CLL cells activated with PMA/ionomycin, although –in contrast to primary T cell samples - total levels of eIF4E remained constant. This and previous reports argue that eIF4E phosphorylation constitutes a limiting step in the induction of translation initiation (3, 11, 19, 49). Induction of apoptosis was also confirmed by increased caspase 3 cleavage compared to untreated cells. This result constitutes the first evidence that VSV oncolysis in $G_0$ arrested CLL can be augmented by pharmacological agents that stimulate exit from $G_0/G_1$.

The current study illustrates that VSV replication in primary lymphocytes is dependent on cell cycle entry and global enhancement of protein synthesis via mTOR and eIF4E activity. The results of this study may provide incentive to explore other combinatorial approaches to overcome the resistance of different cancers to oncolytic virus therapy.

## ACKNOWLEDGMENTS

The authors wish to thank Dr Nathalie Grandvaux, Yan Long, Dr Romieu Raphaëlle, Dr Lézin Agnès, Dr Césaire Raymond and the members of the Molecular Oncology Group, Lady Davis Institute, McGill University for helpful discussions. This research was supported by grants from the National Cancer Institute with funds from the Terry Fox Foundation to JH and JB, the Canadian Institutes of Health Research. SO and PN are supported by Fellowships from the FRSQ, VT is supported by a Studentship from NSERC and JH is the recipient of a CIHR Senior Investigator award.

## REFERENCES


1. **Alberola-Ila, J., and G. Hernandez-Hoyos.** 2003. The Ras/MAPK cascade and the control of positive selection. Immunol Rev **191:**79-96.
2. **Appleman, L. J., A. A. van Puijenbroek, K. M. Shu, L. M. Nadler, and V. A. Boussiotis.** 2002. CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. J Immunol **168:**2729-36.
3. **Averous, J., and C. G. Proud.** 2006. When translation meets transformation: the mTOR story. Oncogene **25:**6423-35.
4. **Balachandran, S., and G. N. Barber.** 2004. Defective translational control facilitates vesicular stomatitis virus oncolysis. Cancer Cell **5:**51-65.
5. **Balachandran, S., and G. N. Barber.** 2000. Vesicular stomatitis virus (VSV) therapy of tumors. IUBMB Life **50:**135-8.
6. **Balachandran, S., P. C. Roberts, L. E. Brown, H. Truong, A. K. Pattnaik, D. R. Archer, and G. N. Barber.** 2000. Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. Immunity **13:**129-41.
7. **Baltzis, D., L. K. Qu, S. Papadopoulou, J. D. Blais, J. C. Bell, N. Sonenberg, and A. E. Koromilas.** 2004. Resistance to vesicular stomatitis virus infection requires a functional cross talk between the eukaryotic translation initiation factor 2alpha kinases PERK and PKR. J Virol **78:**12747-61.
8. **Barber, G. N.** 2005. VSV-tumor selective replication and protein translation. Oncogene **24:**7710-9.
9. **Bell, J. C., K. A. Garson, B. D. Lichty, and D. F. Stojdl.** 2002. Oncolytic viruses: programmable tumour hunters. Curr Gene Ther **2:**243-54.
10. **Bell, J. C., B. Lichty, and D. Stojdl.** 2003. Getting oncolytic virus therapies off the ground. Cancer Cell **4:**7-11.
11. **Beretta, L.** 2004. Translational control in T lymphocytes. Int Rev Immunol **23:**347-63.

12. **Beretta, L., A. C. Gingras, Y. V. Svitkin, M. N. Hall, and N. Sonenberg.** 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. Embo J **15:**658-64.

13. **Bilanges, B., R. Argonza-Barrett, M. Kolesnichenko, C. Skinner, M. Nair, M. Chen, and D. Stokoe.** 2007. Tuberous sclerosis complex proteins 1 and 2 control serum-dependent translation in a TOP-dependent and -independent manner. Mol Cell Biol **27:**5746-64.

14. **Bonni, A., A. Brunet, A. E. West, S. R. Datta, M. A. Takasu, and M. E. Greenberg.** 1999. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. Science **286:**1358-62.

15. **Boyer, D., R. Quintanilla, and K. K. Lee-Fruman.** 2007. Regulation of catalytic activity of S6 kinase 2 during cell cycle. Mol Cell Biochem.

16. **Bunz, F., A. Dutriaux, C. Lengauer, T. Waldman, S. Zhou, J. P. Brown, J. M. Sedivy, K. W. Kinzler, and B. Vogelstein.** 1998. Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science **282:**1497-501.

17. **Cesaire, R., S. Oliere, E. Sharif-Askari, M. Loignon, A. Lezin, S. Olindo, G. Panelatti, M. Kazanji, R. Aloyz, L. Panasci, J. C. Bell, and J. Hiscott.** 2006. Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia. Oncogene **25:**349-58.

18. **Chang, L., and M. Karin.** 2001. Mammalian MAP kinase signaling cascades. Nature **410:**37-40.

19. **Clemens, M. J.** 2004. Targets and mechanisms for the regulation of translation in malignant transformation. Oncogene **23:**3180-8.

20. **Cobrinik, D.** 2005. Pocket proteins and cell cycle control. Oncogene **24:**2796-809.

21. **Colina, R., M. Costa-Mattioli, R. J. Dowling, M. Jaramillo, L. H. Tai, C. J. Breitbach, Y. Martineau, O. Larsson, L. Rong, Y. V. Svitkin, A. P. Makrigiannis, J. C. Bell, and N. Sonenberg.** 2008. Translational control of the innate immune response through IRF-7. Nature **452:**323-8.

22. **Connor, J. H., and D. S. Lyles.** 2002. Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1. J Virol **76:**10177-87.

23. **Danilov, A. V., A. K. Klein, H. J. Lee, D. V. Baez, and B. T. Huber.** 2005. Differential control of G0 programme in chronic lymphocytic leukaemia: a novel prognostic factor. Br J Haematol **128:**472-81.

24. **Durbin, R. K., S. E. Mertz, A. E. Koromilas, and J. E. Durbin.** 2002. PKR protection against intranasal vesicular stomatitis virus infection is mouse strain dependent. Viral Immunol **15:**41-51.

25. **Ebert, O., K. Shinozaki, T. G. Huang, M. J. Savontaus, A. Garcia-Sastre, and S. L. Woo.** 2003. Oncolytic vesicular stomatitis virus for treatment of orthotopic hepatocellular carcinoma in immune-competent rats. Cancer Res **63:**3605-11.

26. **Farassati, F., A. D. Yang, and P. W. Lee.** 2001. Oncogenes in Ras signaling pathway dictate host-cell permissiveness to herpes simplex virus 1. Nat Cell Biol **3:**745-50.

27. **Fernandez, M., M. Porosnicu, D. Markovic, and G. N. Barber.** 2002. Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease. J Virol **76:**895-904.

28. **Fingar, D. C., and J. Blenis.** 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. Oncogene **23:**3151-71.

29. **Fingar, D. C., C. J. Richardson, A. R. Tee, L. Cheatham, C. Tsou, and J. Blenis.** 2004. mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol **24:**200-16.

30. **Gaddy, D. F., and D. S. Lyles.** 2005. Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. J Virol **79:**4170-9.

31. **Garcia-Morales, P., E. Hernando, E. Carrasco-Garcia, M. P. Menendez-Gutierrez, M. Saceda, and I. Martinez-Lacaci.** 2006. Cyclin D3 is down-

regulated by rapamycin in HER-2-overexpressing breast cancer cells. Mol Cancer Ther **5:**2172-81.

32. **Garcia, Z., A. Kumar, M. Marques, I. Cortes, and A. C. Carrera.** 2006. Phosphoinositide 3-kinase controls early and late events in mammalian cell division. Embo J **25:**655-61.

33. **Gebauer, F., and M. W. Hentze.** 2004. Molecular mechanisms of translational control. Nat Rev Mol Cell Biol **5:**827-35.

34. **Gelinas, J. N., J. L. Banko, L. Hou, N. Sonenberg, E. J. Weeber, E. Klann, and P. V. Nguyen.** 2007. ERK and mTOR signaling couple beta-adrenergic receptors to translation initiation machinery to gate induction of protein synthesis-dependent long-term potentiation. J Biol Chem **282:**27527-35.

35. **Gonzalez, J., T. Harris, G. Childs, and M. B. Prystowsky.** 2001. Rapamycin blocks IL-2-driven T cell cycle progression while preserving T cell survival. Blood Cells Mol Dis **27:**572-85.

36. **Gray, N., L. Detivaud, C. Doerig, and L. Meijer.** 1999. ATP-site directed inhibitors of cyclin-dependent kinases. Curr Med Chem **6:**859-75.

37. **Hay, N., and N. Sonenberg.** 2004. Upstream and downstream of mTOR. Genes Dev **18:**1926-45.

38. **Heredia, A., C. Davis, A. Amoroso, J. K. Dominique, N. Le, E. Klingebiel, E. Reardon, D. Zella, and R. R. Redfield.** 2003. Induction of G1 cycle arrest in T lymphocytes results in increased extracellular levels of beta-chemokines: a strategy to inhibit R5 HIV-1. Proc Natl Acad Sci U S A **100:**4179-84.

39. **Jordan, M. A., and L. Wilson.** 2004. Microtubules as a target for anticancer drugs. Nat Rev Cancer **4:**253-65.

40. **Kawamata, S., H. Sakaida, T. Hori, M. Maeda, and T. Uchiyama.** 1998. The upregulation of p27Kip1 by rapamycin results in G1 arrest in exponentially growing T-cell lines. Blood **91:**561-9.

41. **Kim, J. A., S. Hong, B. Lee, J. W. Hong, J. Y. Kwak, S. Cho, and C. C. Kim.** 2007. The inhibition of T-cells proliferation by mouse mesenchymal stem cells

through the induction of p16INK4A-cyclin D1/cdk4 and p21waf1, p27kip1-cyclin E/cdk2 pathways. Cell Immunol **245:**16-23.

42. **Klein, A., O. Miera, O. Bauer, S. Golfier, and F. Schriever.** 2000. Chemosensitivity of B cell chronic lymphocytic leukemia and correlated expression of proteins regulating apoptosis, cell cycle and DNA repair. Leukemia **14:**40-6.
43. **Kopecky, S. A., and D. S. Lyles.** 2003. The cell-rounding activity of the vesicular stomatitis virus matrix protein is due to the induction of cell death. J Virol **77:**5524-8.
44. **Korin, Y. D., and J. A. Zack.** 1998. Progression to the G1b phase of the cell cycle is required for completion of human immunodeficiency virus type 1 reverse transcription in T cells. J Virol **72:**3161-8.
45. **Korz, C., A. Pscherer, A. Benner, D. Mertens, C. Schaffner, E. Leupolt, H. Dohner, S. Stilgenbauer, and P. Lichter.** 2002. Evidence for distinct pathomechanisms in B-cell chronic lymphocytic leukemia and mantle cell lymphoma by quantitative expression analysis of cell cycle and apoptosis-associated genes. Blood **99:**4554-61.
46. **Liang, J., J. Zubovitz, T. Petrocelli, R. Kotchetkov, M. K. Connor, K. Han, J. H. Lee, S. Ciarallo, C. Catzavelos, R. Beniston, E. Franssen, and J. M. Slingerland.** 2002. PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. Nat Med **8:**1153-60.
47. **Lichty, B. D., A. T. Power, D. F. Stojdl, and J. C. Bell.** 2004. Vesicular stomatitis virus: re-inventing the bullet. Trends Mol Med **10:**210-6.
48. **Lichty, B. D., D. F. Stojdl, R. A. Taylor, L. Miller, I. Frenkel, H. Atkins, and J. C. Bell.** 2004. Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy. Hum Gene Ther **15:**821-31.
49. **Mamane, Y., E. Petroulakis, L. Rong, K. Yoshida, L. W. Ler, and N. Sonenberg.** 2004. eIF4E-from translation to transformation. Oncogene **23:**3172-9.

50. **Meloche, S., and J. Pouyssegur.** 2007. The ERK1/2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition. Oncogene **26:**3227-39.

51. **Miron, M., J. Verdu, P. E. Lachance, M. J. Birnbaum, P. F. Lasko, and N. Sonenberg.** 2001. The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signaling and cell growth in Drosophila. Nat Cell Biol **3:**596-601.

52. **Mustelin, T., and K. Tasken.** 2003. Positive and negative regulation of T-cell activation through kinases and phosphatases. Biochem J **371:**15-27.

53. **Nakhaei, P. P., S; Oliere, S; Tumilasci, V; Bell, JC and Hiscott, J.** 2005. Oncolytic virotherapy of cancer with vesicular stomatitis virus. Gene Therapy and Molecular Biology **9:**269-280.

54. **Nethanel, T., and G. Kaufmann.** 1990. Two DNA polymerases may be required for synthesis of the lagging DNA strand of simian virus 40. J Virol **64:**5912-8.

55. **Norman, K. L., K. Hirasawa, A. D. Yang, M. A. Shields, and P. W. Lee.** 2004. Reovirus oncolysis: the Ras/RalGEF/p38 pathway dictates host cell permissiveness to reovirus infection. Proc Natl Acad Sci U S A **101:**11099-104.

56. **Otake, Y., S. Soundararajan, T. K. Sengupta, E. A. Kio, J. C. Smith, M. Pineda-Roman, R. K. Stuart, E. K. Spicer, and D. J. Fernandes.** 2007. Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA. Blood **109:**3069-75.

57. **Pende, M., S. H. Um, V. Mieulet, M. Sticker, V. L. Goss, J. Mestan, M. Mueller, S. Fumagalli, S. C. Kozma, and G. Thomas.** 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol **24:**3112-24.

58. **Pestova, T. V., and C. U. Hellen.** 2001. Functions of eukaryotic factors in initiation of translation. Cold Spring Harb Symp Quant Biol **66:**389-96.

59. **Pestova, T. V., V. G. Kolupaeva, I. B. Lomakin, E. V. Pilipenko, I. N. Shatsky, V. I. Agol, and C. U. Hellen.** 2001. Molecular mechanisms of translation initiation in eukaryotes. Proc Natl Acad Sci U S A **98:**7029-36.

60. **Petroulakis, E., Y. Mamane, O. Le Bacquer, D. Shahbazian, and N. Sonenberg.** 2007. mTOR signaling: implications for cancer and anticancer therapy. Br J Cancer **96 Suppl:**R11-5.

61. **Proud, C. G.** 2007. Signaling to translation: how signal transduction pathways control the protein synthetic machinery. Biochem J **403:**217-34.

62. **Radimerski, T., J. Montagne, F. Rintelen, H. Stocker, J. van der Kaay, C. P. Downes, E. Hafen, and G. Thomas.** 2002. dS6K-regulated cell growth is dPKB/dPI(3)K-independent, but requires dPDK1. Nat Cell Biol **4:**251-5.

63. **Richter, J. D., and N. Sonenberg.** 2005. Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature **433:**477-80.

64. **Rose, J.** 2001. Rhabdoviridae: The Viruses and their Replication 4th edn.

65. **Ruvinsky, I., and O. Meyuhas.** 2006. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. Trends Biochem Sci **31:**342-8.

66. **Sampath, D., and W. Plunkett.** 2000. The role of c-Jun kinase in the apoptotic response to nucleoside analogue-induced DNA damage. Cancer Res **60:**6408-15.

67. **Sanchez-Beato, M., A. Sanchez-Aguilera, and M. A. Piris.** 2003. Cell cycle deregulation in B-cell lymphomas. Blood **101:**1220-35.

68. **Scheid, M. P., and J. R. Woodgett.** 2001. PKB/AKT: functional insights from genetic models. Nat Rev Mol Cell Biol **2:**760-8.

69. **Sherr, C. J., and J. M. Roberts.** 2004. Living with or without cyclins and cyclin-dependent kinases. Genes Dev **18:**2699-711.

70. **Shin, I., F. M. Yakes, F. Rojo, N. Y. Shin, A. V. Bakin, J. Baselga, and C. L. Arteaga.** 2002. PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. Nat Med **8:**1145-52.

71. **Slavik, J. M., D. G. Lim, S. J. Burakoff, and D. A. Hafler.** 2004. Rapamycin-resistant proliferation of CD8+ T cells correlates with p27kip1 down-regulation and bcl-xL induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. J Biol Chem **279:**910-9.

72. **Stojdl, D. F., N. Abraham, S. Knowles, R. Marius, A. Brasey, B. D. Lichty, E. G. Brown, N. Sonenberg, and J. C. Bell.** 2000. The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus. J Virol **74:**9580-5.

73. **Stojdl, D. F., B. Lichty, S. Knowles, R. Marius, H. Atkins, N. Sonenberg, and J. C. Bell. .** 2000. . Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. . Nat Med **6:**821-5.

74. **Stojdl, D. F., B. D. Lichty, B. R. tenOever, J. M. Paterson, A. T. Power, S. Knowles, R. Marius, J. Reynard, L. Poliquin, H. Atkins, E. G. Brown, R. K. Durbin, J. E. Durbin, J. Hiscott, and J. C. Bell.** 2003. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. Cancer Cell **4:**263-75.

75. **Svitkin, Y. V., B. Herdy, M. Costa-Mattioli, A. C. Gingras, B. Raught, and N. Sonenberg.** 2005. Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. Mol Cell Biol **25:**10556-65.

76. **Wang, F., Y. Ma, J. W. Barrett, X. Gao, J. Loh, E. Barton, H. W. Virgin, and G. McFadden.** 2004. Disruption of Erk-dependent type I interferon induction breaks the myxoma virus species barrier. Nat Immunol **5:**1266-74.

77. **Wang, X., F. E. Paulin, L. E. Campbell, E. Gomez, K. O'Brien, N. Morrice, and C. G. Proud.** 2001. Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the epsilon-subunit and their functions in vivo. Embo J **20:**4349-59.

78. **Yang, Q., and K. L. Guan.** 2007. Expanding mTOR signaling. Cell Res **17:**666-81.

79. **Yokosuka, T., K. Sakata-Sogawa, W. Kobayashi, M. Hiroshima, A. Hashimoto-Tane, M. Tokunaga, M. L. Dustin, and T. Saito.** 2005. Newly generated T cell receptor microclusters initiate and sustain T cell activation by recruitment of Zap70 and SLP-76. Nat Immunol **6:**1253-62.

# ~ CHAPTER III ~

## *DISCUSSION*

***Importance of identifying cellular and molecular changes in HTLV-1-infected CD4+ T-cells***

While the AIDS epidemic justifiably captured the interest of the most talented researchers in the world, scientific attention to HTLV-1 was considerably reduced. Yet, the advent of globalization has provided HTLV-1 with unprecedented opportunities to spread worldwide. Thirty years after its discovery, HTLV-1 remains a poorly recognized concern for human health. Although many HTLV-1-infected individuals remain lifelong asymptomatics, they contribute to the silent spread of the virus. ATL and HAM/TSP represent the two most devastating pathologies associated with HTLV-1 infection and until now neither of these diseases can be effectively treated, nor cured. Thus, preventing transmission is fundamental for control of HTLV-1 incidence; however, safe alternatives to breastfeeding - the major route of transmission - are difficult to provide in many resource-limited countries endemic with HTLV-1. This underlines the need for developing cost-effective, novel therapeutic strategies which could i) prevent productive infection after transmission of the virus, ii) prevent the pathologic events that occur prior to clinical symptoms or iii) improve treatment for late-stage disease outcomes. A cure for HTLV-1 infection is unlikely, as once the provirus integrates into the host genome, it cannot be removed – although it may be "inactivated", as in the case of endogenous retroviruses [1-3].

Even though the clinical symptoms associated with HTLV-1 diseases are well defined, the precise nature of HTLV-1 pathogenesis remains elusive. Thus, a better understanding of the cellular and molecular events following HTLV-1 infection should provide us additional clues for the development of effective therapies and ultimately reduce morbidity and mortality in HTLV-1-infected populations. In the presented original research articles, we established for the first time a comparative genome-wide analysis to characterize immune-related gene expression profiles for each of the HTLV-1-associated disease states and acquire a better understanding of the molecular changes associated with HTLV-1 pathogenesis. We identified gene signatures characteristic of each HTLV-1

disease (**Manuscript I**) (Oliere *et al.*, *Plos Pathogens*, in revision) and ultimately propose new avenues for the development of therapies against HAM/TSP (**Manuscript I**) and ATL diseases (**Manuscript II and III**) [4-5].

***Expression profiling of genes differentially expressed in HTLV-1-associated diseases: identification of particular genes of interest for the early diagnosis of ATL and HAM/TSP pathologies***

It is estimated that 15-20 million of people worldwide are infected with HTLV-1. Although only 2-5% of HTLV-1-carriers develop ATL or HAM/TSP, evaluation of the individual risk for developing diseases in each AC would certainly be of considerable importance especially in HTLV-1 endemic areas such as Japan, the Caribbean, Central and South America, the Middle East, Melanesia, and Africa [6-7]. The complexity of gene expression deregulation in ATL or HAM/TSP diseases has been highlighted in a large number of studies involving transcriptional profiling using microarray analysis [8-12] and protein profiling employing a range of proteomics approaches [13]. However, comparative analysis of gene expression profiles between NI, AC, ATL and HAM/TSP individuals had never been investigated comprehensively. Herein, we used genomic approaches followed by bioinformatic analysis to identify gene expression profile characteristic of HTLV-1 infected individuals and particular disease states. Despite the infrequent cases of HTLV-1-infected individuals worldwide, we were able to gather a unique cohort of 8 NI and 30 HTLV-1 infected individuals from the Caribbean basin. Using state-of-the-art Affymetrix microarray technology, we identified ~1000 significant immune-related genes that were differentially regulated in CD4+ T-cells from 11 AC, 7 ATL, 12 HAM/TSP patients and 8 NI donors from Martinique and French Guyana **(Manuscript I)**. Based on their gene expression profiles, unique clustering of gene signatures could be identified among the ATL, HAM and NI groups – in accordance with their clinical status. Although clear class discrimination was observed between ATL, HAM and NI patients, the AC patients segregated among all groups.

Notably, NI and HAM/TSP displayed greater similarity in the gene expression pattern than with ATL. The ATL group presented a distinct profile compared to the NI group, suggesting that the profile of CD4+ T-cells from HAM/TSP are genetically less altered than CD4+ T-cells from ATL patients. This clear difference between HAM and ATL is in agreement with the previous observation that CD4+ T-cells from ATL patients undergo multiple alterations during the transformation process [14-15]. Several studies reported that the viral transcriptional activator Tax and the HTLV-I bZIP factor (HBZ) play a critical role in cellular transformation through deregulation of genes encoding i) proteins involved in cell growth and cell death (including proto-oncogenes) and/or ii) growth factors and their receptors [16-18]. In contrast to ATL, evolution to HAM/TSP does not involve cell transformation but is rather characterized by pro-inflammatory cytokine production and a high HTLV-1 proviral load [19]. An important caveat to consider in the interpretation of the present results is the percentage of non-infected versus HTLV-1-infected CD4+ T-cells circulating in the peripheral blood of HAM/TSP and ATL individuals. In HAM/TSP individuals the percentage of CD4+/CD25+ T-cells subset containing an integrated provirus ranges from 0.5% to 5%, as compared to the 5% to 90% of atypical cells found in ATL. However, our microarray study was performed on the entire population of CD4+ T-cells without discriminating between the HTLV-1-infected versus the non-infected CD4+ T-cell population and thus could explain why HAM/TSP and NI individuals exhibit distinct yet more similar gene expression patterns. Thus, the HAM/TSP profile may be due to substantial gene expression changes in a small proportion of total CD4+ T-cells or conversely moderate gene expression changes in a broad population of CD4+ T-cell due to specific paracrine effects occurring in HAM/TSP. Therefore, it would have been also appropriate to isolate the specific HTLV-1-infected CD4+/CD8-/CD25+ in order to identify genes that are specifically modulated within HTLV-1-infected cells during HAM/TSP disease state. Furthermore, neuropathological studies demonstrated focal infiltrate of HTLV-1-infected CD4+ T-cells, CD8+ T-cells and macrophages in the central nervous system [20-21]. It has been suggested that together with viral gene expression and cellular signaling mechanisms, HTLV-1 infected T-cells trigger a strong virus-specific T-cell response, leading to CNS

inflammation and autologous tissue damage [22]. Since HTLV-1 sequences have been detected in the cerebrospinal fluid (CSF) cells from HAM/TSP patients, and infiltrating CD4+ T-cells appear to be the main reservoir of HTLV-1 in spinal cord lesions, it is possible that HTLV-1-infected CD4+ T-cells from the peripheral blood differ from the "pathogenic" HTLV-1 infected CD4+ T-cells subset in the CNS [23]. It would be of particular interest to compare the gene expression profile of HTLV-1 infected CD4+ T-cells subset from the peripheral blood with "pathogenic" HTLV-1 infected CD4+ T-cells circulating in the CSF from HAM/TSP patients.

Another interesting aspect of this study arises from the observation that AC patients distributed among NI, ATL and HAM/TSP patients, revealing that the gene expression profile in circulating CD4+ T-cells from AC is not unique, but rather mimicked that of uninfected donors or one of the HTLV-1-induced diseases. HTLV-1 infected individuals remain AC for many decades before a fraction of them develop either of the HTLV-1-associated pathologies (reviewed in [24]. Although establishment of a diseased state is slow, once either HAM/TSP or ATL are diagnosed, disease progression is inevitable. It would therefore be advantageous to identify genes important for the preclinical or early diagnosis of HTLV-1 diseases - ATL or HAM/TSP - so that appropriate follow-up and treatment can be initiated. Our microarray data support the idea that a global disruption of the T-cell function – and therefore the immune response at large - occurs following HTLV-1 infection. Indeed, we show that the ATL phenotype was associated with the deregulation of genes involved in cell cycle, DNA repair and apoptosis including *CDC7*, *CDC16*, *BCL3*, *CFLAR*, *ATM*. This result is in agreement with previous studies showing that the HTLV-1 oncoprotein - Tax - induces leukemogenesis, through functional modulation of molecules and genes involved in growth signaling, cell cycle control and DNA repair [25-26]. For example, overexpression of the *BCL3* gene in ATL is consistent with a previous report demonstrating that Tax induces BCL3 mRNA expression to stimulate proliferation of ATL cells [27-29]. Tax also inhibits Fas-mediated apoptosis by upregulating CFLAR - or c-FLIP - in HTLV-1-infected T-cells via NF-κB activation [30-31]. These studies are in

agreement with the 3.1-fold increase obtained for *CFLAR* mRNA expression in ATL compared to NI patients. A characteristic manifestation of ATL disease is the extensive infiltration of leukemic cells into various organs, including lymph nodes, liver, spleen, lungs, and skin [32]. Tissue infiltration likely reflects certain unique biologic properties of the leukemic cells, and although these events are poorly understood, they may be related to the expression and function of chemokines, chemokine receptors [33-36] and adhesion molecules [37]. Strikingly, our microarray study revealed that genes important for cell adhesion (*ITGA2*, *SEPLG*), chemokine activity (*CCL14*, *GDF10*, *CXCR4*) are deregulated in ATL individuals. The chemokine receptor CXCR4 is upregulated about 8.5-fold in the ATL group compared to NI group. This target represents an interesting gene signature for ATL disease since a previous study from Lee *et al.* demonstrated that the CXCR4 signal pathway might play a role in the metastasis of breast cancer cells by inducing chemotactic and invasive responses, and thus could be involved in the widespread distribution of ATL cells into visceral organs and skin [38]. Tax has been shown to be involved in CXCR4 promoter activation through nuclear respiratory factor 1, suggesting a potential for amplification of CXCR4-mediated signaling via its agonist Stromal Derived Factor-1 (SDF-1/ CXCL12) [31]. In addition, the cell surface markers adhesion molecules CD2, CD48, CD63 were found to be highly up-regulated in ATL individuals (~20.1, 36.5, 7.1-fold, respectively) and could serve as potential gene signature for ATL disease. The utility of a gene signature depends on the ability to detect alterations in its expression pattern by routine laboratory testing (FACS, ELISA). CD2 and its ligand CD48 have been identified as markers of cell adhesion and T-cell activation [39-41]. Interestingly, it has been proposed that CD2 ligation might play an important role in the HTLV-1 life cycle by potentially promoting viral transcription and thus enhancing transmission of cell-associated HTLV-1. This hypothesis is particularly relevant since HTLV-1-infected lymphocytes are known to be mitogenic to resting T-cells and thymocytes via activation of CD2 signaling pathway [42-43]. Therefore, CD2, CD48 and CD63 expression on CD4+ T cells may be useful candidate markers to monitor disease progression from AC to ATL – and should be tested for their positive predictive value in ATL development.

In contrast to ATL, our microarray experiment confirms that the HAM/TSP phenotype is associated with a deregulation of genes involved in the pro-inflammatory response such as *SOCS1*, *IKBKG*, *ENG*, *STAT3*, *IFN-γ* and *SPN*. Elevated levels of IFN-γ have been previously observed in the serum of HAM/TSP and AC patients and therefore could constitute an important immunological marker in HAM/TSP pathogenesis [44]. IFN-γ, which is secreted by activated T-lymphocytes and NK cells, is considered to play a deleterious role in immune-mediated demyelinating disorders such as multiple sclerosis [45-46]. However, the exact role(s) of IFN-γ in the multiple sclerosis remains controversial, with evidence suggesting protective role in early MS and deleterious effects in late stage of MS disease [47]. Interestingly, SPN (sialophorin also known as CD43) is one of the most abundant CD4+ and CD8+ T-cell surface glycoproteins and although its function remains controversial, it has been suggested to play a role in T-cell activation and to have pro-adhesive properties in T-cell trafficking [48-50]. Furthermore, CD43 might also play a role in T-cell trafficking to the central nervous system. Onami *et al*. reported that antigen-specific CD8 + T-cells do not migrate into the brain of CD43 knockout mice that are intracerebrally infected with Lymphocytic Choriomeningitis Virus (LCMV) [51]. In agreement with this idea, *CD43* was shown to promote the infiltration of CD8+ T-cells into the brain of mice infected with Dengue virus [52]. Thus, *CD43* represents an interesting gene signature for early diagnosis of HAM/TSP disease since it could have an important role in the infiltration of HTLV-1-infected CD8+ or CD4+ T-cells, leading to inflammation, myelin loss, and axonal damage in the central nervous system (CNS) [22, 53-54]. Of particular interest, *ENG* (endoglin) gene was highly up-regulated in AC (6.3-fold) and HAM/TSP (5.4-fold) and not in ATL patients. ENG (CD105) is a membrane glycoprotein considered as a specific marker of proliferating and endothelial cells, which are characteristic of angiogenesis. CD105 has been reported to act as a co-receptor for transforming growth factor β (TGF-β), which is found in multiple sclerosis (MS) lesions [55]. Recently Holley *et al.* found that vessels containing proliferating endothelial cells were more present in MS tissue than control tissue suggesting that angiogenesis may play a role in lesion progression, failure of repair and scar formation found in brain tissue of individuals with MS and HAM/TSP patients [56].

Although ENG is mainly found in endothelial cells, Schmidt-Weber *et al.* demonstrated that TCR engagement enhances expression of ENG at the surface of CD4+ T-cells. TGF-β inhibits T-cell activation and alters differentiation of naive T-cells into effector cells. In contrast to the suppressive signal mediated by the TGF-β, cross-linking of ENG substantially enhances T-cell proliferation, indicating that CD105 by itself mediates signal transduction. Therefore, ENG acts as a regulatory receptor, counteracting TGF-β-mediated T-cell suppression [57]. *ENG* could represent an interesting gene signature for the early diagnosis of HAM/TSP disease. However, as discussed previously, it would be worthwhile to validate these genes in the circulating HTLV-1-infected CD4+ T-cells from the CSF, since this subset of cells is more likely representative of the pathogenic population of CD4+ T-cells from HAM/TSP patients.

A comparison of gene expression profiling of the 11 AC clearly identified that 3 AC had a similar profile to the ATL group. In fact, these 3 AC were from French Guyana where ATL predominates among the black population [58-59]. Since the progression of ATL versus HAM/TSP is expected to be influenced by unidentified racial determinants and environmental triggers [60-61], it is possible that these patients are more likely to progress to an ATL phenotype. Annual long-term (~20 years +) follow-up of all AC enrolled in this study would reveal if any of the patients develop the predicted HTLV-1 associated diseases. As well, the correlation (if any) between disease stage and gene signature expression could be established.

It is important to notice that hierarchical clustering of genes from microarray studies may be confounded by multiple variables – such as i) demographics of patient cohort, and ii) stage of disease progression versus clinical diagnosis. Our case-control study was not designed to adjust for demographic confounders nor the effect of disease progression on gene expression – questions that would be best addressed in a prospective longitudinal study. Moreover, we are aware that it would be worthwhile to validate the

identified genes of interest in a separate set of patient samples from the Caribbean basin, but at the time of the study, we had limited access to patient samples, which restricted our ability to validate these genes in a sufficiently sized cohort.

The risk for HTLV-I-carriers to develop ATL and HAM/TSP varies mainly and markedly across Japanese (15-30%) and Caribbean populations (3-6%) [62]. In Japanese carriers, the annual incidence of ATL is comparatively high, peaks around 60 years old, and is approximately 3 times greater in men than in women. The incidence of ATL among Caribbean carriers is less than 1/3 the rate in Japan, peaks in the forties, and does not vary by gender. In contrast, the incidence of HAM/TSP is relatively high among Caribbean carriers, whereas in Japan HAM/TSP occurs at 1/10 the rate in the Caribbean. HAM/TSP demonstrates a female predominance in both populations. Since the array data has given us a set of potential biomarkers that may be predictive of disease onset and progression, it would be of particular interest to validate these biomarkers in another patient cohort such as Japanese cohort and further characterize their molecular involvement in HTLV-1 pathogenesis.

Our understanding of human biology and disease is ultimately dependent on a complete understanding of the genome and its functions. New technologies are rapidly expanding our analytical power. Among the technical innovations developed in the past few years, cDNA and oligonucleotide microarrays have revolutionized the way we look at and understand gene expression, allowing the rapid quantification of a panel of genes at once in a given cell population. Recently, technological advancements such as “Direct RNA sequencing” (DRNA) for in-depth quantitative characterization of transcriptomes have emerged as valuable tools for understanding cellular physiology as well as human diseases biology, and have begun to be used in clinical diagnostic applications [63-64]. These new platforms allow sequencing of RNA molecules directly without prior synthesis of cDNA or the need of ligation and amplification. Since this new technology has several applications ranging from chromatin immune-precipitation, mutation mapping and polymorphism discovery to non-coding RNA discovery, it would be interesting to use

these platforms to establish a genome wide comparison between Japanese and Caribbean HTLV-1 cohorts and ultimately provide functional annotations for gene variants that may predict disease susceptibility and genetic risk factors [65-66].

The purpose of this microarray study was to identify genes of interest which could be tested at a molecular level for a specific role in the innate immune response to HTLV-1 infection. This goal was accomplished with the publication **Manuscript I**, and further shown to be a reliable method to identify salient genes involved in the immunologic deregulation during HTLV-1 infection.

### *Mechanisms of SOCS1-mediated suppression of the IFN antiviral response in HTLV-1-infected cells*

Among the many genes modulated during HTLV-1 infection, the suppressor of cytokine signaling – SOCS1 – was upregulated in AC (5-fold) and HAM/TSP (3.8-fold) compared to NI patients. SOCS1 was discovered as a cytokine-inducible intracellular negative regulator that inhibits IFN-γ signaling by triggering ubiquitination and proteasomal degradation of various components of the JAK-STAT signaling cascade [67-72]. SOCS1 is induced during virus infection and binds directly to the type I IFN and/or II IFN receptors to suppress IFN signaling, thereby preventing chronic inflammation. However, SOCS1 can be subverted to enhance viral replication via untimely inhibition of the IFN response [73-77]. For instance, Potlichet *et al.* reported that Influenza A virus suppresses the antiviral response by inducing SOCS1 and SOCS3 via TLR3-independent but RIG-I/IFNAR dependent pathways [78]. SOCS1 plays also an important role in the inhibition of the antiviral effect of IFN-γ in keratinocytes infected with HSV-1 [79]. Thus, it has become apparent that virus-induced upregulation of SOCS1 constitutes a powerful immune evasion mechanism employed by several viruses to inhibit the early IFN response to infection.

Initiation of the type I IFN response upon RNA virus infection occurs via a well-defined signaling cascade involving viral recognition by cytoplasmic sensors, activation of the IKK and IKK-related kinases and downstream transcription factors IRF-3 and NF-κB, which mediate *IFN-β* gene transcription (reviewed in [80]. Secreted IFN-β is then recognized by IFN receptors in neighboring cells. This activates the JAK/STAT pathway and stimulates the expression of IRF-7, IFN-α and hundreds of IFN-stimulated genes, resulting in the establishment of a strong antiviral state. Although only a few reports have documented the effect of IFN-α/β on HTLV-1, it is clear that type I IFN constitutes a potent anti-retroviral mechanism that dramatically affects HTLV-1 replication in the host [81-82].

Reciprocally, accumulating evidence indicates that HTLV-1 possesses evasion mechanisms to block type I IFN signaling. Feng *et al.* reported that HTLV-1 suppress IFN-α-stimulated JAK-STAT activation by reducing phosphorylation of tyrosine kinase 2 and STAT2, possibly through a Gag- or Pr-mediated mechanism [83]. Another study suggested that the HTLV-1 Tax protein negatively modulates IFN-α-induced JAK/STAT signaling by competing with STAT2 for CBP/p300 coactivators [84].

Herein, we demonstrated that SOCS1 gene expression positively correlated with HTLV-1 mRNA levels in HAM/TSP patient samples. In PBMCs expressing an HTLV-1 proviral clone, we observed a transient induction of IFN-α/β expression, followed by SOCS1 up-regulation, robust HTLV-1 replication and extinction of IFN production. From these observations two hypotheses emerged: 1) HTLV-1 infection in PBMCs is detected through yet unidentified pathogen recognition receptors (TLR or RLR) resulting in an early synthesis IFN-β production; and 2) SOCS1 plays a key role in HTLV-1 infection by dampening the IFN response and thus favoring viral replication. Since antigen presenting cells (APCs) have been shown to become infected with HTLV-1 *in vivo*, it is possible that in PBMCs, HTLV-1-infected pDCs are partly responsible for the transient activation of

the type I IFN response. Colisson *et al.* demonstrated that upon *ex vivo* recognition of cell free HTLV-1 particles through TLR7, pDCs produce large amounts of IFN-α 24h post-infection, in agreement with our experiment in PBMCs [85]. However, another group reported that *ex vivo* pDCs from HTLV-1-infected patients are impaired in their response to TLR7 agonists and in production of IFN-α, suggesting a role for pDCs in viral persistence and possibly disease progression. Unpublished observations from our group reveal that HIV RNA triggers both RIG-I and TLR signaling pathways (Solis *et al*., manuscript in revision), therefore it is possible that the HTLV-1 mRNA genome activates the same signaling pathways. Another interesting candidate gene stemming from our microarray is the upregulation of the newly identified DNA sensor - Absent in melanoma 2 (AIM2) - in ATL (2.7-fold) and HAM/TSP (2.7-fold) patients. AIM2 is involved in the recognition of cytosolic DNA produced during infection by DNA viruses and bacterial DNA [86]. This sensor might recognize HTLV-1 DNA after the reverse transcription step (prior to provirus integration into the host genome) and subsequently trigger pro-inflammatory cytokine production. It is therefore our hypothesis that HTLV-1 activation of SOCS1 functions as a multi-pronged mechanism to evade the IFN antiviral response initiated by PRRs. However, additional research is required to further delineate which PRRs are responsible for the recognition of HTLV-1 in APCs such as macrophages, conventional dendritic cells and CD4+ T-lymphocytes.

To corroborate the idea that HTLV-1-induced SOCS1 expression functions as a viral evasion mechanism to limit the antiviral immune response, we used siRNA technology and showed that depletion of SOCS1 in MT-2 cells - a non-leukemic cell line chronically infected with HTLV-1 -restored IFN production and reduced HTLV-1 replication. HTLV-1 induced SOCS1 expression limited IFN production by targeting IRF3 for ubiquitin-mediated proteasomal degradation. SOCS1 protein - through its SOCS box domain - acts as a scaffold to the ubiquitin ligase complex formed of Elongin B/C, Cullin 5, and Rbx1 to trigger K48-linked ubiquitination of lysine residues and proteasomal degradation of target proteins. Mutant lacking the SOCS-box – SOCS1ΔB/C box – prevents proteasomal degradation of IRF3. Similarly, IRF3 turnover is completely reversed when SOCS1 is overepressed in conjunction with ub-K48R – an ubiquitin

mutated so that it is unable form a K48-polyubiquitin chain. Thus, our finding of SOCS1-induced K48-linked ubiquitination of IRF3 reveals a previously unrecognized mechanism to inhibit antiviral responses. Further studies, such as identification of the lysines residues of IRF3 targeted by ubiquitination, are required to extensively elucidate the details of SOCS1-mediated proteasomal degradation of IRF3. These results also raise the possibility of a cross-talk mechanism between type I and type II interferon signaling mediated by SOCS1. Although we have established this negative regulatory loop in HTLV-1 infected cells, it is possible that this mechanism also operates in non-infected activated T-cells that produce high levels of IFN-γ. In this context, T-cell activation would limit the induction of type I IFN and its downstream effects: diminished protein synthesis, absence of cell proliferation and induction of apoptosis. If HTLV-1 virus highjacks this regulatory loop through constitutive induction of IFN-γ or by directly inducing SOCS1 expression through Tax, this would increased viral replication. Preliminary data was suggestive of Tax being responsible for SOCS1 induction. Bioinformatics analysis of the SOCS1 promoter region reveal the presence of CRE, AP-1 and NF-κB binding regions, suggesting the possible involvement of Tax in the induction of SOCS1 expression (data not shown). Another possibility is that SOCS1 transcriptional activation is not directly regulated by viral proteins, but rather by recognition of viral RNA and downstream signaling events. Viral RNA recognition by PRRs may induce cytokine(s) which in turn enhance the expression of SOCS1 [78].

***Induction of SOCS1 enhances viral mRNA synthesis in HTLV-1-infected cells***

Another interesting aspect of our study stems from the observation that SOCS1 enhances HTLV-1 mRNA load. Expression of the HTLV-1 provirus together with SOCS1 in T-cells resulted in enhanced HTLV-1 mRNA synthesis. Interestingly, a report from Ryo *et al.* showed that SOCS1 is an inducible host factor during HIV-1 infection and enhances HIV-1 particle production in infected cells. SOCS1 directly associates with HIV-1 Gag to facilitate its stability and intracellular trafficking and ultimately promotes

efficient production of HIV-1 particles [87]. Since SOCS1 has been shown to possess pleiotropic functions, it is possible that SOCS1 plays a dual role during HTLV-1 infection. First, SOCS1 would limit type I IFN signaling by mediating IRF3 degradation; second, SOCS1 would bind to HTLV-1 Gag and facilitates its cellular trafficking and contribute to particle production. This hypothesis based on what has been observed during HIV-1 infection, and needs verification in HTLV-1 infection models.

Among the many candidate genes identified as potential marker for the early diagnosis of HTLV-1 associated diseases, SOCS1 appears to have physiologic implications for the pathobiology and clinical outcome of HAM/TSP disease. Although SOCS1 has been clearly identified as key regulator of the type I/II IFN response, it remains subject to speculation whether it is involved in reducing the responsiveness of HAM/TSP patients to IFN-α therapy. Exploring individual differences in SOCS gene expression and correlating this intermediate phenotype with single nucleotide polymorphisms in the SOCS1 gene from HAM/TSP responders and non-responders to IFN-α therapy might help to predict who will respond best to therapy. Overall, this strategy would provide insights into the development of improved IFN-α treatment regimens for HAM/TSP disease.

***VSV a potential therapy for ATL***

Although targeting SOCS1 could represent a new approach to enhance the therapeutic efficacy of type I IFNs in HAM/TSP, there is a strong need for novel ATL treatments. Standard chemotherapeutics regimens have failed miserably in the treatment of ATL. Since ATL is a rare disease, clinical trials to establish treatment standards have been sparse. As a result, the common frontline therapy to treat ATL mirrors that which is used to cure other types of T-cell lymphomas such as CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) or EPOCH (etoposide, vincristine, doxorubicin,

cyclophosphamide and prednisone) regimes. Other treatments include the nucleoside analog azidothymidine (AZT, Zidovudine) in combination with IFN-α, which induce a partial, but temporary remission. New drugs currently in clinical trials for other T-cell lymphomas are emerging as potential treatments for ATL: PDX (pralatrexate), the HDI Vorinostat (Zolinza) and Bortezomib (Velcade). These current therapies are nevertheless ineffective in prolonging the survival rate of acute ATL patients, which has a median prognosis of 6 -10 months. The aggressive phenotype of ATL, coupled with its resistance to standard chemotherapeutic strategies, suggests that this leukemia may be an appropriate target for oncolytic virus (OVs) therapy. OVs constitute a novel biotherapeutic approach to treat malignancies refractory to standard therapy. Replication-competent OVs selectively infect and replicate in tumor cells that harbor defects in key antiviral, survival, and growth signaling pathways – effectively leading to their destruction. Normal cells and tissues are spared because intact antiviral responses naturally limit OV replication. Thus, OVs are considered highly targeted biological anti-cancer weapons with limited toxicity in comparison to conventional treatments. Vesicular Stomatitis Virus (VSV) is an enveloped, negative strand RNA virus that serves as a prototype for OV therapy. VSV infection has been reported to selectively kill a large panel of human tumour cell lines, clear bone marrow of leukemic AML cells and effectively arrest metastatic spread of murine tumors in immunocompetent animals [88-90]. While most studies of VSV oncolysis *in vitro* and/or in animal models have been performed with cell lines, an important question that remains to be addressed is the ability of this virus to infect and kill primary cancer cells. Herein, we investigated the oncolytic activity of VSV in primary PBMCs from individuals with acute ATL versus PBMCs from healthy volunteers (**Manuscript II**). VSV replicates and induces cell death selectively in *ex vivo* leukemic cells from ATL patients, whereas PBMCs from healthy donors remain unaffected. This suggests that VSV represents a potential strategy to specifically kill leukemic cells in mixed cultures with relatively minimal impact on normal cells. Similar results were obtained with VSV-AV1 – an attenuated laboratory strain of VSV (data not shown). Both VSV wild-type and VSV-AV1 are able to induce the expression of the genes encoding interferon, but the mutant virus fails to block the export and translation of

the interferon mRNA – resulting in a robust IFN response [91-92]. The induction of IFN and other antiviral genes by this attenuated virus generates what is been termed a "cytokine cloud" that protects the host not only from the mutant virus but also from any wild-type virus presents in the inoculum. The strategy produces an effective oncolytic virus that is less toxic than a recombinant virus engineered to express interferon [92-93]. At the time of this study, VSV-AV1 was not an available resource in our laboratory; however, the oncolytic properties of this VSV strain were later tested in PBMCs from ATL patients and gave similar results to those obtained with VSV-wt (data not shown). Due to its low toxicity index in normal cells, VSV-AV1 would be appropriate for future *in vivo* studies. Although we demonstrate the *ex vivo* oncolytic potential of VSV in primary ATL cells, we cannot predict the oncolytic efficiency of VSV *in vivo*. Further studies in immunocompetent ATL mouse model would provide some indication of the anti-tumoral response following VSV injection and would help determine the contribution of the adaptive immune system for treatment efficacy *in vivo*.

Alternatively, VSV could be used as an *ex vivo* strategy to purge contaminating tumour cells from autografts. High-dose cytotoxic chemotherapy followed by autologous heamatopoietic stem cell transplantation (ASCT) has been used for the treatment of cancers that are refractory to standard therapeutic regimes [94]. However, a major challenge with ASCT for patients with hematological malignancies, such as ATL, is disease relapse due to either contamination with cancerous hematopoietic stem and progenitor cells within the autograft, or the persistence of residual therapy-resistant disease niches within the patients [95-96]. The purging potential of VSV has already been tested in acute myelogenous leukemia (AML) cell line OCI/AML3 using VSV-wt. Stojdl *et al*. reported that OCI/AML3 cell lines co-cultured with normal bone marrow cells at 10% tumour burden challenged with VSV-wt show complete destruction of tumours and sparing of bone marrow progenitors [88, 97]. In addition, Lichtly *et al.* demonstrated that VSV-AV1 is able to selectively eliminate a leukemic cell line with only minimal effects on the colony-forming ability of normal peripheral blood progenitor cells [98]. Thus, VSV could represent a promising therapeutic approach to prevent cancer relapse by

eliminating tumour-initiating cells that contaminate the autograft prior to transplantation in ATL individuals and ultimately cure the patient.

### *VSV replicates in highly proliferating cells, but not in CLL arrested in the $G_0$ phase*

VSV infection selectively kills a large panel of human tumour cell lines, including 80% of the NCI 60 tumor cell bank. However, 20% of tumour cells tested were partially or completely refractory to VSV oncolysis, suggesting that in the clinical setting many primary cancers may not respond to VSV treatment. To investigate the specificity of VSV oncolysis, we tested the ability of VSV to replicate in and kill another type of primary leukemia – Chronic Lymphocytic Leukemia (CLL). Surprisingly, primary *ex vivo* B-CLL samples were resistant to VSV replication and subsequent cell death, even when infected at high multiplicity of infection (MOI) (10 MOI). The discrepancy in VSV susceptibility may be due to the fact that CLL cells are non-proliferating B-cells arrested in the $G_0$/early $G_1$ phase of the cell cycle, whereas ATL cells are highly proliferating cells [99-100]. Since activation of the IL-2/IL-2R loop during the acute phase of HTLV-1 infection contributes to the activation and proliferation of HTLV-1 infected CD4+/CD25+ subset of ATL cells [101-102], we hypothesized that activation and proliferation of lymphocytes could render them susceptible to VSV-mediated oncolysis. In support of this notion, HTLV-1-infected non-leukemic CD4+ T-cells from HAM/TSP patients - known to spontaneously proliferate *in vitro* - should be permissive to VSV-mediated oncolysis [61, 103]. However, PBMCs from HAM/TSP individuals appear resistant to VSV cytolytic activity. This result might be due to the sensitivity of our assay. In PBMCs from HAM/TSP the HTLV-1-infected subpopulation ranges from 1-5% compared to the 40-80% found in PBMCs from acute ATL donors. Therefore, to ascertain whether VSV replication and cell death is linked to the state of cell growth or activation induced by HTLV-1 infection, it would be of interest to assess the differential sensitivities of the HTLV-1-infected and non-infected cell compartments in PBMCs from HAM/TSP patients. *Ex vivo* infection of PBMCs from HAM/TSP patients with VSV-encoding a green fluorescent protein (GFP) and Annexin V as well as CD4+, CD25+, HTLV-1 env

(envelope) staining of the HTLV-1-infected T-cell population could help to assess whether the non-leukemic HTLV-1-infected cells from HAM/TSP are susceptible to VSV replication and lysis.

Both VSV permissive and non-permissive primary cells and cell lines have been identified. However, due to their stable phenotype, these distinct cell types or cell lines do not allow a complete study of the molecular basis for differences in the permissive state. The ability to monitor VSV infection in a single cell type during the conversion from the non-permissive to the permissive state provides insight into the basis of host-imposed regulation of virus replication. Therefore, we examined the effect of cell activation on VSV oncolytic activity by treating *ex vivo* primary CD4+ T-cells from healthy volunteers with anti-CD3 and anti-CD28, which reproduced a similar activation/proliferation state to that of CD4+ T-cells infected with HTLV-1. VSV replicated and induced cell death in primary CD4+ T-cells compared to non-stimulated cells, revealing a relationship between T-cell activation status and VSV replication. Our data indicate that VSV replication is time-dependent and related to the intensity of T-cell activation; yet at the same intensity of activation, primary *ex vivo* ATL cells remain more permissive to VSV oncolysis than activated CD4+ T-cells. This further supports the idea that in addition to cell activation status, VSV replication in ATL cells might be also influenced by defects unique to tumour cells including deficiencies in antiviral IFN-α/β, p53 dysfunction, myc overexpression [88, 92, 104-105]. Studies demonstrated that translation control downstream of PKR activation, frequently deregulated in transformed cells, can cooperate with the attenuated IFN antiviral activity to facilitate VSV oncolysis. High levels of the translation initiation factor - eIF2Bε - are essential for increased permissiveness of transformed cells to VSV replication and cytolysis [106]. However, no change in the expression of eIF2B or Bε has been observed in resting or activated CD4+ T-cells (data not shown), suggesting that other cellular components restricting VSV replication to cancer and leukemic cells are likely to be accentuated during T-cell activation. Accordingly, we characterized the signaling events governing VSV permissiveness in primary leukemia and normal activated T-cells (**Manuscript III**).

***VSV oncolysis requires cell cycle entry and translation initiation in lymphocytes***

TCR-CD3 engagement mediates signaling pathways that can result in activation, anergy or apoptosis - the outcome depending upon T-cell differentiation state and the nature of co-stimulatory transmitted by the antigen presenting cell. Stimulation of CD4+ T-lymphocytes by TCR plus co-receptor engagement triggers the activation of two distinct signaling pathways: Ras and PI3K. The Ras pathway signals through the mitogen-activated protein kinases (MAPKs) - the extracellular signal-regulated kinase (ERK), the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 - whereas PI3K activates Akt (reviewed in [107]). The signals mediated by these upstream kinases, but not p38, culminate in the activation of transcription factors such as NF-AT, AP-1, NF-κB and ultimately lead to the upregulation of genes involved in protein translation, cell survival and cell cycle progression [108]. We show that VSV replication requires activation of ERK, JNK and Akt but not p38, in primary CD4+ T-lymphocytes (**Manuscript III**). Inhibition of any one of these kinases by using specific pharmacological inhibitors prior activation of the CD4+ T-cells blocks VSV proteins synthesis but not VSV mRNA expression. This clearly indicates that suppression of these signaling pathways has no effect on virus uptake, uncoating or transcription but rather affects VSV mRNA translation. These results are in agreement with a previous study from Rose *et al.* that showed that VSV can infect both permissive and non-permissive cells to produce viral mRNAs - VSV protein synthesis occurring only in permissive cells [109]. This further supports our idea that cell permissiveness depends on post-transcriptional regulation of viral protein synthesis. While our experiments were being conducted, Noser *et al.*, reported that activation of the RAS/Raf/MEK/ERK signaling pathway enhances VSV oncolysis in NIH-3T3 cells via negative regulation of IFN-*α*-mediated antiviral response [110]. Our data demonstrate that although activated CD4+ T-cells synthesize high levels of IFN-γ, they are paradoxically poor producer of type I IFN compared to human epithelial cells infected with VSV. It is therefore possible that the amount of type I IFN produced by activated CD4+ T-cells is not sufficient to mount an appropriate antiviral response against VSV. Further studies are required to elucidate why IFN-γ has undetectable or minimal antiviral effect on VSV replication in activated T-

cells. We speculate that IFN-γ may limit the antiviral response by enhancing the expression of negative regulator of cytokine signaling. Another possible explanation is that IFN-γ is a positive regulator of growth and differentiation for CD4+ T-cells and thus enhances VSV replication.

Noser *et al.* also showed that suppression of the RAS/Raf/MEK/ERK signaling pathway in RAS-overexpressing cells has no effect on VSV replication. Accordingly, our results demonstrated that MEK1/2 inhibitor does not block VSV protein synthesis when added to pre-activated CD4+ T-cells. Thus, VSV replication in CD4+ T-lymphocyte is dependent on global activation events and not on the direct action of ERK, JNK or Akt kinases on their downstream target genes. It is well accepted that following CD4+ T-cells stimulation, MAP kinases and PI3K signaling pathways induce cell proliferation by acting at the transcriptional and post-transcriptional levels on cdk inhibitory proteins as well as cyclin molecules. Because inhibition of one of these signaling pathways arrests the cell in the $G_0/G_1$ phase and blocks VSV protein synthesis, we hypothesized that VSV replication is dependent on cell cycle entry. The cdk inhibitor $p27^{kip1}$ is considered an important regulator of T-cell cycle progression: high levels of $p27^{kip1}$ are present in resting T-cells and prevent $G_1$ to S transition by inhibiting cyclin E/cdk2 complex [111]. Mitogenic signals increase cyclin D protein levels and cyclin D/cdk4,6 kinase activity leading to the hyperphosphorylation of the retinoblastoma tumor suppressor gene product (Rb) which in turn prevents Rb binding to E2F family transcription factors. E2F heterodimers are then released and stimulate transcription of genes such as cyclin E, required for entry into S phase. $p27^{kip1}$ protein is subsequently released and degraded via the proteasome pathway by cyclin E/cdk2-dependent and -independent mechanisms that require MEK and PI3K activation [112-114]. Once $p27^{kip1}$ is degraded, newly synthesized cyclin E/cdk2 heterodimers are released and orchestrate $G_1/S$ transition. The use of different cell cycle inhibitors - oloumicine ($G_0$/early $G_1$), aphidicolin ($G_1$/S), taxol and nocodazol ($G_2$/M) - to specifically block cell cycle progression independently of MAPK and PI3K activity confirm that entry into $G_1$ phase, but not progression through S or $G_2$/M, allows VSV replication in activated CD4+ T-cells. To corroborate the idea that

VSV-mediates oncolysis of highly proliferating cells, Chakraborty *et al*. demonstrated that VSV preferentially kills tumour cells that have a high mitotic index [115]. During mitosis, VSV M protein interacts with Rae1-Nup98 and RNP complex, causing spindle abnormalities and triggers cell death. Based on this observation, we speculate that increasing mitotic events, by inducing CD4+ T-cell proliferation, could render proliferating CD4+ T-cells more susceptible to VSV-mediated cell death during mitosis, however further assessment of VSV-induced apoptosis in CD4+ T-cells synchronized at the $G_0/G_1$, S and $G_2$/M phase of the cell cycle are required to confirm or refute this hypothesis.

Activation of the ERK, JNK, or AKT signaling pathways appears to confer VSV permissiveness to activated CD4+ T-cells by mediating cell cycle entry, which is characterized by a sharp increase in protein translation. Cell cycle progression is tightly linked to protein synthesis and ribogenesis. Translation rates are high in cancer cells, and deregulation of the RAS/MEK/ERK and PI3K/AKT/mTOR signaling pathways has been reported to contribute to cancer development and maintenance [116]. The mammalian target of rapamycin (mTOR) plays a central and coordinate role in protein synthesis and cell cycle progression. mTOR regulates cell growth by phosphorylating the ribosomal S6 Kinase 1 (S6K1) and eIF4 binding protein 1 (4EBP1); its activity is controlled through phosphorylation by the serine/threonine kinase Akt (reviewed in [117-118]). Phosphorylation of 4EBP1 leads to its dissociation from the translation initiation factor eIF4E, which in turns is phosphorylated by MAPK-interacting protein 1 (Mnk1). In CD4+ T-cells ERK is an upstream regulator of MNK and thus is directly involved in the activation of eIF4E throughout the cell cycle [119]. Phosphorylation and activation of eIF4E enhances ribosome recruitment to the translational start site and initiates protein synthesis. Our data show that ERK, Akt, and JNK signaling pathways regulate translation initiation and ribosome biogenesis since their specific inhibition by pharmacological inhibitors prevented mTOR and eIF4E phosphorylation. Moreover, considering that VSV mRNAs are capped and polyadenylated, we suggest that eIF4E is directly involved in VSV replication. In support of this notion, pretreatment of activated CD4+ T-cells with

rapamycin - which induces arrest of the cell in the $G_0/G_1$ phase and blocks mRNA translation by binding mTOR – and also inhibits VSV protein synthesis. Although we show that mTOR-mediated translation initiation and ribosome biogenesis during $G_1$ phase is essential for VSV replication in primary activated CD4+ T-cells, we are not able to attribute a direct role of eIF4E in VSV replication since depletion of eIF4E leads to cell cycle arrest in $G_0/G_1$ phase.

Contradictory results by Connor *et al.* showed that rapamycin has no effect on VSV replication in HeLa cells. This discrepancy might be explained by the fact that the antiproliferative effect of rapamycin is maximal in quiescent CD4+ T-cells that express high levels of $p27^{kip1}$, but not in exponentially growing cells [120]. Recently, Marozin *et al.* reported that cell cycle progression or translation is not essential for VSV oncolysis of hepatocellular carcinoma [121]. This observation emphasizes the importance of cell-type specificity for VSV oncolysis. Activated CD4+ T-cells appear as a good model to study the oncolytic properties of VSV. Indeed, activated CD4+ T-cells have a similar molecular make-up to cancer cells, especially concerning their inability to mount an efficient innate immune response, which is irreparably compromised during malignant transformation.

Overall, the present study clearly shows that activation of ERK, JNK and Akt but not p38 signaling pathways - leading to $G_0$ to $G_1$ phase transition - is crucial for VSV replication in primary CD4+ T-cells, due to a global increase in protein translation mediated by the activation of mTOR and eIF4E. As a supporting model, treatment of quiescent *ex vivo* primary B-CLL with PMA/ionomycin induces cell cycle entry concomitantly with eIF4E phosphorylation and thereby restores VSV-mediated oncolysis. A cDNA microarray analysis of the differences between resting and activated CLL cells, either infected or not with VSV, would provide significant insights into the genes/pathways that might be affected during CLL activation and ultimately rendering this leukemia permissive to VSV oncolysis.

In conclusion, the application of oncolytic viruses as novel agents in cancer therapy should be based on the understanding of cancer cell biology. Identification and characterization of host factors that facilitate tumor-specific OV replication is critical for the design of viral vectors with effective and selective anti-tumor activity, while protecting normal tissue from toxicity. Combination therapy represents a promising avenue for ongoing translation of oncolytic viruses into clinical practice (reviewed in [122]).

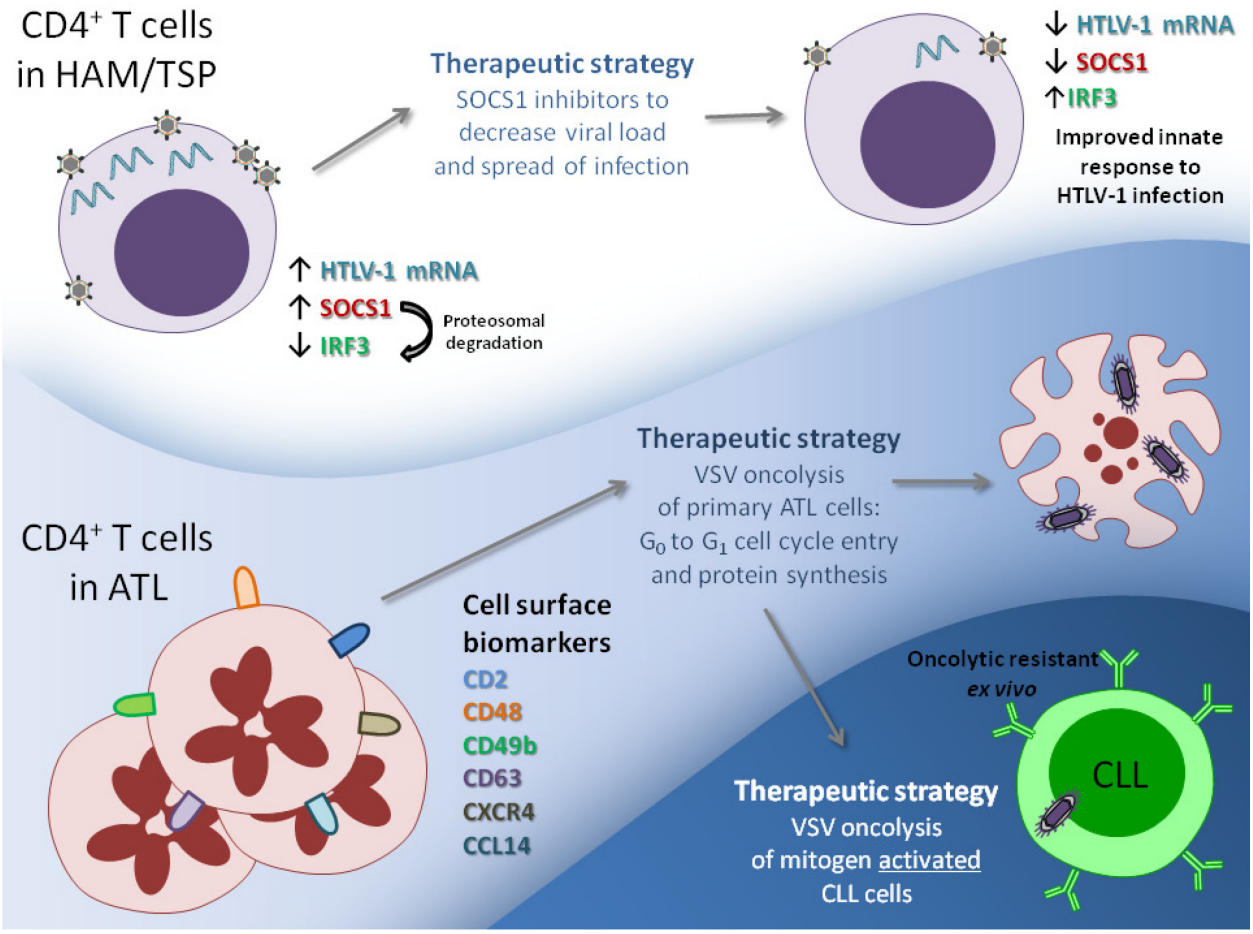



**Figure 1. Summary of the candidate's research findings. Novel therapeutic strategies for HAM/TSP and ATL.** These interventions are either aimed at 1) increasing the antiviral immune response and to limit HTLV-1 mRNA expression in HAM/TSP or at 2) exploiting the defective control of cell cycle - which is characterized by a sharp increase in ribogenesis and protein synthesis - to treat ATL and CLL with VSV, and 3) ultimately provide incentive to explore other combinatorial approaches - mitogenic agent and VSV - to overcome the resistance of different cancers such as quiescent CLL cells to oncolytic virus therapy.

# REFERENCES


1. Fujita, F., et al., *Inactivation of porcine endogenous retrovirus by human serum as a function of complement activated through the classical pathway.* Hepatol Res, 2003. **26**(2): p. 106-113.
2. Golding, M.C., L. Zhang, and M.R. Mann, *Multiple epigenetic modifiers induce aggressive viral extinction in extraembryonic endoderm stem cells.* Cell Stem Cell, 2010. **6**(5): p. 457-67.
3. Perez-Caballero, D., S.J. Soll, and P.D. Bieniasz, *Evidence for restriction of ancient primate gammaretroviruses by APOBEC3 but not TRIM5alpha proteins.* PLoS Pathog, 2008. **4**(10): p. e1000181.
4. Oliere, S., et al., *Vesicular stomatitis virus oncolysis of T lymphocytes requires cell cycle entry and translation initiation.* J Virol, 2008. **82**(12): p. 5735-49.
5. Cesaire, R., et al., *Oncolytic activity of vesicular stomatitis virus in primary adult T-cell leukemia.* Oncogene, 2006. **25**(3): p. 349-58.
6. Kaplan, J.E., et al., *The risk of development of HTLV-I-associated myelopathy/tropical spastic paraparesis among persons infected with HTLV-I.* J Acquir Immune Defic Syndr, 1990. **3**(11): p. 1096-101.
7. Gessain, A., et al., *Antibodies to human T-lymphotropic virus type-I in patients with tropical spastic paraparesis.* Lancet, 1985. **2**(8452): p. 407-10.
8. de La Fuente, C., et al., *Gene expression array of HTLV type 1-infected T cells: Up-regulation of transcription factors and cell cycle genes.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1695-700.
9. Pise-Masison, C.A., et al., *Transcription profile of cells infected with human T-cell leukemia virus type I compared with activated lymphocytes.* Cancer Res, 2002. **62**(12): p. 3562-71.
10. Ng, P.W., et al., *Genome-wide expression changes induced by HTLV-1 Tax: evidence for MLK-3 mixed lineage kinase involvement in Tax-mediated NF-kappaB activation.* Oncogene, 2001. **20**(33): p. 4484-96.
11. Sasaki, H., et al., *Overexpression of a cell adhesion molecule, TSLC1, as a possible molecular marker for acute-type adult T-cell leukemia.* Blood, 2005. **105**(3): p. 1204-13.
12. Baba, M., et al., *Highly enhanced expression of CD70 on human T-lymphotropic virus type 1-carrying T-cell lines and adult T-cell leukemia cells.* J Virol, 2008. **82**(8): p. 3843-52.
13. Wu, K., et al., *Protein profile of tax-associated complexes.* J Biol Chem, 2004. **279**(1): p. 495-508.
14. Peloponese, J.M., Jr., T. Kinjo, and K.T. Jeang, *Human T-cell leukemia virus type 1 Tax and cellular transformation.* Int J Hematol, 2007. **86**(2): p. 101-6.
15. Yasunaga, J. and M. Matsuoka, *Leukaemogenic mechanism of human T-cell leukaemia virus type I.* Rev Med Virol, 2007. **17**(5): p. 301-11.
16. Durkin, S.S., et al., *HTLV-1 Tax oncoprotein subverts the cellular DNA damage response via binding to DNA-dependent protein kinase.* J Biol Chem, 2008. **283**(52): p. 36311-20.

17. Chandhasin, C., et al., *Human T-cell leukemia virus type 1 tax attenuates the ATM-mediated cellular DNA damage response.* J Virol, 2008. **82**(14): p. 6952-61.
18. Matsuoka, M., *HTLV-1 bZIP factor gene: Its roles in HTLV-1 pathogenesis.* Mol Aspects Med, 2010.
19. Kubota, R., et al., *Increased HTLV type 1 tax specific CD8+ cells in HTLV type 1-asociated myelopathy/tropical spastic paraparesis: correlation with HTLV type 1 proviral load.* AIDS Res Hum Retroviruses, 2000. **16**(16): p. 1705-9.
20. Yamano, Y., et al., *Abnormally high levels of virus-infected IFN-gamma+ CCR4+ CD4+ CD25+ T cells in a retrovirus-associated neuroinflammatory disorder.* PLoS One, 2009. **4**(8): p. e6517.
21. Izumo, S., et al., *Neuropathology of HTLV-1-associated myelopathy (HAM/TSP).* Leukemia, 1997. **11 Suppl 3**: p. 82-4.
22. Jacobson, S., *Immunopathogenesis of human T cell lymphotropic virus type I-associated neurologic disease.* J Infect Dis, 2002. **186 Suppl 2**: p. S187-92.
23. Lezin, A., et al., *Human T lymphotropic virus type I (HTLV-I) proviral load in cerebrospinal fluid: a new criterion for the diagnosis of HTLV-I-associated myelopathy/tropical spastic paraparesis?* J Infect Dis, 2005. **191**(11): p. 1830-4.
24. Gallo, R.C., *Human retroviruses after 20 years: a perspective from the past and prospects for their future control.* Immunol Rev, 2002. **185**: p. 236-65.
25. Marriott, S.J. and O.J. Semmes, *Impact of HTLV-I Tax on cell cycle progression and the cellular DNA damage repair response.* Oncogene, 2005. **24**(39): p. 5986-95.
26. Mamane, Y., et al., *Repression of DNA repair mechanisms in IRF-4-expressing and HTLV-I-infected T lymphocytes.* J Interferon Cytokine Res, 2005. **25**(1): p. 43-51.
27. Saito, K., et al., *Activation of the PI3K-Akt pathway by human T cell leukemia virus type 1 (HTLV-1) oncoprotein Tax increases Bcl3 expression, which is associated with enhanced growth of HTLV-1-infected T cells.* Virology, 2010. **403**(2): p. 173-80.
28. Hishiki, T., et al., *BCL3 acts as a negative regulator of transcription from the human T-cell leukemia virus type 1 long terminal repeat through interactions with TORC3.* J Biol Chem, 2007. **282**(39): p. 28335-43.
29. Koga, H., T. Ohshima, and K. Shimotohno, *Enhanced activation of tax-dependent transcription of human T-cell leukemia virus type I (HTLV-I) long terminal repeat by TORC3.* J Biol Chem, 2004. **279**(51): p. 52978-83.
30. Okamoto, K., et al., *Human T-cell leukemia virus type-I oncoprotein Tax inhibits Fas-mediated apoptosis by inducing cellular FLIP through activation of NF-kappaB.* Genes Cells, 2006. **11**(2): p. 177-91.
31. Krueger, A., et al., *HTLV-1 Tax protects against CD95-mediated apoptosis by induction of the cellular FLICE-inhibitory protein (c-FLIP).* Blood, 2006. **107**(10): p. 3933-9.
32. Matsuoka, M., *Human T-cell leukemia virus type I and adult T-cell leukemia.* Oncogene, 2003. **22**(33): p. 5131-40.

33. Mori, N., et al., *Human T-cell leukemia virus type I tax activates transcription of the human monocyte chemoattractant protein-1 gene through two nuclear factor-kappaB sites.* Cancer Res, 2000. **60**(17): p. 4939-45.
34. Imaizumi, Y., et al., *Natural course of HTLV-1 carriers with monoclonal proliferation of T lymphocytes ("pre-ATL") in a 20-year follow-up study.* Blood, 2005. **105**(2): p. 903-4.
35. Ruckes, T., et al., *Autocrine antiapoptotic stimulation of cultured adult T-cell leukemia cells by overexpression of the chemokine I-309.* Blood, 2001. **98**(4): p. 1150-9.
36. Shimauchi, T., et al., *Production of thymus and activation-regulated chemokine and macrophage-derived chemokine by CCR4+ adult T-cell leukemia cells.* Clin Cancer Res, 2005. **11**(6): p. 2427-35.
37. Tatewaki, M., et al., *Constitutive overexpression of the L-selectin gene in fresh leukemic cells of adult T-cell leukemia that can be transactivated by human T-cell lymphotropic virus type 1 Tax.* Blood, 1995. **86**(8): p. 3109-17.
38. Lee, B.C., et al., *Involvement of the chemokine receptor CXCR4 and its ligand stromal cell-derived factor 1alpha in breast cancer cell migration through human brain microvascular endothelial cells.* Mol Cancer Res, 2004. **2**(6): p. 327-38.
39. Muhammad, A., et al., *Sequential cooperation of CD2 and CD48 in the buildup of the early TCR signalosome.* J Immunol, 2009. **182**(12): p. 7672-80.
40. Nunes, R.J., et al., *Protein interactions between CD2 and Lck are required for the lipid raft distribution of CD2.* J Immunol, 2008. **180**(2): p. 988-97.
41. Assarsson, E., et al., *2B4/CD48-mediated regulation of lymphocyte activation and function.* J Immunol, 2005. **175**(4): p. 2045-9.
42. Zhang, Z., et al., *Effective therapy for a murine model of adult T-cell leukemia with the humanized anti-CD2 monoclonal antibody, MEDI-507.* Blood, 2003. **102**(1): p. 284-8.
43. Guyot, D.J., G.C. Newbound, and M.D. Lairmore, *Signaling via the CD2 receptor enhances HTLV-1 replication in T lymphocytes.* Virology, 1997. **234**(1): p. 123-9.
44. Montanheiro, P.A., et al., *The elevated interferon gamma production is an important immunological marker in HAM/TSP pathogenesis.* Scand J Immunol, 2009. **70**(4): p. 403-7.
45. Miyatake, Y., et al., *Role of neuronal interferon-gamma in the development of myelopathy in rats infected with human T-cell leukemia virus type 1.* Am J Pathol, 2006. **169**(1): p. 189-99.
46. Popko, B., et al., *The effects of interferon-gamma on the central nervous system.* Mol Neurobiol, 1997. **14**(1-2): p. 19-35.
47. Lees, J.R. and A.H. Cross, *A little stress is good: IFN-gamma, demyelination, and multiple sclerosis.* J Clin Invest, 2007. **117**(2): p. 297-9.
48. Matsumoto, M., et al., *CD43 plays both antiadhesive and proadhesive roles in neutrophil rolling in a context-dependent manner.* J Immunol, 2008. **181**(5): p. 3628-35.
49. Matsumoto, M., et al., *CD43 functions as a ligand for E-Selectin on activated T cells.* J Immunol, 2005. **175**(12): p. 8042-50.

50. Ziltener, H.J. and D.A. Carlow, *CD43 in T cell-DC conjugate formation?* Nat Immunol, 2001. **2**(12): p. 1087.
51. Onami, T.M., et al., *Dynamic regulation of T cell immunity by CD43.* J Immunol, 2002. **168**(12): p. 6022-31.
52. van der Most, R.G., K. Murali-Krishna, and R. Ahmed, *Prolonged presence of effector-memory CD8 T cells in the central nervous system after dengue virus encephalitis.* Int Immunol, 2003. **15**(1): p. 119-25.
53. Nagai, M., et al., *CD8(+) T cells are an in vivo reservoir for human T-cell lymphotropic virus type I.* Blood, 2001. **98**(6): p. 1858-61.
54. Yamano, Y., et al., *Correlation of human T-cell lymphotropic virus type 1 (HTLV-1) mRNA with proviral DNA load, virus-specific CD8(+) T cells, and disease severity in HTLV-1-associated myelopathy (HAM/TSP).* Blood, 2002. **99**(1): p. 88-94.
55. De Groot, C.J., et al., *Expression of transforming growth factor (TGF)-beta1, -beta2, and -beta3 isoforms and TGF-beta type I and type II receptors in multiple sclerosis lesions and human adult astrocyte cultures.* J Neuropathol Exp Neurol, 1999. **58**(2): p. 174-87.
56. Holley, J.E., et al., *Increased blood vessel density and endothelial cell proliferation in multiple sclerosis cerebral white matter.* Neurosci Lett, 2010. **470**(1): p. 65-70.
57. Schmidt-Weber, C.B., et al., *TGF-{beta} signaling of human T cells is modulated by the ancillary TGF-{beta} receptor endoglin.* Int Immunol, 2005. **17**(7): p. 921-30.
58. Kazanji, M. and A. Gessain, *Human T-cell Lymphotropic Virus types I and II (HTLV-I/II) in French Guiana: clinical and molecular epidemiology.* Cad Saude Publica, 2003. **19**(5): p. 1227-40.
59. Gerard, Y., et al., *Clustering and clinical diversity of adult T-cell leukemia/lymphoma associated with HTLV-I in a remote black population of French Guiana.* Int J Cancer, 1995. **60**(6): p. 773-6.
60. Suruki, R., et al., *Alanine aminotransferase level as a predictor of hepatitis C virus-associated hepatocellular carcinoma incidence in a community-based population in Japan.* Int J Cancer, 2006. **119**(1): p. 192-5.
61. Mortreux, F., A.S. Gabet, and E. Wattel, *Molecular and cellular aspects of HTLV-1 associated leukemogenesis in vivo.* Leukemia, 2003. **17**(1): p. 26-38.
62. Proietti, F.A., et al., *Global epidemiology of HTLV-I infection and associated diseases.* Oncogene, 2005. **24**(39): p. 6058-68.
63. Ozsolak, F., et al., *Direct RNA sequencing.* Nature, 2009. **461**(7265): p. 814-8.
64. Mardis, E.R., *Next-generation DNA sequencing methods.* Annu Rev Genomics Hum Genet, 2008. **9**: p. 387-402.
65. Bonetta, L., *Whole-genome sequencing breaks the cost barrier.* Cell, 2010. **141**(6): p. 917-9.
66. Zagordi, O., et al., *Error correction of next-generation sequencing data and reliable estimation of HIV quasispecies.* Nucleic Acids Res, 2010.

67. DiGiandomenico, A., L.S. Wylezinski, and J. Hawiger, *Intracellular delivery of a cell-penetrating SOCS1 that targets IFN-gamma signaling.* Sci Signal, 2009. **2**(80): p. ra37.
68. Letourneur, M., et al., *Sp2 regulates interferon-gamma-mediated socs1 gene expression.* Mol Immunol, 2009. **46**(11-12): p. 2151-60.
69. Kamio, M., et al., *SOCS1 [corrected] inhibits HPV-E7-mediated transformation by inducing degradation of E7 protein.* Oncogene, 2004. **23**(17): p. 3107-15.
70. Starr, R., et al., *A family of cytokine-inducible inhibitors of signalling.* Nature, 1997. **387**(6636): p. 917-21.
71. Alexander, W.S. and D.J. Hilton, *The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response.* Annu Rev Immunol, 2004. **22**: p. 503-29.
72. Mansell, A., et al., *Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation.* Nat Immunol, 2006. **7**(2): p. 148-55.
73. Yokota, S., et al., *Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 confers efficient viral replication.* Virology, 2005. **338**(1): p. 173-81.
74. Yokota, S., et al., *Induction of suppressor of cytokine signaling-3 by herpes simplex virus type 1 contributes to inhibition of the interferon signaling pathway.* J Virol, 2004. **78**(12): p. 6282-6.
75. Bode, J.G., et al., *IFN-alpha antagonistic activity of HCV core protein involves induction of suppressor of cytokine signaling-3.* FASEB J, 2003. **17**(3): p. 488-90.
76. Zhao, D.C., et al., *Respiratory syncytial virus inhibits interferon-alpha-inducible signaling in macrophage-like U937 cells.* J Infect, 2007. **54**(4): p. 393-8.
77. Pauli, E.K., et al., *Influenza A virus inhibits type I IFN signaling via NF-kappaB-dependent induction of SOCS-3 expression.* PLoS Pathog, 2008. **4**(11): p. e1000196.
78. Pothlichet, J., M. Chignard, and M. Si-Tahar, *Cutting edge: innate immune response triggered by influenza A virus is negatively regulated by SOCS1 and SOCS3 through a RIG-I/IFNAR1-dependent pathway.* J Immunol, 2008. **180**(4): p. 2034-8.
79. Frey, K.G., et al., *HSV-1-induced SOCS-1 expression in keratinocytes: use of a SOCS-1 antagonist to block a novel mechanism of viral immune evasion.* J Immunol, 2009. **183**(2): p. 1253-62.
80. Hiscott, J., *Triggering the innate antiviral response through IRF-3 activation.* J Biol Chem, 2007. **282**(21): p. 15325-9.
81. Kinpara, S., et al., *Stromal cell-mediated suppression of human T-cell leukemia virus type 1 expression in vitro and in vivo by type I interferon.* J Virol, 2009. **83**(10): p. 5101-8.
82. Mahieux, R. and O. Hermine, *In vivo and in vitro treatment of HTLV-1 and HTLV-2 infected cells with arsenic trioxide and interferon-alpha.* Leuk Lymphoma, 2005. **46**(3): p. 347-55.
83. Feng, X. and L. Ratner, *Human T-cell leukemia virus type 1 blunts signaling by interferon alpha.* Virology, 2008. **374**(1): p. 210-6.

84. Zhang, J., et al., *Human T-cell leukemia virus type 1 Tax modulates interferon-alpha signal transduction through competitive usage of the coactivator CBP/p300.* Virology, 2008. **379**(2): p. 306-13.
85. Colisson, R., et al., *Free HTLV-1 induces TLR7-dependent innate immune response and TRAIL relocalization in killer plasmacytoid dendritic cells.* Blood, 2010. **115**(11): p. 2177-85.
86. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.* Nat Immunol, 2010. **11**(5): p. 395-402.
87. Ryo, A., et al., *SOCS1 is an inducible host factor during HIV-1 infection and regulates the intracellular trafficking and stability of HIV-1 Gag.* Proc Natl Acad Sci U S A, 2008. **105**(1): p. 294-9.
88. Stojdl, D.F., et al., *Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus.* Nat Med, 2000. **6**(7): p. 821-5.
89. Balachandran, S., M. Porosnicu, and G.N. Barber, *Oncolytic activity of vesicular stomatitis virus is effective against tumors exhibiting aberrant p53, Ras, or myc function and involves the induction of apoptosis.* J Virol, 2001. **75**(7): p. 3474-9.
90. Fernandez, M., et al., *Genetically engineered vesicular stomatitis virus in gene therapy: application for treatment of malignant disease.* J Virol, 2002. **76**(2): p. 895-904.
91. Ahmed, M., et al., *Ability of the matrix protein of vesicular stomatitis virus to suppress beta interferon gene expression is genetically correlated with the inhibition of host RNA and protein synthesis.* J Virol, 2003. **77**(8): p. 4646-57.
92. Stojdl, D.F., et al., *VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents.* Cancer Cell, 2003. **4**(4): p. 263-75.
93. Peyman Nakhaei, S.P., Stephanie Oliere, Vanessa Tumilasci, John C. and B.a.J. Hiscott, *Oncolytic virotherapy of cancer with vesicular stomatitis virus.* Gene Therapy and Molecular Biology, 2005. **9**: p. 269-280.
94. Rahman, M.M., et al., *Oncolytic viral purging of leukemic hematopoietic stem and progenitor cells with Myxoma virus.* Cytokine Growth Factor Rev, 2010. **21**(2-3): p. 169-75.
95. Phillips, A.A., et al., *A multi-institutional experience of autologous stem cell transplantation in North American patients with human T-cell lymphotropic virus type-1 adult T-cell leukemia/lymphoma suggests ineffective salvage of relapsed patients.* Leuk Lymphoma, 2009. **50**(6): p. 1039-42.
96. Fujiwara, H., et al., *Interferon-alpha therapy following autologous peripheral blood stem cell transplantation for adult T cell leukemia/lymphoma.* Acta Haematol, 2002. **107**(4): p. 213-9.
97. Thirukkumaran, C.M., et al., *Viral purging of haematological autografts: should we sneeze on the graft?* Bone Marrow Transplant, 2007. **40**(1): p. 1-12.
98. Lichty, B.D., et al., *Vesicular stomatitis virus: a potential therapeutic virus for the treatment of hematologic malignancy.* Hum Gene Ther, 2004. **15**(9): p. 821-31.
99. Caligaris-Cappio, F., *Role of the microenvironment in chronic lymphocytic leukaemia.* Br J Haematol, 2003. **123**(3): p. 380-8.

100. Hamblin, T.J. and D.G. Oscier, *Chronic lymphocytic leukaemia: the nature of the leukaemic cell.* Blood Rev, 1997. **11**(3): p. 119-28.
101. Maruyama, M., et al., *Evidence for aberrant activation of the interleukin-2 autocrine loop by HTLV-1-encoded p40x and T3/Ti complex triggering.* Cell, 1987. **48**(2): p. 343-50.
102. Satoh, M., et al., *Involvement of IL-2/IL-2R system activation by parasite antigen in polyclonal expansion of CD4(+)25(+) HTLV-1-infected T-cells in human carriers of both HTLV-1 and S. stercoralis.* Oncogene, 2002. **21**(16): p. 2466-75.
103. Cavrois, M., et al., *Proliferation of HTLV-1 infected circulating cells in vivo in all asymptomatic carriers and patients with TSP/HAM.* Oncogene, 1996. **12**(11): p. 2419-23.
104. Stojdl, D.F., et al., *The murine double-stranded RNA-dependent protein kinase PKR is required for resistance to vesicular stomatitis virus.* J Virol, 2000. **74**(20): p. 9580-5.
105. Takaoka, A. and T. Taniguchi, *New aspects of IFN-alpha/beta signalling in immunity, oncogenesis and bone metabolism.* Cancer Sci, 2003. **94**(5): p. 405-11.
106. Balachandran, S. and G.N. Barber, *Defective translational control facilitates vesicular stomatitis virus oncolysis.* Cancer Cell, 2004. **5**(1): p. 51-65.
107. Reuter, C.W., M.A. Morgan, and L. Bergmann, *Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies?* Blood, 2000. **96**(5): p. 1655-69.
108. Alberola-Ila, J. and G. Hernandez-Hoyos, *The Ras/MAPK cascade and the control of positive selection.* Immunol Rev, 2003. **191**: p. 79-96.
109. Rose, J.H., and M. A. Whitt *Rhabdoviridae: the viruses and their replication.* Fields virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, PA., 2001. **1**(In D. M. Knipe and P. M. Howley (ed.)): p. 1121-1244.
110. Noser, J.A., et al., *The RAS/Raf1/MEK/ERK signaling pathway facilitates VSV-mediated oncolysis: implication for the defective interferon response in cancer cells.* Mol Ther, 2007. **15**(8): p. 1531-6.
111. Nourse, J., et al., *Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin.* Nature, 1994. **372**(6506): p. 570-3.
112. Cobrinik, D., *Pocket proteins and cell cycle control.* Oncogene, 2005. **24**(17): p. 2796-809.
113. Appleman, L.J., et al., *CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression.* J Immunol, 2000. **164**(1): p. 144-51.
114. Garcia, Z., et al., *Phosphoinositide 3-kinase controls early and late events in mammalian cell division.* EMBO J, 2006. **25**(4): p. 655-61.
115. Chakraborty, P., et al., *Vesicular stomatitis virus inhibits mitotic progression and triggers cell death.* EMBO Rep, 2009. **10**(10): p. 1154-60.
116. Silvera, D., S.C. Formenti, and R.J. Schneider, *Translational control in cancer.* Nat Rev Cancer, 2010. **10**(4): p. 254-66.
117. Sonenberg, N. and A.G. Hinnebusch, *Regulation of translation initiation in eukaryotes: mechanisms and biological targets.* Cell, 2009. **136**(4): p. 731-45.

118. Gibbons, J.J., R.T. Abraham, and K. Yu, *Mammalian target of rapamycin: discovery of rapamycin reveals a signaling pathway important for normal and cancer cell growth.* Semin Oncol, 2009. **36 Suppl 3**: p. S3-S17.
119. Pyronnet, S., *Phosphorylation of the cap-binding protein eIF4E by the MAPK-activated protein kinase Mnk1.* Biochem Pharmacol, 2000. **60**(8): p. 1237-43.
120. Connor, J.H. and D.S. Lyles, *Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1.* J Virol, 2002. **76**(20): p. 10177-87.
121. Marozin, S., et al., *Cell cycle progression or translation control is not essential for vesicular stomatitis virus oncolysis of hepatocellular carcinoma.* PLoS One, 2010. **5**(6): p. e10988.
122. Nguyen, T.L., et al., *The emergence of combinatorial strategies in the development of RNA oncolytic virus therapies.* Cell Microbiol, 2009. **11**(6): p. 889-97.

# ~ CHAPTER IV ~

## *CANDIDATE'S CONTRIBUTIONS*

## *TO ORIGINAL KNOWLEDGE*

**The results presented in this thesis have contributed to a more thorough understanding of the host immune response to HTLV-1 infection leading to HAM/TSP or ATL clinical outcomes. The candidate proposes Vesicular Stomatitis Virus as therapeutic approach to target leukemic cells from ATL individuals. Ultimately, the candidate delineates host factors important for VSV oncolysis in primary *ex vivo* ATL cells. The candidate's major contributions to original knowledge are listed below:**

1. The candidate was the first to establish a comparative genome-wide array analysis to characterize gene expression profiles of HTLV-1-associated diseases. In addition the candidate was the first to identify several gene signatures (*SOCS1*, *CD43, ENG*, *CD48*, *CD2*, etc.) in CD4+ T-cells for the pre-symptomatic and clinical diagnosis of ATL and HAM/TSP diseases.

2. The candidate was the first to show that the Suppressor of Cytokine Signaling - SOCS1 - is induced by HTLV-1 to negatively regulate the antiviral response. In addition, the candidate was the first to demonstrate that induction of SOCS1 protein during HTLV-1 infection mediates proteasomal degradation of IRF-3.

3. The candidate was the first to investigate oncolytic properties of Vesicular Stomatitis Virus in primary *ex vivo* PBMCs from ATL patients.

4. The candidate was the first to demonstrate the resistance of quiescent primary *ex vivo* CLL cells to VSV oncolysis.

5. The candidate was the first to show that VSV replication in primary activated CD4+ T-cells relies on cell cycle transition from $G_0$- to $G_1$- phase which is characterized by a sharp increase in ribogenesis and protein synthesis.

6. The candidate was the first to demonstrate that the resistance of quiescent primary *ex vivo* CLL cells to VSV oncolysis can be overcome by mitogenic agents that stimulate exit from $G_0$- to $G_1$ phase of the cell cycle.

**Together, the candidate's original contributions have advanced our knowledge of molecular mechanisms governing HTLV-1-mediated diseases. These novel findings provide a basis for new therapeutic strategies for treating HAM/TSP and ATL.**