Molecular Mechanisms of Monocyte Depletion and $CD4^+$ T-cell Persistence during Human T-cell Leukemia Virus Infection

By

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To my friends, you guys were great. Thank you so much for listening when I started rambling off about my work, and being supportive when things were not going great. I have met a lot of you here while working on my degree and wish you all the luck on your own projects. Special thanks to my D&D group for giving me the chance to create and explore completely ridiculous scenarios while having a blast.

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Abstract

Human T-cell Leukemia Virus type 1 (HTLV-1) was the first human retrovirus discovered in 1980. It is the causal agent of two well characterized human diseases, Adult T-cell Leukemia (ATL) and HTLV-1-Associated Myelopathy/Tropical Spastic Paraparesis (HAM/TSP). Few treatment options have been developed for ATL and HAM/TSP; the median survival times for ATL remains under a year, and treatment options of HAM/TSP remain palliative in nature. Thankfully the incidence of disease in HTLV-1 infected patients is relatively low, with only approximately 5% of individuals developing ATL and 2% HAM/TSP.

One major obstacle in the development of effective therapies is a lack of understanding regarding the factors that determine HTLV-1 associated pathology. The route of transmission, contaminated breast milk and blood, almost exclusively lead to ATL or HAM/TSP development respectively, but it is unclear as to why. This suggests that the early events of HTLV-1 infection may be key in discerning pathological outcomes. Unfortunately this time point is understudied, in part due to the fact that *de novo* infection is asymptomatic. The activity of cytotoxic T-cell lymphocytes seems to be vital in controlling viral replication, and is likely a key determinant in disease progression. We thus set out to study the early events of HTLV-1 infection in two important immunologically relevant peripheral blood mononuclear cell populations, monocytes and $CD4^+$ T-cells.

HTLV-1 infection of primary human monocytes resulted in the depletion of this cell type. This was not mediated by viral accessory proteins, as host restriction factor SAMHD1 prevented the completion of reverse transcription. The DNA by-products of this inhibition induced a potent STING-mediated immunological response, that triggered Bax and IRF3 activation and complex formation that led to apoptosis.

Infection of activated $CD4^+$ T-cells on the other hand, resulted in persistent cellular survival. This was mediated by the viral accessory protein Tax, known to have oncogenic properties. Tax expression activated the AKT pathway, which resulted in the inactivation of the pro-apoptotic FOXO3a transcription factor. This led to the long-term survival of an activated $CD4^+$ T-cell population that was capable of viral transmission.

Overall this work has demonstrated the molecular consequences of HTLV-1 infection in two important cell types, monocytes and $CD4^+$ T-cells. These events likely shape the subsequent immunological events that control viral replication and likely influence HTLV-1 disease pathology.

Résumé

Le virus T-lymphotropique humain de type 1 (HTLV-1) a été le premier rétrovirus humain à avoir été découvert en 1980. Il est l'agent causal de deux maladies humaines bien caractérisées, la leucémie lymphocytaire de l'adulte (ATL) et la myélopathie associée au HTLV-1/Paraparésie spastique tropicale (HAM / TSP). Peu d'options de traitement ont été développées pour l'ATL et la HAM/TSP; les temps de survie médians pour l'ATL restent inférieurs à un an, et les options thérapeutiques de la HAM/TSP sont de nature palliative. Cependant, l'incidence de la maladie chez les patients infectés par le HTLV-1 est relativement faible, avec seulement environ 5% des individus développant l'ATL et 2% la HAM/TSP.

L'obstacle majeur au développement de thérapies efficaces est le manque de compréhension des facteurs qui déterminent la pathologie associée au HTLV-1. Les voies de transmission, le lait maternel contaminé et le sang, entraînent presque exclusivement le développement de l'ATL ou de la HAM/TSP, mais la cause de ceci n'est pas connue. Cela suggère que les premiers événements de l'infection par le HTLV-1 peuvent être la clé pour révéler les conséquences pathologiques de la maladie. Malheureusement, les étapes précoces de l'infection sont peu étudiées, dû au fait que l'infection de novo est asymptomatique. L'activité des lymphocytes T cytotoxiques semble être essentielle pour contrôler la réplication virale et est probablement un facteur clé dans la progression de la maladie. Nous avons donc entrepris d'étudier les premiers événements de l'infection par le HTLV-1 dans deux populations cellulaires mononuclées importantes et immunologiquement pertinentes du sang périphérique, les monocytes et les lymphocytes T $CD4^+$.

L'infection des monocytes primaires humains par le HTLV-1 a entraîné la déplétion de ce type cellulaire. Cela n'est pas médiée par les protéines accessoires virales, car le facteur de restriction SAMHD1 a empêché l'achèvement de la transcription inverse. Les sous-produits de l'ADN produits lors de cette inhibition ont induit une réponse immunologique puissante médiée par STING. Ceci a déclenché l'activation des protéines Bax et IRF3 et la formation de complexes qui ont conduit à l'apoptose des cellules.

D’autre part, l’infection par le virus des cellules T $CD4^+$ activées, les a fait entrer dans un état de survie cellulaire persistante. Le transactivateur viral Tax en est responsable car il est connu pour ses propriétés oncogéniques. La seule expression de Tax a activé la voie AKT, entraînant l’inactivation du facteur de transcription pro-apoptotique FOXO3a. Cela a conduit à la survie à long terme d’une population de lymphocytes T $CD4^+$ activés capables de transmettre le virus.

Ce travail de doctorat a permis de démontrer les conséquences moléculaires de l'infection par HTLV-1 dans deux types cellulaires importants, les monocytes et les cellules T $CD4^+$. Ces événements ont une conséquence sur les évènements immunologiques qui en découlent et qui contrôlent la réplication virale et influencent probablement la pathologie de la maladie dû au HTLV-1.

Preface and Contributions

In accordance with departmental guidelines, I have chosen to present the results of my work in a manuscript format. Chapter 1 is a general literature review on HTLV-1 that also briefly touches upon innate immune signaling. Chapter 2 is a published review and an original scholarship paper, which should be considered as an extension of the literature review. It covers a significant amount of knowledge regarding SAMHD1 and retroviral infection, and can be found in its published form here:

1. **Sze, A.**; Olagnier, D.; Lin, R.; van Grevenynghe, J.; Hiscott, J. Samhd1 host restriction factor: A link with innate immune sensing of retrovirus infection. *Journal of molecular biology* 2013, *425*, 4981-4994.

Author contributions: Sze wrote the manuscript. Olagnier produced the figures. Lin, and van Grevenynghe reviewed the manuscript. Hiscott helped write and reviewed the manuscript.

My results are presented in Chapter 3 and 4, and each constitute as a significant contribution to original scientific research. They are each published peer reviewed articles that can be found in the following journals:

2. **Sze, A.**; Belgnaoui, S.; Olagnier, D.; Lin, R.; Hiscott, J.; van Grevenynghe, J. Host restriction factor SAMHD1 limits Human T-Cell Leukemia Virus Type 1 infection of monocytes via sting-mediated apoptosis. *Cell host & microbe* 2013, *14*.

Author contributions: Sze conceived the project, performed experiments and wrote the manuscript. Belgnaoui provided technical assistance. Olagnier helped produce the figures. Lin supervised the project and provided technical assisstance. Hiscott helped concieve the study and helped write the paper. van Grevenynghe designed and performed experiments, supervised and conceived of the project, and helped to write the manuscript.

3. Olagnier, D.; **Sze, A.**; Bel Hadj, S.; Chiang, C.; Steel, C.; Han, X.; Routy, J.P.; Lin, R.; Hiscott, J.; van Grevenynghe, J. Htlv-1 tax-mediated inhibition of foxo3a activity is

critical for the persistence of terminally differentiated cd4+ t cells. *PLoS pathogens* 2014, *10*, e1004575.

Author contributions of this co-first author study: Olagnier performed experiments. Sze performed experiments and wrote the paper. Bel Hadj, Chiang, Steel, and Han provided technical assisstance. Routy provided materials. Lin supervised the project. Hiscott helped write the manuscript. van Grevenynghe conceived, designed and performed experiments.

I have also been invovled with other research not presented in this thesis. The work has been published in the following:

1. Oliere, S.; Douville, R.; **Sze, A.**; Belgnaoui, S.M.; Hiscott, J. Modulation of innate immune responses during Human T-Cell Leukemia Virus (HTLV-1) pathogenesis. *Cytokine & growth factor reviews* 2011, *22*, 197-210.
2. Liu, Y.; Goulet, M.L.; **Sze, A.**; Hadj, S.B.; Belgnaoui, S.M.; Lababidi, R.R.; Zheng, C.; Fritz, J.H.; Olagnier, D.; Lin, R. RIG-I-mediated sting upregulation restricts herpes simplex virus 1 infection. *Journal of virology* 2016, *90*, 9406-9419.
3. Olagnier, D.; Lababidi, R.R.; Hadj, S.B.; **Sze, A.**; Liu, Y.; Naidu, S.D.; Ferrari, M.; Jiang, Y.; Chiang, C.; Beljanski, V.*, et al.* Activation of nrf2 signaling augments vesicular stomatitis virus oncolysis via autophagy-driven suppression of antiviral immunity. *Molecular therapy : the journal of the American Society of Gene Therapy* 2017, *25*, 1900-1916.
4. **Sze, A.**; Olagnier, D.; Hadj, S.B.; Han, X.; Tian, X.H.; Xu, H.T.; Yang, L.; Shi, Q.; Wang, P.; Wainberg, M.A.*, et al.* Sophoraflavenone G restricts dengue and zika virus infection via rna polymerase interference. *Viruses* 2017, *9*.

I have helped edit other studies and I have been included in the acknoledgments in the following published work:

1. Liu, Z.; Pan, Q.; Liang, Z.; Qiao, W.; Cen, S.; Liang, C. The highly polymorphic cyclophilin a-binding loop in HIV-1 capsid modulates viral resistance to MXB. *Retrovirology* 2015, *12*, 1.
2. Aubouy, A.; Olagnier, D.; Bertin, G.; Ezinmegnon, S.; Majorel, C.; Mimar, S.; Massougbodji, A.; Deloron, P.; Pipy, B.; Coste, A. Nrf2-driven CD36 and HO-1 gene expression in circulating monocytes correlates with favourable clinical outcome in pregnancy-associated malaria. *Malaria journal* 2015, *14*, 358.
3. Liu, Y.; Olagnier, D.; Lin, R. Host and viral modulation of RIG-I-mediated antiviral immunity. *Frontiers in immunology* 2016, *7*, 662.

List of Abbreviations

3’ (3 prime end)

5’ (5 prime end)

ABC (abacavir)

AGS (Aicardi–Goutières syndrome)

AID (activation-induced cytidine deaminase)

AIM2 (absent in melanoma)

AP-1 (activator protein 1)

APC/C (Anaphase-promoting complex/cyclosome)

APOBEC-A3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3)

ATL (adult T-cell leukemia)

ATM (ataxia telangiectasia mutated)

AZT (azidothymidine)

BER (base excision repair)

C/EBPα (CCAAT-enhancer-binding proteins)

CADM1 (cell adhesion molecule 1)

CARD (caspase activation and recruitment domain)

Cdc25 (cell division cycle protein 25)

CDK (cyclin-dependent kinases)

c-FLIP (Cellular FLICE-inhibitory protein)

cGAMP (dinucleotide cyclic GMP–AMP)

cGAS (cGAMP synthetase)

Chk1 (check point kinase 1)

Chk2 (check point kinase 2)

cIAP-2 (cellular inhibitor of apoptosis 2)

CKI (cyclin-dependent kinase inhibitors)

cPPT (central PPT)

CRE (cAMP response)

CREB-regulated transcription coactivator (CRTC)

CTL (cytotoxic T lymphocytes)

DC (dendritic cell)

dN (deoxynucleosides)

dNTP (deoxynucleoside triphosphates)

ds (double stranded)

ERK (extracellular signal–regulated kinases)

FADD (FAS-associated death domain protein)

FLICE (FADD-like IL-1β-converting enzyme)

Foxp3 (forkhead box P3)

FSCN-1 (Fascin)

GAS (gamma activation sequences)

GLUT1 (glucose transporter-1)

gp21 (TM)

gp46 (SU)

gRNA (genomic RNA)

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

HBZ (HTLV-1 basic leucine zipper factor)

HIV-1 (human immunodeficiency virus type 1)

HSPG (heparin sulfate proteoglycans)

HTLV-1 (Human T-cell Leukemia virus type-1)

ICAM-1 (intercellular adhesion molecule 1)

IFI16 (IFN-inducible protein 16)

IFN (interferon)

IFNAR (IFNα/β receptor)

IKK (IκB kinases)

IL (interleukin)

IN (integrase)

IRAK (IL-1R1-associated protein kinases)

IRF (IFN regulatory factor)

ISGF3 (IFN-stimulated gene factor 3)

ITIM (immunoreceptor tyrosine-based inhibition motif)

iT_{reg} (induced T_{reg})

LFA-1 (leukocyte function antigen 1)

LPS (lipopolysaccharide)

LTR (long terminal repeat)

MAPK (mitogen-activated protein kinase)

MAVS (mitochondrial antiviral-signaling protein)

MDDC (monocyte-derived DC)

MDM (monocyte-derived macrophage)

MEKK1 (MAPK/ERK kinase kinase 1)

MHC-1 (major histocompatibility complex class I)

MLV (Murine Leukemia Virus)

MMR (mismatch repair)

MOMP (mitochondrial outer membrane permeabilization)

MTOC (microtubule organizing center)

MyD88 (myeloid differentiation factor 88)

NB (nuclear bodies)

NEMO (NF-κB essential modulator)

NER (nucleotide excision repair)

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)

NHEJ (non-homologous end joining)

NIK (NF-κB-inducing kinase)

NK (natural killer)

NLR (NOD-like receptors)

NPC (nuclear pore complexes)

NRP (NEMO-related protein)

NRP-1 (neuropilin-1)

NRTI (Nucleoside reverse transcriptase inhibitors)

NTP (nucleotide triphosphates)

NUPs (nucleoporins)

P/CAF (p300/CBP-associated factor)

p15 (NC)

p19 (MA)

p24 (CA)

p300/CRB (CREB binding protein)

p30RE (p30 response element)

PAMPS (pathogen-associated molecular patterns)

PAS (primer activation sequence)

PBS (primer binding site)

PD-1 (programmed cell death 1)

pDC (plasmacytoid dendritic cells)

PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase)

PIC (preintegration complex)

PPT (polypurine tract)

PRR (pathogen recognition receptors)

PRR (proline rich region)

Rb (retinoblastoma susceptibility protein)

RBD (receptor binding domain)

RIG (retinoic acid-inducible gene)

RLR (RIG-like receptors)

RNF4 (Really Interesting New Gene Finger Protein 4)

rNTP (ribonucleoside triphosphates)

ROS (reactive oxygen species)

RT (reverse transcriptase)

RTC (reverse transcription complex)

RTI (reverse-transcribed intermediaries)

RxRE (Rex responsive element)

SAMHD1 (sterile alpha motif and histidine-aspartic domain containing protein 1)

sHBZ (spliced HBZ)

SL (stem loop)

smHBZ (silent mutation HBZ)

SOCS (suppressor of cytokine signaling)

SRE (serum response element)

SRF (serum response factor)

ss (single stranded)

STAT (signal transducer and activator of transcription)

STING (stimulator of IFN genes)

TAB1 (TAK1-binding protein 1)

TAB2 (TAK1-binding protein 2)

TAF4 (TBP-associated factor 4)

TAK1/MAP3K7 (TGF-β activating kinases 1)/(mitogen-activated protein kinase kinase kinase 7)

TANK (TRAF family member-associated NF-κB activator)

Tax1BP1 (Tax1 binding protein 1)

TBK1 (TANK-binding kinase 1)

TCR (T-cell receptor)

TF (transcription factor)

TFIID (general transcription factor II D)

TGF-β (transforming growth factor-β)

TIGIT (T cell immunoglobulin and ITIM domain)

TIR (toll IL-1 receptor)

TLR (toll-like receptors)

TNF (tumor necrosis factor)

TNFR (TNF-α receptor)

TRAF (TNF receptor associated factor)

TRAILR (TNF-related apoptosis-inducing ligand receptor)

TRE-1 (Tax-response element-1)

Treg (regulatory T-cells)

TRIF (TIR domain–containing adaptor–inducing IFN-β)

TRIM (tripartite motif protein)

TSS (Tax speckled structures)

TYK2 (tyrosine kinase 2)

usHBZ (unspliced HBZ)

VEGF (vascular endothelial growth factor)

wtHBZ (wild type HBZ)

wtT_{reg} (wild type T_{reg})

XIAP (X-chromosome-linked inhibitor of apoptosis)

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Chapter 1: Literature Review

1.1. HTLV-1

HTLV-1 (Human T-cell Leukemia virus type-1) was identified in 1980, and was the first human retrovirus discovered. This was done by Dr. Robert Gallo, who isolated it from a patient diagnosed with cutaneous T-cell lymphoma[1]. This patient had no family history of lymphoma, leukemia or immune deficiency, and had not been exposed to any known carcinogens. Several cell lines were established from samples the patient provided, and the first human retroviral particles were isolated soon after. HTLV-1 viral products were found in many T-cell malignancies, but not in healthy human tissues, suggesting that HTLV-1 was not an endogenous virus[2]. Eventually a link was made between the presence of HTLV-1 and a distinct form of T-cell leukemia called ATL (adult T-cell leukemia) that was characterized by Dr. Kiyoshi Takatsuki[3]. ATL samples were infected with HTLV-1, and the oncogenic ability of HTLV-1 was demonstrated when healthy cells from an uninfected donor were transformed *in vitro* following exposure to the virus[4, 5]. HTLV-1 infection is also associated with an autoimmune-like disorder called HAM/TSP (HTLV-1-associated myelopathy/tropical spastic paraparesis).

1.1.1. Epidemiology of HTLV

HTLV-1 is thought to infect between 15 and 20 million people throughout the world and is endemic only in certain areas such as Japan, the African continent and the Caribbean basin[6, 7]. This information however, was difficult to estimate as HTLV-1 distribution is not homogenous; one region may have very high prevalence of HTLV-1, but a neighboring one will not. Furthermore, the spread of the virus is mainly monitored by serological assays of blood donations, which may not accurately represent human demographics depending on the region. During the 1980s and 1990s, those tests lacked specificity and likely overestimated the rate of HTLV-1[8, 9]. Current diagnostics usually confirm infection with a different assay.

Africa contains several regions with endemic HTLV-1, including; Morocco, Cameroon, Gabon and South Africa[8, 10-12]. However, there is no information available for several African countries, making accurate estimates of African HTLV-1 difficult. Certain islands in the Caribbean contain large populations of infected individuals, such as Jamaica, Haiti, Martinique,

Trinidad and Tobago and Barbados[13-17]. Japan likely has the largest number of HTLV-1 infected individuals in the world[18], and is the most important center for studying the virus and its associated diseases. Even here however, there are large discrepancies; some regions report 40% of individuals over the age of 50 are infected, while other regions are mostly clear[19]. Other regions in Asia, such as Korea and China do not seem to have high levels of seropositivity[20, 21]. In the Middle-East, certain regions of Iran are endemic[22], although many other countries in the area have not been studied. The majority of cases of HTLV-1 infection in Europe are from individuals who are native from endemic regions such as the African continent or the Caribbean basin. The one exception to this is Romania[23], where the prevalence is significantly higher than the rest of Europe. HTLV-1 is found in the Americas in certain populations such as immigrants from endemic regions, and some native American populations[24, 25]. Overall, newer estimates of HTLV-1 prevalence are between 5 and 10 million people, but this is based on known endemic regions which only represent about a quarter of the total world population[10]. It is likely that the true levels of HTLV-1 are higher.

An interesting feature of HTLV-1 spread, is that it possesses remarkable genetic stability[10, 26]. The few nucleotide differences observed between strains does not seem to lead to any differences in terms of disease type or progression[27], and is a mark of geography and source of infection rather than pathogenesis[28]. Even the pressure from the immune system does not lead to viral mutations[29]. There still exist numerous genotypes of HTLV-1; subtype A is the most prevalent and is further divided into other geographical subtypes. Ultimately, the stability of the HTLV-1 genome is largely attributed to the fact that it replicates primarily through mitosis, whereby infected cells replicate and copy its genome[30]. This allows HTLV-1 to employ host polymerases that have high fidelity and proofreading capabilities.

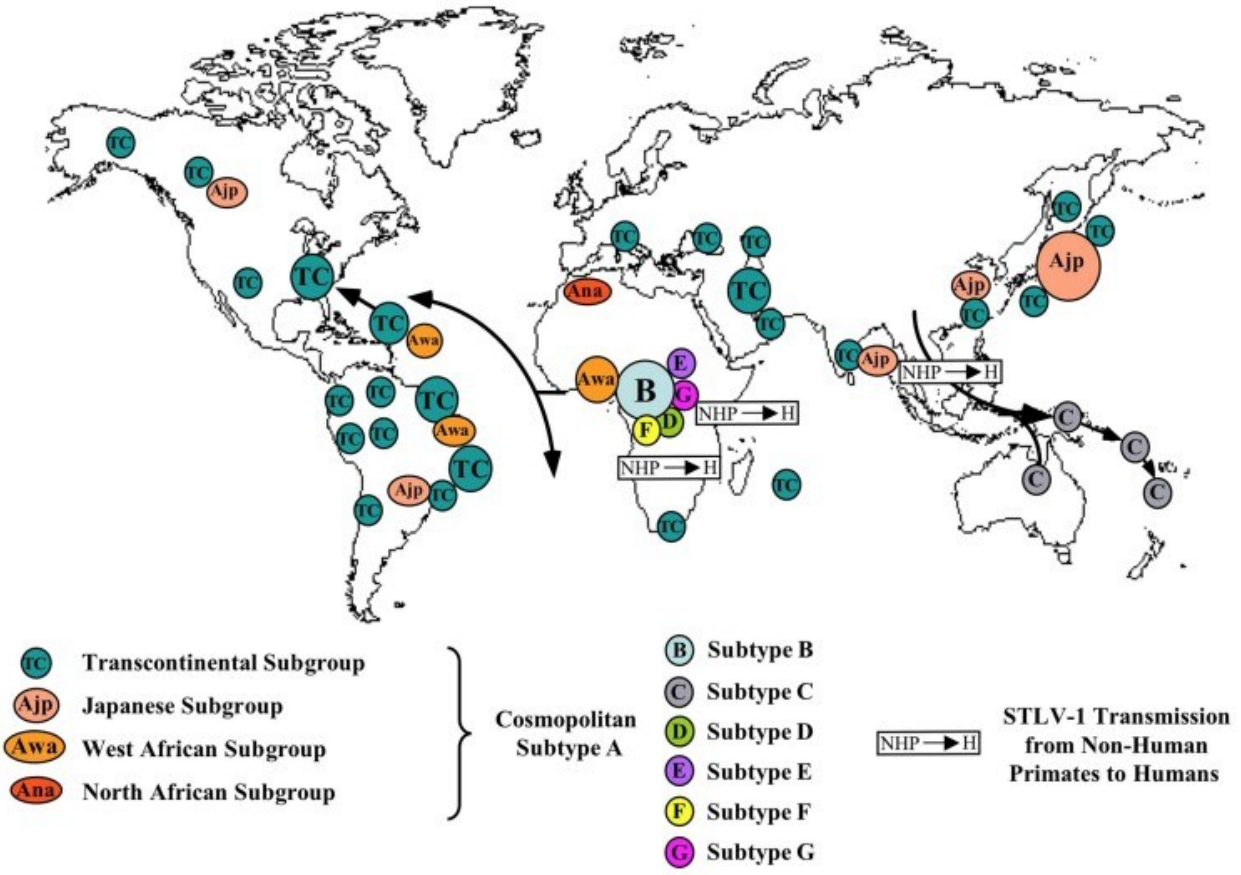

Figure 1. Worldwide geographical distribution of various HTLV-1 genotypes. The most common subtype throughout the world is subtype A. The majority of these classifications are based on geographical localization rather than divergent retroviral DNA sequences due to the stability of the HTLV-1 genome. Adapted from[10].

1.1.2. Virion Structure

HTLV-1 is a human retrovirus belonging to the deltaretrovirus genus, which also contains HTLV-2, 3, 4, Simian-T-Lymphotrophic Virus 1, 2, 3, 4 and Bovine Leukemia Virus. The HTLV-1 virion is generally polyhedral in shape[31], and display a wide range of sizes; between 85 to over 145 nm[32]. The outer layer of the virus is composed of a lipid bilayer from the host cells and contains trimers the viral glycoprotein surface gp46 (SU) and transmembrane gp21 (TM), in which the former is attached to the membrane via the latter. The viral matrix protein p19 (MA) interacts with the inner layer of the bilayer at raft microdomains[33], and helps form the inner capsid made of p24 (CA). This icosahedral capsid houses the two positive ssRNA of HTLV-1 gRNA (genomic RNA) that are bound non-covalently and coated with p15 (NC) nucleocapsid proteins. Host $tRNA^{pro}$ that initiates reverse transcription is also found within this structure[34], along with reverse transcriptase, and integrase.

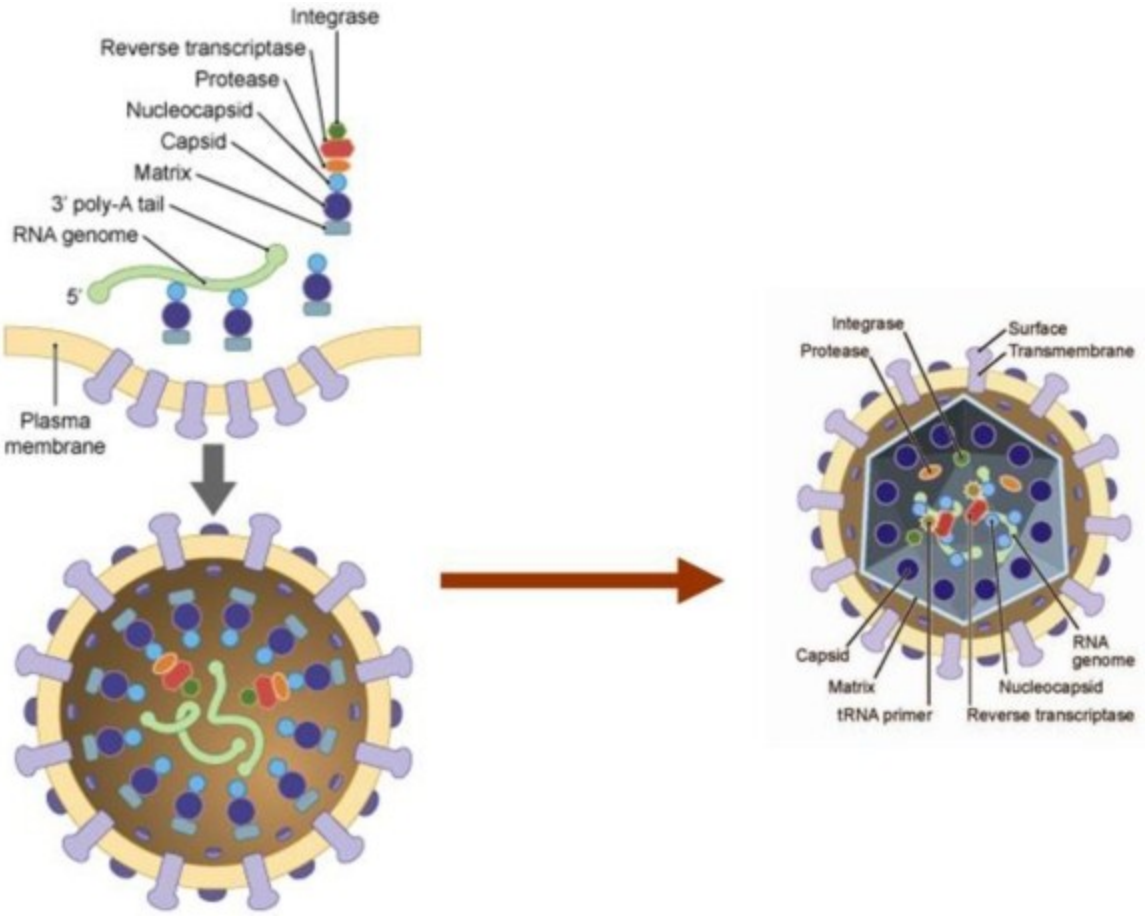

Figure 2: Components and assembly of the HTLV-1 virion. Gag and Gag-Pol precursor proteins are assembled along with two copies of gRNA into nascent viral particles. PR cleavage will mature the particle after release. HTLV-1 does not package any of its accessory proteins. Adapted from[35].

1.1.3. Genomic Organization

The packaged RNA genome of HTLV-1 contains a 5' cap and a poly(A) tail. The genome itself is approximately 9kb in length, and is flanked by a 5' and 3' LTR (long terminal repeat). As an integrated DNA sequence, called a provirus, the 5' and 3' LTR are identical, and each is comprised of U3, R and U5 regions, which are required for retroviral reverse transcription, integration, and transcription. However, as an RNA molecule, the 5' LTR only contains the R and U5 region, while the 3' contains a R and U3 region. The genome contains other typical retroviral components such as the *gag*, *pro*, *pol*, and *env* genes, in addition to a deltaretroviral-unique region known as pX near the 3' end, which encodes many regulatory proteins. The *gag* gene is translated into the polyprotein p55, and is subsequently cleaved by the viral protease to form p19, p24 and p15 (MA, CA and NC). *Pol* and *Env* are similarly processed to produce reverse transcriptase (RT) and integrase (IN), and SU and TM respectively. SU contains three domains; N-terminal RBD (receptor binding domain), a Proline rich region (PRR), and a C-terminal domain[36]. *Pro* overlaps *gag* and *pol*, but is produced via frameshifting[37, 38].

Between the 5' LTR and *gag*, is the primer binding site (PBS) that interacts with the packaged cellular $tRNA^{pro}$. The sequence of the PBS is UGGGGGCUCGUCCGGGAU[39]. Functionally associated with the PBS, is the primer activation sequence (PAS) whose sequence is AAUUU and is located just a few bases upstream of the PBS[40]. The PAS helps initiate reverse transcription, although the HTLV-1 PAS has not been functionally characterized like HIV-1 (human immunodeficiency virus type 1)[41].

Another important motif is the Psi or packaging element[42], which includes numerous stem loops. One structure is the dimerization initiation site, also located just upstream of the primer binding site. It forms a 14 nucleotide stem loop whose sequence is CUCUCCAGGAGAG[43-45]. This stem loop forms non-covalent bonds with a different strand of genomic RNA, and forms the genomic dimer that is found in viral particles. This stem loop seems to be dispensable for HTLV-1 replication *in vitro*, but leads to reduced infectivity[46]. The Psi element also contains other stem loops that are responsible for interacting with Gag proteins during gRNA packaging. It is currently unclear for HTLV-1 what those sequences are, but it likely contains stem loop structures that span the 5' LTR and the beginning of the *gag* gene[47, 48]. Lastly, near

the 3’ U3 region lays the polypurine tract (PPT); a region that contains many purine bases, that is required for reverse transcription.

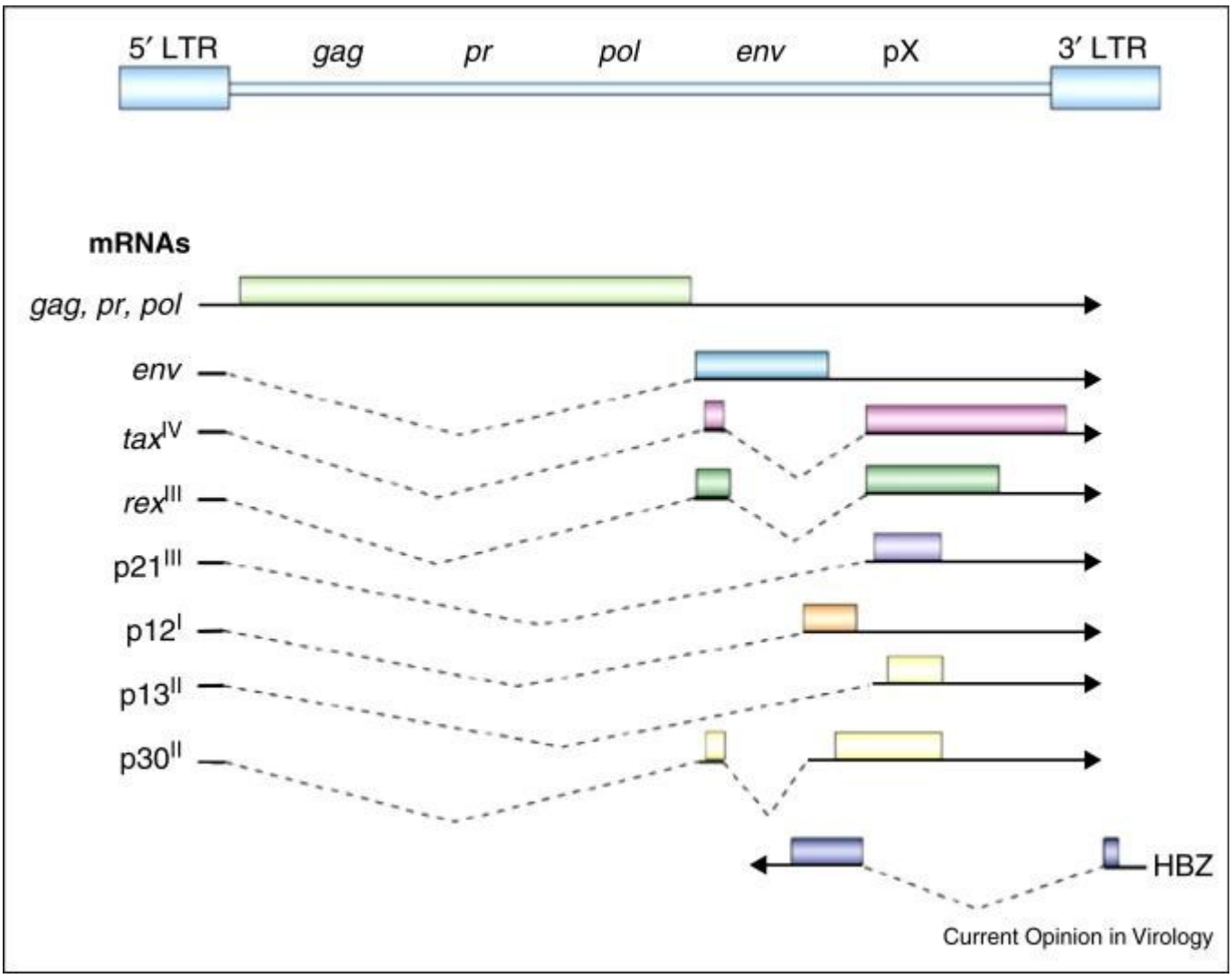

Figure 3: Genomic structure and mRNA produced from the integrated HTLV-1 genome. The gRNA of HTLV-1 follows a relatively standard format, but its accessory proteins are all encoded in the pX region. It can produce several different forms of mRNA, including one produced from its 3' LTR. Adapted from[49].

1.1.4. Life Cycle of HTLV-1

Integrated HTLV-1 DNA is primarily found in $CD4^+$ T-cells *in vivo*[50], although many other cells types have been shown to be infected *in vivo* and *in vitro*. Other cells types include: $CD8^+$ T-cells, regulatory T-cells (Tregs), B-cells, plasmacytoid dendritic cells, myeloid cells, fibroblasts, endothelial cells, etc., but it is not always clear how or if they contribute to HTLV-1 associated diseases[51-57]. It is hypothesized that the large diversity of potential targets is due to the ubiquity of the host molecules that interact with TM and SU. Following this interaction, the viral and host membranes fuse and allow the virus entry into the cytoplasm. Here, reverse transcription occurs and converts the viral ssRNA genome into a dsDNA molecule. This process is associated with many proteins that also give the viral DNA access to the nucleus, where it is integrated into the host DNA. Once integrated, the HTLV-1 DNA is referred to as a provirus, and can be transcribed to generate mRNA to produce viral proteins, or gRNA to be packaged into new viruses and restart the viral lifecycle.

1.1.4.1. Attachment

Viral attachment begins with the interaction between the C-terminal domain of SU and heparin sulfate chains of HSPG (heparin sulfate proteoglycans) on the cell surface[58, 59]. Higher numbers of HSPG syndecan core proteins and a shorter heparin sulfate chain length is associated with increased infection, possibly due to reducing the distance between the virion and the cell, and increasing the likelihood of interaction with the other HTLV-1 receptors[60, 61]. The RBD of SU[62] then interacts with neuropilin-1 (NRP-1)[63] via its KPxR motif[64]. These two receptors, HSPG and NRP-1, normally function as co-receptors for VEGF (vascular endothelial growth factor) 165, and initiate binding to the growth factor prior to its interaction with its receptor VEGF-R[65]. VEGF-165 interacts with NRP-1 via the same KPxR motif, suggesting that HTLV-1 employs molecular mimicry to bind to its target cells[64]. The interactions with HSPG and NRP-1 likely lead to conformational changes in SU and expose its glucose transporter-1 (GLUT1) binding sites[66-68]. The binding of GLUT1 with SU disrupts the disulfide bonds between SU and TM, and causes TM refolding[69]. These conformational changes lead to the exposure of the fusogenic peptide on the N-terminal domain of TM which mediates membrane fusion and allows the viral capsid entry into the cell cytoplasm[66, 70].

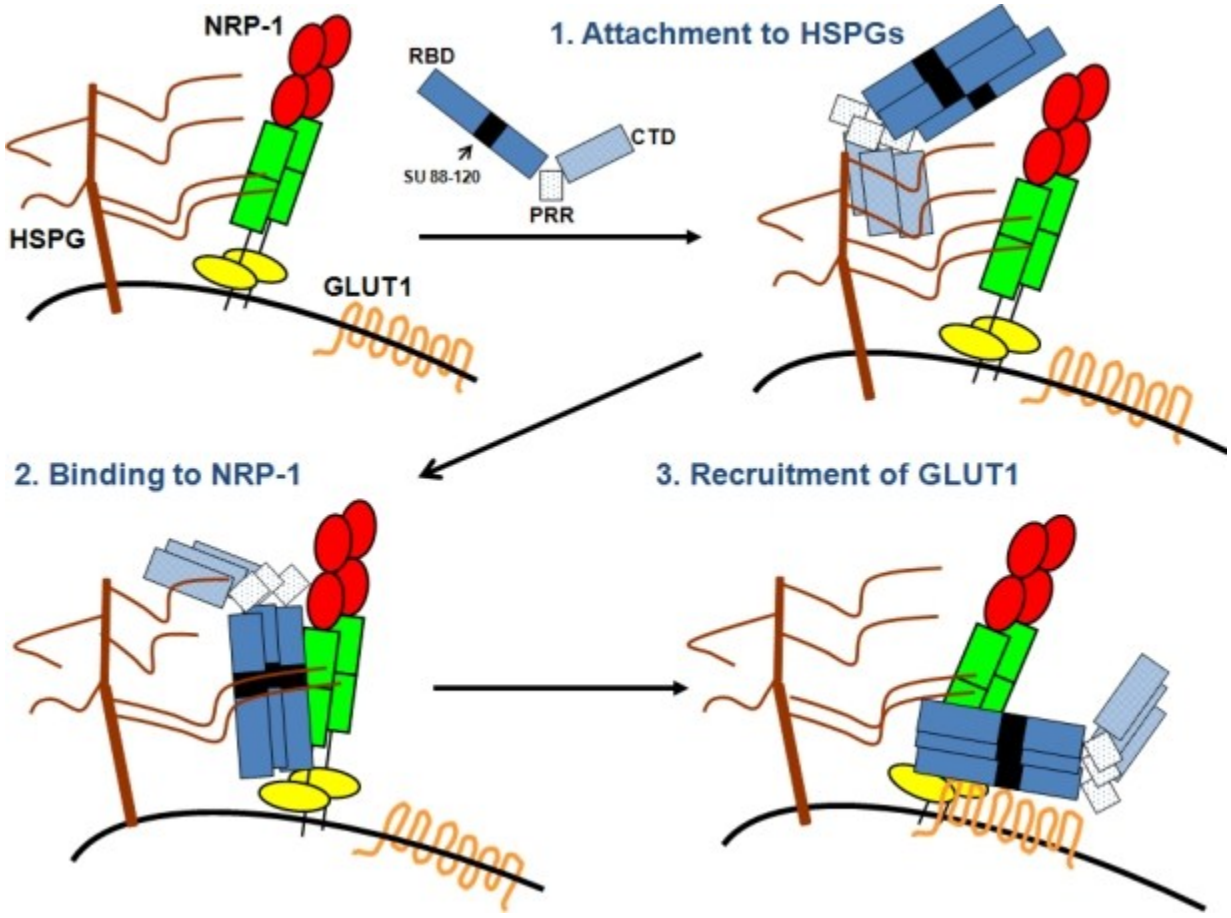

Figure 4: Schematic representation of the cellular receptors involved in the binding to HTLV-1 SU. Initially SU and HSPG will interact via the CTD of SU. HSPG will then bring the virus in close contact with NRP-1, which will interact with SU via its RBD. These interactions will expose the GLUT1 binding site on SU and lead to their interaction and viral entry. Adapted from[66].

Other molecules have also been reported to facilitate viral infection, such as DC-SIGN which also interacts with SU to greatly enhance cell-free HTLV-1 infection of myeloid cells[71, 72]. It has also been shown that hDlg, a common scaffold protein involved with cell adhesion, can interact with TM and SU/TM complexes, and enhances cell-to-cell infection[73]. It was further shown that hDlg can also induce GLUT1 clustering, suggesting an important role in infection[74].

A careful balance between the surface expression of GLUT1 and SU must be achieved in infected cells in order for them to produce infectious viral particles. If this does not occur, it may lead to a phenomenon known as superinfection[75], where an infected cell is infected again with another viral particle. This seemingly occurs very rarely if ever *in vivo* in infected patients[76]. Overexpression of GLUT1 prevents proper maturation of HTLV-1 Env, and greatly reduces the infectivity of viral particles[77]. Under normal circumstances, the Env polyprotein is kept in a separate cellular compartment than GLUT1, as HTLV-1 does not seem to encode a protein that alters the expression of its receptor, unlike HIV-1[78, 79]. Interestingly, $CD4^+$ T-cells express relatively low levels of GLUT1 compared to $CD8^+$ T-cells and other cell types[61, 80, 81], but express higher levels of HSPG[61]. In the end, this may be an unexpected benefit to the virus, as $CD4^+$ T-cells could be more likely to produce functional viral particles once infected, due to low GLUT1 expression. It has also been reported that GLUT1 does not play an important role in viral attachment, which is mostly mediated by HSPG and NRP-1, and therefore HTLV-1 would not require high surface levels of GLUT1 to infect cells[60, 72, 80].

An interesting aspect regarding cell-free viral attachment was demonstrated when several viruses including HIV-1, but not HTLV-1 that was not used in the experiment, can interact with filopodia in a receptor-dependent manner, and “surf” down the filopodia towards the cell body[82]. Data showed that viral surfing was based on myosin and the actin cytoskeleton. Of particular note was that polarized epithelial cells, used here as a model of the mucosal epithelium, were particularly susceptible to infection via viral surfing on their microvilli-rich apical side, but not their basolateral side. Intriguingly, infection via the basolateral side could be enhanced via myosin or actin inhibitors. Viral surfing has currently not been observed during HTLV-1 infection[83], but in this case infection was mediated in a cell-to-cell manner, and the filopodia was the source of infection rather than the receiver.

1.1.4.2. Entry

The current model[84, 85] of membrane fusion supports the notion that the fusogenic peptide imbeds itself into the target cell membrane, while the C-terminal of TM is anchored in the virion[86, 87]. Overall a trimer of TM forms a rod-like structure[88-90], also called the pre-hairpin intermediate, that is stabilized by a coiled-coil formed from other residues on the N-terminal of TM. The rod-like structure then folds itself into a trimer-of-hairpins, or six-helix bundle structure, which brings the viral and cellular envelopes together to induce the formation of a fusion pore through which the viral capsid enters[91, 92].

The formation of the fusion pore during infection is not an immediate or low-energy process, and begins with the joining of the outer layers of the lipid membranes that form a stalk structure. Expanding the initial point of contact brings the inner layers of the lipid membranes together and forms a very stable intermediate hemifusion diaphragm, which can lead to fusion pores[93-95]. The energy required to deform the lipid membrane and cause hemifusion formation could be derived from as little as a single transmembrane fusogenic peptide[96], but likely requires three transmembrane trimers[97]. Small reversible pores may form before the complete folding of TM, but large and stable pores require complete trimer-of-hairpins formation[92]. The speed of this process depends on the virus, but in the context of HIV-1 takes approximately 15 minutes[98]. Much of the knowledge regarding viral-mediated membrane fusion comes from studies with Influenza and HIV-1 viruses.

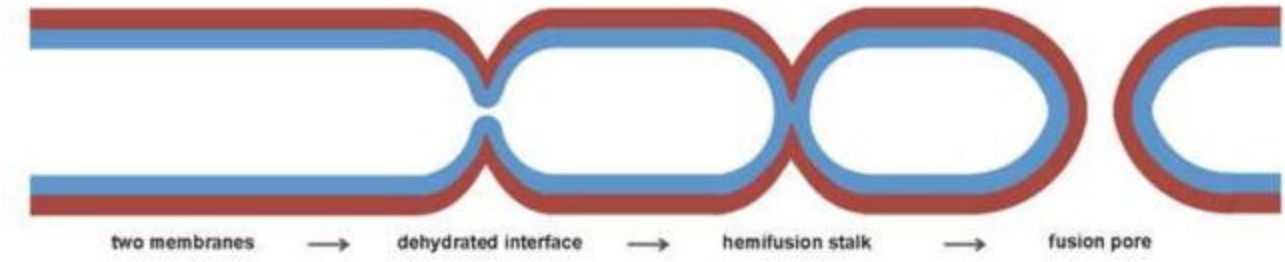

Figure 5: Representation of formation of a fusion pore that occurs during infection. A fusion pore is a complex and energy dependent structure that is formed through various stable intermediaries. Adapted from[99].

A possible physical barrier to viral entry is the host actin cortex that lies just underneath the cell membrane that could prevent the viral core from entering the cytoplasm[100-102]. Little is known regarding the modulation of the actin network by HTLV-1 to facilitate entry, but HIV-1 can interact with actin binding proteins to promote receptor clustering in order to facilitate entry[103-105]. The HIV-1 accessory protein Nef is also known to cause actin depolarization[106, 107] and increase viral replication[100, 108]. Indeed, virions that entered the cell via endocytosis, which bypasses the actin cytoskeleton, are not affected by a lack of Nef, which suggest that its activity is required to overcome the actin cortex[100, 109, 110]. It is also possible that the matrix protein of HIV also mediates entry through its modulation of the actin cytoskeleton. Phosphorylated HIV p17 interacts with actin and is critical for the early stages of infection[107, 111].

HTLV-1 does not package its regulatory proteins, such as Tax that has known actin-modulating properties[112-116], but interaction with its receptors may alter the actin network. NRP-1 and GLUT1 interact with GLUT1CBP which associates with cytoskeletal proteins[117-119]. This interaction is mediated through the PDZ domain on GLUT1 and NRP-1, which is also found on HSPG syndecan core proteins[120]. Alternatively, if HTLV-1 does make use of viral surfing, it may target itself to sections of the cell body that are undergoing actin remodeling to avoid actin-rich areas[82].

Whether or not the actin cortex is a barrier or useful tool to be exploited[82] by HTLV-1 may ultimately depend on a multitude of factors, including the specific mechanism of entry; multiple categories of endocytosis, direct fusion at the outer cell membrane, etc. A recent study has shed light on this issue: during cell-to-cell infection of dendritic cells (DC) actin polymerization was required for HTLV-1 entry into cytoplasmic vesicular compartments[121]. Pharmacological acidification of these vesicles greatly reduced infection, suggesting that productive entry was mostly mediated via endocytosis and not fusion at the plasma membrane. While the details of these vesicles have not been elucidated, they are $CD82^{+}$, which is expressed on the cell surface and is already known to interact with SU[122]. Indeed, the overexpression of CD82 leads to reduced viral infectivity, and is reminiscent of GLUT1 overexpression. CD82 therefore, may be vital for endocytosis-mediated entry of HTLV-1 particles. Another study comparing the transmission of cell-to-cell HTLV-1 with HIV-1, found that HTLV-1 infection was significantly

susceptible to chemical agents that interfered with cytoskeletal remodeling[123]. A functional actin cytoskeleton therefore, seems to be vital for HTLV-1 infection, which has evolved methods to co-opt its functions.

1.1.4.3. Reverse Transcription

Following entry of the viral capsid into the cell cytoplasm, a reverse transcription complex (RTC) is formed around the gRNA to enhance infection. Many features of HTLV-1 reverse transcription and the formation of the RTC are not known, and currently rely on studies on HIV-1 to fill certain gaps in knowledge. It is known that the RTC contains multiple proteins, including Gag proteins, RT and IN, which remain assembled during the uncoating process. In the context of HIV-1, the RTC contains MA which may play a role in the nuclear import step[124], and CA which targets the RTC to the nucleus[125-127] and maintains the shell structure of RTC and allows for nucleotides to enter the core[128, 129]. The CA structure seems to be required for RTC stability to ensure infection, but may leave the RTC as reverse transcription is completed and uncoating progresses[130-132]. IN also plays an interesting role, as its direct interaction with RT may be required for reverse transcription[133, 134].

There is also evidence that the actin cytoskeleton is crucial for uncoating and RTC formation as well as entry. Actin may interact with several important members of the RTC, including RT[135], IN[136] and Gag[137, 138]. Inhibitors of actin polymerization are also associated with reduced early retroviral DNA products[101, 107, 139]. It has been suggested that the interactions between the retroviral core and the actin cortex affects uncoating and therefore RTC formation and reverse transcription[140].

The process of reverse transcription converts the ssRNA genome into a dsDNA molecule. It begins with priming: when the packaged host tRNA binds to the PBS on the 5' end of the gRNA. It is possible to prime the reaction *in vitro* with tRNA fragments instead of tRNA, both of which are found in HTLV-1 virions[39]. Following tRNA/PBS interaction, the antiPAS sequence on the tRNA is exposed leading to further interaction between the tRNA and gRNA[141]. It is believed that these two RNA/RNA interactions at the PBS and PAS with tRNA stimulate reverse transcription[40, 142].

Reverse transcription initiation is linked to uncoating, as delays in the former result in delays in the later[143, 144]. It was recently shown that uncoating was mechanically linked to reverse transcription, as the stress inside the viral core increased due to the conversion of a flexible ssRNA molecule into a dsDNA one[145]. The entire process is also strongly influenced by NC; virions made with mutated NC are more likely to contain viral DNA[146-148]. In those cases, reverse transcription likely began in virus producing cells[149]. Overall, it is thought that tRNA/PBS interaction may occur early in the virus producing cell[150], but that the tRNA/PAS interaction is more tightly controlled by NC[141, 151]. These proteins likely repress tRNA/PAS interaction, and therefore reverse transcription, until the virus infects a new cell[152]. While the exact mechanisms are not clear, NC has the ability to destabilize double stranded nucleic acid structures in gRNA, induce tRNA unwinding, and enhance tRNA binding to gRNA[153].

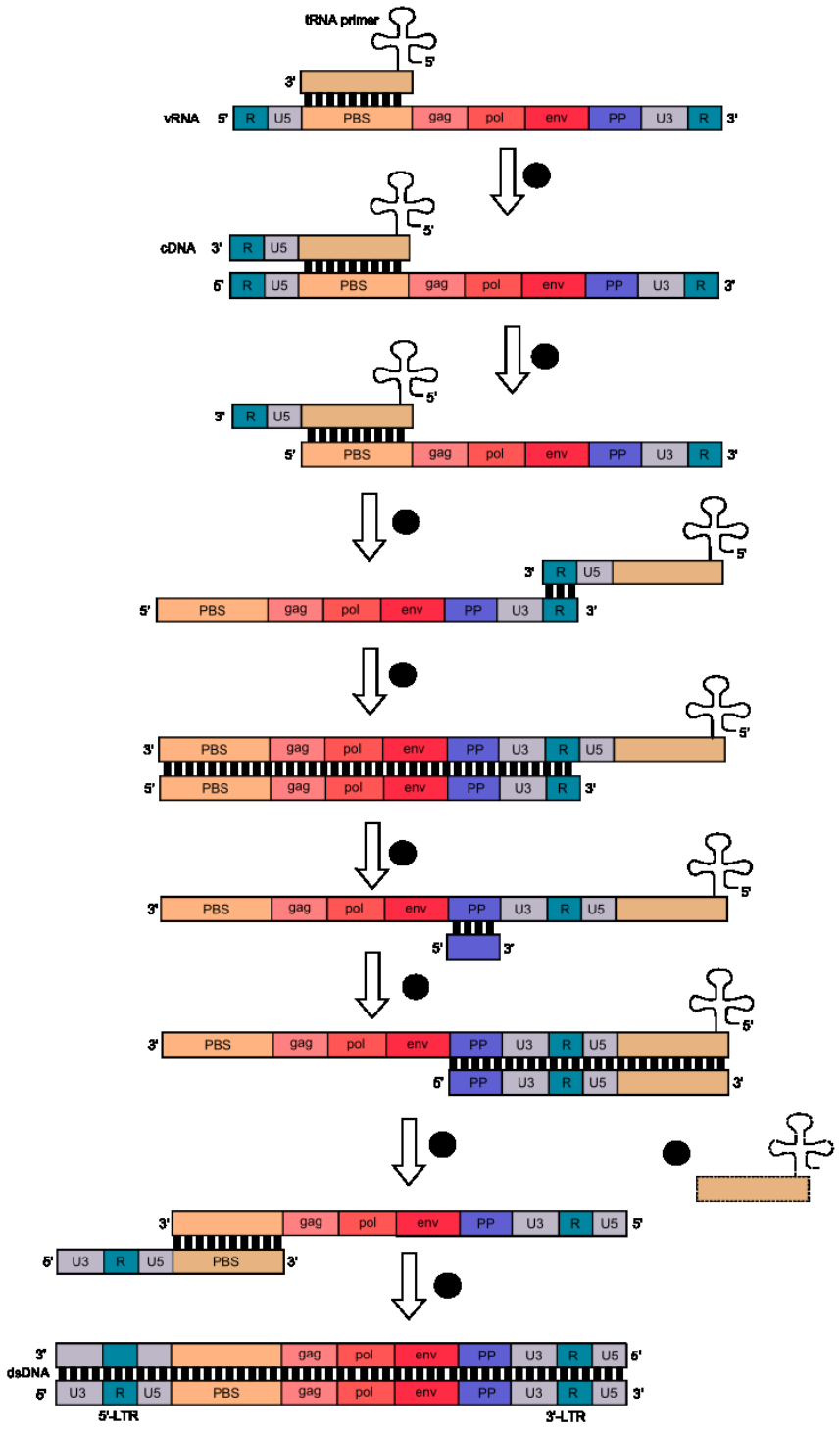

Figure 6: Representation of the key steps that occur during reverse transcription. These steps including primer binding and elongation, completion of the negative strand, strand transfer, cleavage of the gRNA, second strand transfer and completion.

Once initiated, the packaged viral reverse transcriptase catalyzes the production of viral DNA in a 5' to 3' manner, and quickly finishes transcribing the U5 and R regions. The dNTP required to build the DNA are provided by the newly infected cell. To copy the rest of the gRNA, the nascent viral DNA and reverse transcriptase complex must transfer itself over to the 3' end of the gRNA, using the complementary sequence on the R region it produced and the R region on the 3' end of the gRNA as a guide. This negative strand transfer is significantly enhanced by the presence of NC, which aggregates the nucleic acids involved to facilitate the transfer[153]. This event is also highly associated with capsid maturation, as delays in negative strand transfer are associated with deferred uncoating[154].

Retroviral NC has several RNA chaperon activities that enhance reverse transcription: nucleic acid duplex destabilization and aggregation. However the NC of different retroviruses exhibit these functions at varying strengths. HTLV-1 NC was shown to display stronger duplex destabilization than HIV-1, but significantly weaker RNA binding and aggregation due to an overall neutral charge and slow dissociation kinetics[155-159]. Weak nucleic acid binding suggests that HTLV-1 NC does not strongly promote the strand transfer step during reverse transcription. It is currently unclear if this has any effect on HTLV-1 reverse transcription, or if other viral proteins, such as MA, alleviate this potential defect. It has been shown that other deltaretroviruses use MA to facilitate gRNA binding to compensate for the weak NC/gRNA interactions[160]. Additionally, the R region of the HTLV-1 LTR is particularly long, over twice as long as in HIV-1, and may be another mechanism employed by HTLV-1 to mediate strand transfer. There is a correlation between the length of a retroviral R region and duplex destabilizing strength of its NC protein[155].

As the rest of the gRNA is reverse transcribed to produce the negative DNA strand, the RNase H domain of the reverse transcriptase enzyme degrades any gRNA in a gRNA/DNA hybrid via its endonuclease activity in a sequence independent manner. This occurs during pauses in DNA synthesis[161], but it is unclear which reverse transcriptase enzyme performs this function. Multiple RT enzymes are packaged into a virion, and studies have shown that the DNA producing RT need not be the one that degrades the gRNA[162, 163]. The PPT of the gRNA is resistant to RNase H activity and is left intact, and it serves as an RNA primer to begin the synthesis of the positive DNA strand. Positive strand synthesis begins with the 3' LTR in

addition to the PBS of the tRNA. In the context of HIV-1, whose primer is $tRNA^{lys}$, reverse transcription stops at the first modified nucleotide present in the tRNA, which lies just after the PBS sequence[164, 165]. The pausing and subsequent interruption of RT here is likely due to the stability provided by the unconventional nucleotide. Once the PBS region has been reverse transcribed, the tRNA is removed via RNase H which allows for a second strand transfer to occur, also known as the positive strand transfer. This second transfer occurs more slowly than the first, likely due to the requirement of RNase H activity[166]. During positive strand transfer, the complementary PBS sequence is used as a guide, and the transfer is again mediated by NC[167]. Reverse transcription can continue, using both strands as templates to complete the dsDNA retroviral genome.

HIV-1 contains two identical PPT; a central PPT (cPPT) and one near the 3' end. The PPT of HTLV-1 has not been functionally characterized, but a 3' PPT is required for reverse transcription to occur. The cPPT of HIV-1 is vital for the subsequent step of nuclear entry[168], but it is not known if HTLV-1 employs similar mechanisms or uses multiple PPT. There is evidence that suggests that positive strand synthesis occurs at multiple sites including the cPPT, and not just at 3' PPT[166, 169, 170]. This causes the positive strand to be completed faster than the negative strand. The nature of the priming at these multiple sites remains unclear; if these are specific sequences that are resistant to RNase H activity or random gRNA fragments that have bound to the negative strand.

Reverse transcriptase often pauses and even dissociates from the nucleic acid chain it produces and reads. This is due to secondary structures in the RNA template[171, 172]. Interruptions can also occur due to gRNA fragmentation or damage, and during strand transfer events. These events, as well as others, may lead to retroviral recombination, resulting in a DNA product that contains sequences from both packaged gRNA strands[173]. In the context of superinfection, this can lead to rapid changes in the viral genome if the two packaged gRNA come from two different strains. While superinfection has not been reported with HTLV-1[76], there is evidence that this has occurred in the past[174]; a recombination between a Senegal and West African strain of HTLV-1 occurred approximately 4000 years ago, and is presently active in North Africa. The data strongly suggested a recombination event between the U3 and R regions of the LTR, suggesting that a negative strand transfer induced recombination event took place during

reverse transcription. To put this in perspective, the main HTLV-1 genotype most prevalent around the world (cosmopolitan a subtype) arose over 12,000 years ago, and the earliest human strain of HTLV-1 could have arisen as late as 60,000 years ago[175].

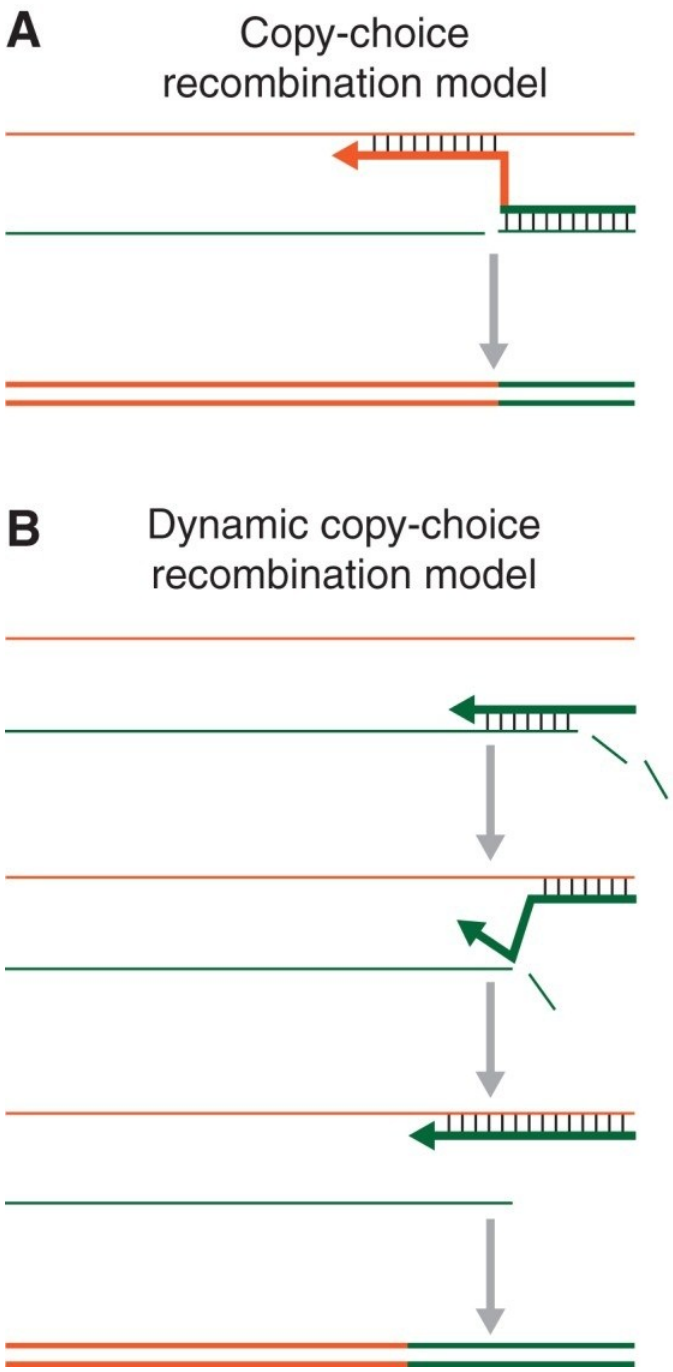

Figure 7: Representation of the two known methods of strand recombination that can occur during reverse transcription. Adapted from[164].

For HIV-1, error-prone RT is an important source of viral mutations, as well as host RNA pol II that produces the gRNA to be packaged[164]. In both cases neither enzyme has proofreading capabilities. While the genetic stability of HTLV-1 is largely attributed to host DNA polymerase II, its RT enzyme also produces significantly less errors than HIV-1 RT[176, 177]. HTLV-1 RT is produced from an mRNA that contains both *gag* and *pro*. Two frameshifts are required to produce RT; one following *gag* and another following *pro*, resulting in a Gag-Pro-Pol polyprotein that requires PR mediated cleavage. Overall this results in a very small amount of RT being present in virions. There is a small peptide that separates uncleaved Pro and Pol, p1, which must be removed from the N-terminus of RT for it to function[178]. Pol is also processed by PR to produce IN and RT. The latter can be further cleaved to p49 that only contains the polymerase domain, while RT, also called p62, contains both the polymerase and RNase H domains[179]. p62 and p49 can form a heterodimer *in vitro*, and is reminiscent of HIV-1 RT that forms a similar p66/p51 heterodimer.

1.1.4.4. Nuclear Transport

Following the formation of the RTC, active transport is required to bring it towards the nucleus. Many details of this step remain elusive, even in the context of HIV-1 infection. The mechanisms involved are further obscured by the type of cell targeted and the mode of entry. It is believed that actin and/or microtubule dependent movement is required for the retroviral core to reach the nucleus[101, 128, 139, 180], an interaction that likely begins during RTC formation. Ultimately, dissecting the differences between transport through the actin cortex and the cytoplasm are difficult to discover, as any modulation to the former would likely have impacted the latter.

Several retroviral proteins included in the RTC such as MA[124], IN[181] and Vpr[182] in the case of HIV-1, contain NLS or have been reported to localize cytoplasmic proteins to the nucleus when bound. It has also been reported that IN can interact with nucleoporins (NUPs) found in nuclear pore complexes (NPC) that regulate traffic through the nuclear envelope[183]. The presence of the cPPT during reverse transcription also influences nuclear entry through the formation of a triple stranded DNA flap which serves as an import signal at the NPC[168, 184].

However, the requirements of these features has been challenged multiple times, as many mutations in MA, IN, Vpr and cPPT do not necessarily inhibit infection[185-187].

Studies using MLV (Murine Leukemia Virus), a retrovirus that cannot infect resting cells due to its inability to get past the nuclear envelope, have indicated that CA may be primarily responsible for the nuclear localization of the RTC[125-127]. Specific mutations in HIV-1 CA have shown no defects in reverse transcription but defects in DNA nuclear translocation[188]. Ultimately it appears that CA in the viral core plays an important role at multiple steps of the retroviral lifecycle. Uncoating is a carefully regulated process important for reverse transcription and transportation of the viral dsDNA into the nucleus[189].

Several proteins have been identified for the transport of the HIV-1 retroviral core into the nucleus. Such proteins include importin proteins such as TNPO3, and several NUP proteins such as NUP358 and NUP 153[189, 190] that interact with IN[191] and CA[192, 193]. It is unclear if HTLV-1 uses similar proteins for entry, as its CA functions much differently than the HIV-1 CA. The HTLV-1 capsid core forms a spherical shape[31], while in HIV-1 it forms a conical structure[194]. Furthermore, HTLV-1 CA does not interact with cyclophilin A[195, 196], which in the case of HIV-1 plays an important role in uncoating[197] and nuclear transport via NUP358[192]. However, considering how HTLV-1 is also able to infect non-dividing cells like HIV-1, it is likely that it could employ similar mechanisms for entry through NUP interactions.

1.1.4.5. Integration

As reverse transcription is completed, uncoating progresses and the RTC transforms into the preintegration complex (PIC). During this time, IN performs a number of important steps in the cytoplasm[198] to prepare the dsDNA for integration. Multiple IN will interact with the ends of the completed retroviral dsDNA and nick their 3' ends, removing two to three nucleotides[199]. This 3'-end processing will lead to the formation of CA dinucleotide OH-3'-hyroxyl reactive ends. This reactive complex is likely stabilized by host proteins, as the 3' ends may aberrantly integrate themselves internally within the same dsDNA molecule, leading to the formation of circular rearranged, or truncated, retroviral DNA, which is not infectious[200, 201]. Several

retroviruses have evolved different mechanisms to prevent this, such as BAF for MLV[202] or the SET complex for HIV-1[203].

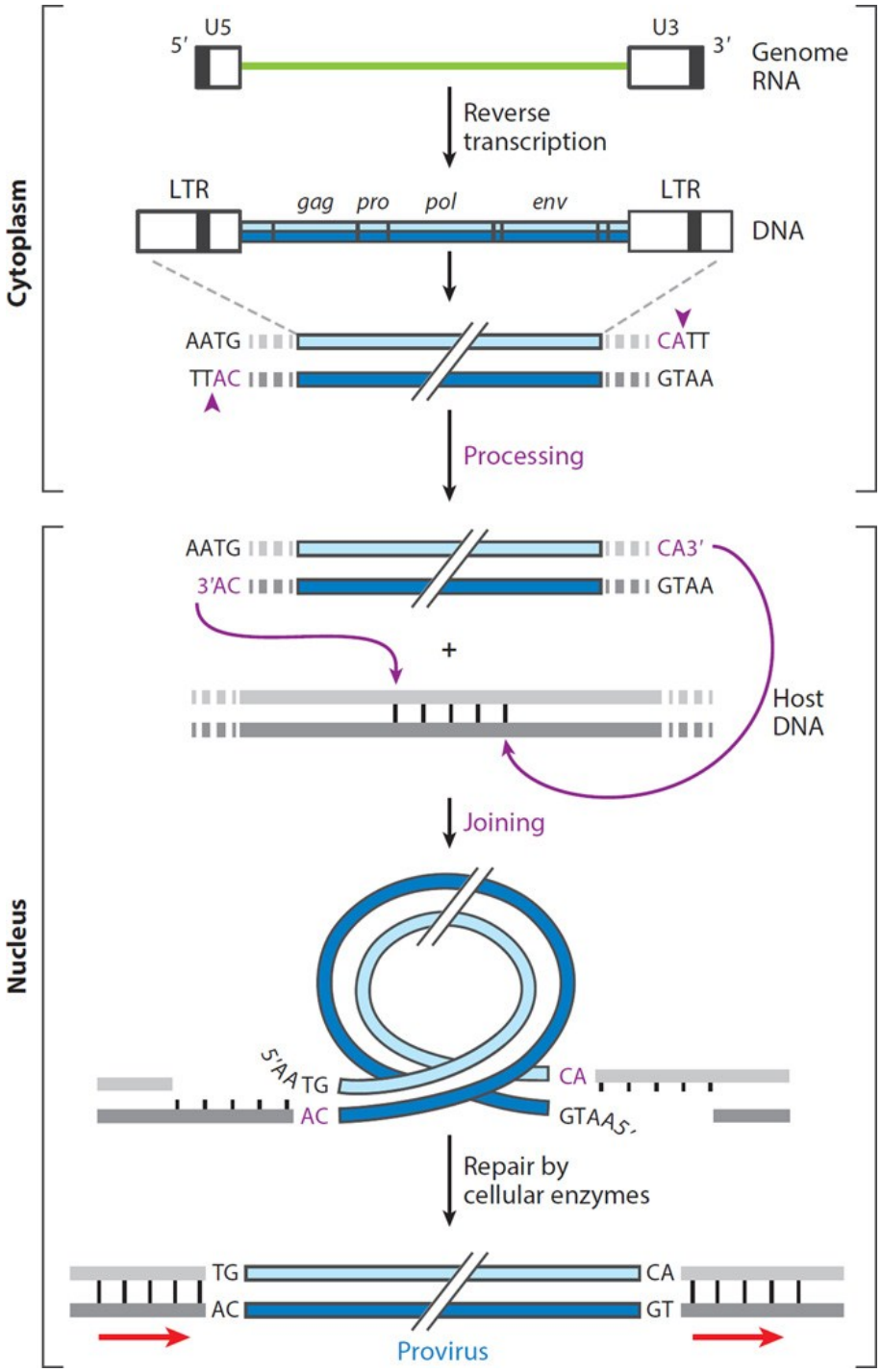

Figure 8: Schematic representation of all the enzymatic reactions carried out by the viral enzyme integrase, depending on its subcellular localization. In the cytoplasm, integrase removes two bases to form 5' overhangs. In the nucleus, integrase cleaves the host DNA, creating a 6 base overhang in the context of HTLV-1. Integrase of HIV-1 represented here. Adapted from[204].

Once inside the nucleus, several broad mechanisms are proposed to target the retroviral dsDNA to the host DNA. The first is the tethering of specific chromatin proteins to IN. In the context of HIV-1, LEDGF binds to IN[205] and mediates the interaction between chromatin and IN[206]. HIV-1 integration will then occur in and around genes bound with LEDGF[207].

While HTLV-1 does not seem to integrate near any specific genes it does prefer open chromatin and prefers integration near transcription start and active sites[208-211]. However, it was reported that PP2A can bind to the IN of deltaretroviruses, including HTLV-1, and stimulate IN enzymatic activity[212]. PP2A does not directly bind to chromatin, but interacts with several DNA binding proteins such as p53, STAT1 and HDACs[213, 214]. Intriguingly, newer methods of integration site analysis have identified enriched sites of HTLV-1 integration which include p53 and STAT1 binding sites[211].

Another method of targeting was thought to be mediated by palindromic sequences in the host DNA that are targeted by IN[209]. Recent work has demonstrated that this is not the case for HIV-1 and HTLV-1, but a non-palindromic DNA motif is recognized that is present on both DNA strands[215]. However it is thought that the DNA specificity of IN is quite weak and it can bind multiple sequences.

The next catalytic step performed by IN is called strand transfer, whereby the retroviral DNA is inserted into the host DNA. IN catalyzes a break in the host DNA, creating a six base pair overhang in the context of HTLV-1[216], and joins the 3' processed ends of the retroviral DNA to the overhang. This causes a gap on both sides on the 5' end of the retroviral LTR, that once repaired generates a six base pair repeat that flanks the integrated provirus. In addition to this gap, unligated DNA segments on the positive strand and the DNA flap, are repaired by host DNA repair machinery including FEN1 [217-219]. Prior to integration, the host repair machinery may interact with the retroviral genome and cause the formation of 1 or 2-LTR circles in the nucleus via homologous recombination and non-homologous end joining respectively[220].

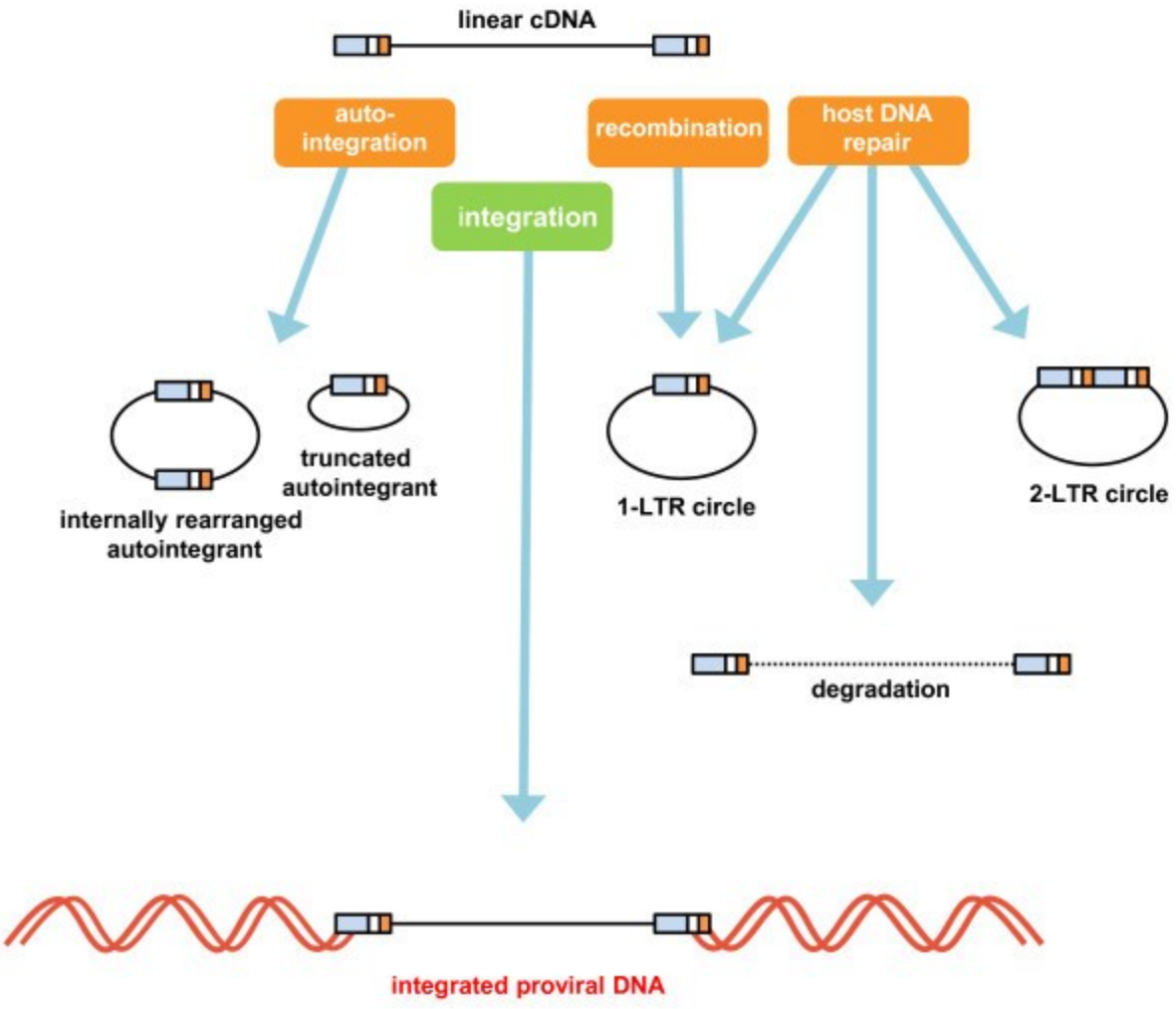

Figure 9: A diagram that demonstrates the various possible states that retroviral DNA can find itself in. During normal pauses in the retroviral lifecycle, integrase may accidentally mediate autointegration. On the other hand, the primed ends may also be detected by cellular enzymes that catalyze the formation of LTR circles. Adapted from[220].

As HTLV-1 replicates primarily through mitosis, beneficial integration sites will accumulate *in vivo*, and provide new insight into disease progression. Individuals suffering from HAM/TSP are more likely to display increased HTLV-1 integration into transcriptionally active regions compared to asymptomatic carriers[208]. In the context of ATL, a large number of unique clones increases the risk of disease, but not integration near oncogenes or oligoclonal expansion[221]. Studies of *in vivo* HTLV-1 integration sites suggest that the long arm of acrocentric chromosomes, 13, 14, 15 and 21, are more likely to harbor a provirus, but it remains unclear why this occurs[221].

1.1.4.6. Viral Transcription

Following integration and DNA repair, the integrated HTLV-1 DNA, now referred to as a provirus, may being producing viral mRNA via RNA pol II. The initial round of transcription is dependent on cellular proteins. The U3 region of the viral 5' LTR contains several important transcriptional activation sites, such as a TATA box and a Tax-response element-1 (TRE-1). The TATA box is a general transcription factor binding site that contains the TATA nucleotide sequence, while the TRE-1 is made up of three imperfect 21bp repeats. Tax augments CREB binding to the TRE[222-224], while also enhancing TBP-mediated transcription from the TATA box[225, 226]. Overall Tax and CREB function together to enhance retroviral transcription[227-229]. Further details on Tax-mediated transcription of the viral and cellular genes will be included in the later section on Tax.

The U3 region of the 3' LTR contains the polyadenylation signal that is required for proper mRNA processing[230, 231]. The RxRE (Rex responsive element) can be found in the U3 and R regions of the LTR, and is found in HTLV-1 mRNA[232]. It influences viral mRNA splicing and nuclear export.

The HTLV-1 provirus can produce differently spliced mRNA that encode several different proteins from its 5' LTR. Unspliced mRNA can produce a large Gag-Pol polyprotein; it requires ribosomal frameshifts for Gag, PR and RT-IN expression, and requires PR to cleave the proteins. A single spliced mRNA encodes for Env proteins, and PR similarly separates SU from TM. The pX region of HTLV-1 encodes numerous regulatory proteins produced via several different

mRNA. Tax and Rex proteins are produced by a doubly spliced dicistronic mRNA. p8/p12 is produced as p12 from a singly spliced mRNA, which can be cleaved to p8. p30 is produced by its own doubly spliced mRNA, but p13, which is identical to 87 amino acids of the C-terminal of p30, is produced from its own singly spliced mRNA. The HTLV-1 genome may also produce other mRNA, such as one coding for $p21^{Rex}$, but these truncated proteins have unknown or unconfirmed biological significance[233, 234].

The HTLV-1 genome also utilizes its 3' LTR for transcription; it encodes HBZ in an anti-sense orientation[235-237]. It was recently shown that Tax mediated sense transcription had no effect and was not affected by strong antisense Tax independent transcription from the 3' LTR[238].

The site of retroviral integration can also modulate viral gene expression. It was shown that same sense integration 1kb upstream of a transcription start site was associated with spontaneous viral gene expression, whereas integration downstream led to silencing likely due to transcriptional interference[211]. There were however, significant differences on retroviral gene expression based on the transcription factor investigated.

1.1.4.7. Viral Translation and Rex

The first mRNA produced by the HTLV-1 provirus is the doubly spliced *tax/rex* dicistronic mRNA. Tax is more readily produced from this, and activates further retroviral transcription to produce more Tax and Rex. Eventually, enough Rex is produced to bind and stabilize HTLV-1 mRNA[239-241] through the RxRE that forms secondary structures[230]. This results in the cytoplasmic export of singly spliced and unspliced HTLV-1 mRNA through Rex and is associated with a decrease, but not a shutdown, in the export of *tax/rex* mRNA[233, 239]. Recent work has demonstrated that retroviral mRNA exported this way contains a 75 nucleotide long hairpin intronic sequence[242].

p30 mRNA, which is doubly spliced but expressed at low levels, produces a protein that binds to *tax/rex* mRNA and keeps it in the nucleus through its NLS[243-245]. This further suppresses Tax and Rex expression, and together generates an overall switch towards the expression of other genes required for virion production such as *gag*, *pol* and *env*[246, 247].

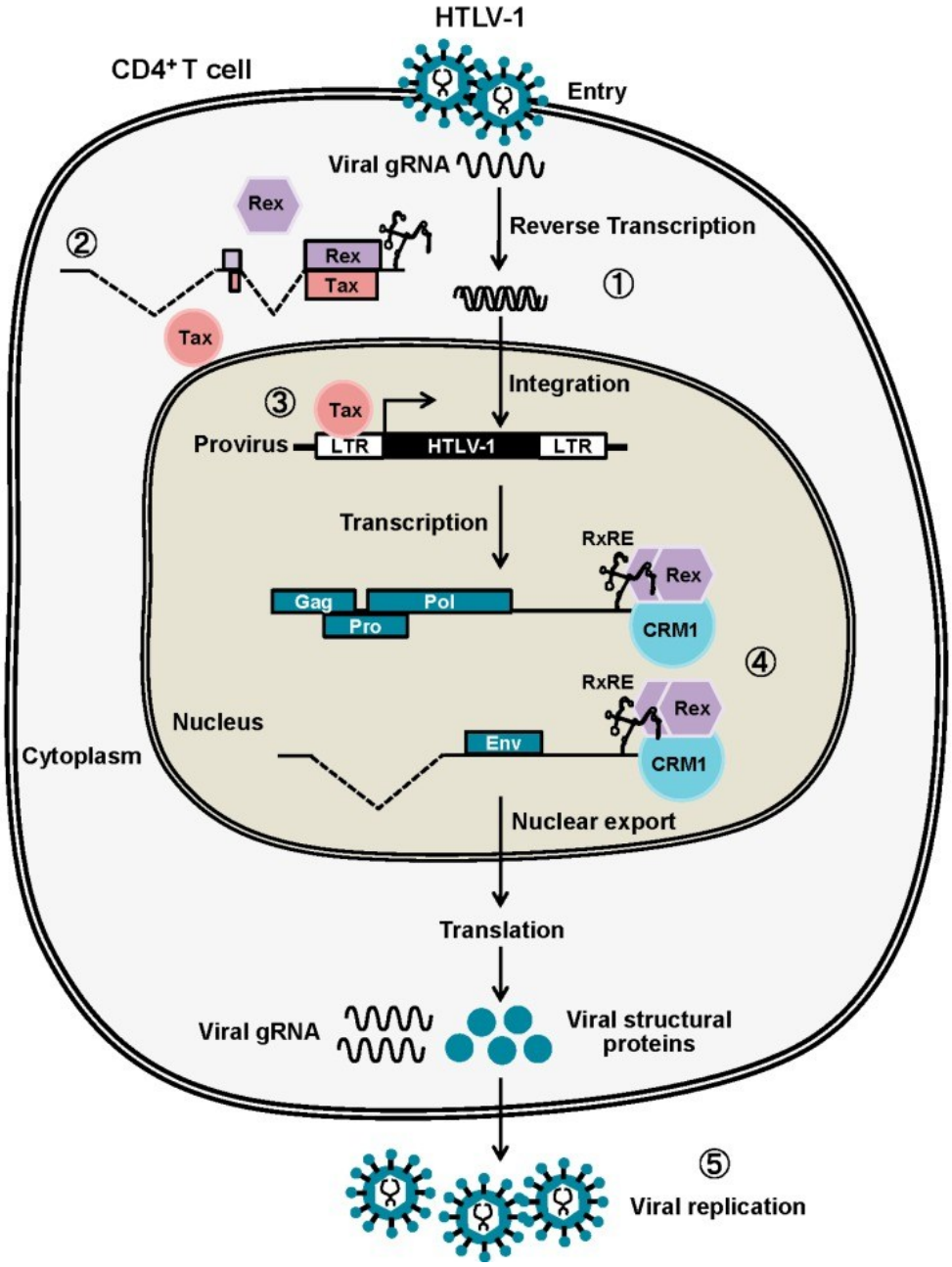

Figure 10: Overall representation of the HTLV-1 retroviral lifecycle. Rex expression following Tax trans-activation leads to the stability of HTLV-1 mRNA that code for non-Tax/Rex mRNA. Adapted from[247].

Rex performs its functions through several important domains, and is positively regulated via phosphorylation[248]. Its N-terminal contains RNA binding domain and NLS which allows Rex to enter the nucleus. Rex interacts with CRM1 through its NES for nuclear export[249]. CRM1 binding also promotes oligomerization of Rex, which is required for mRNA binding and is mediated through two multimerization domains.

Once in the cytoplasm, most HTLV-1 mRNA is translated by free ribosomes, with the exception of the singly spliced *env* mRNA that is translated by membrane-bound ribosomes[30]. In order to induce translation from host machinery, it is believed that HTLV-1 employs an IRES element in its 5' LTR[250, 251], but there is evidence that other mechanisms, such as DHX9 cap-mediated translation, may be involved[252].

Unspliced retroviral RNA that contains a Psi element diffuses through the cytoplasm towards the plasma membrane for dimerization and incorporation into new virions[253]. There the gRNA dimer likely interacts with both MA and NC of Gag for particle assembly[160]. This is in contrast to HIV-1, whose NC can interact with RNA much more easily[158]. MA also guides new particle formation at lipid rafts at the plasma membrane[254, 255]. Assembled particles then bud from the host membrane as immature virions. PR will then process the Gag and Gag-Pol polyproteins for virus maturation and infectivity.

1.1.5. Transmission

HTLV-1 is mainly transmitted vertically; from mother to child via breastmilk, but can also be contracted through contaminated blood or sexual contact[256]. It usually causes diseases in less than 10% of infected individuals[257, 258], and is the causal agent of ATL and HAM/TSP, as well as other associated diseases that will be discussed in a later section. Intriguingly, it appears that the route of infection is a key determinant for disease progression[259]. An infection that begins during breastfeeding may develop into ATL[260], while one acquired due to contaminated blood could result in HAM/TSP[261]. The molecular reasons for this are still being investigated, but early infection is asymptomatic making it difficult to study *de novo* transmission.

HTLV-1 is not a very infectious virus compared to HIV-1. Transmission of whole blood or cellular blood products will lead to infection in about 10%[262, 263] and 50%[264] of cases respectively, but the transfer of plasma[264], non-cellular blood products[265], or refrigerated blood will not[266]. The rates of sexual transmission are skewed towards male-to-female transmission[267]; approximately 60% of women in serodiscordant couples will become infected over a 10 year period versus only 0.4% of men[268]. The rates of mother-to-child vary on the viral load of the mother and the duration of the breastfeeding, but are between 5% and 25%[260, 269-271]. The source of the virus appears to be infected lymphocytes in the blood, semen or breastmilk[272, 273], which are not present in the plasma or non-cellular blood products, and will not survive refrigeration. However, in the context of semen and breastmilk, it is not clear how the virus actually spreads to the uninfected individual *in vivo*.

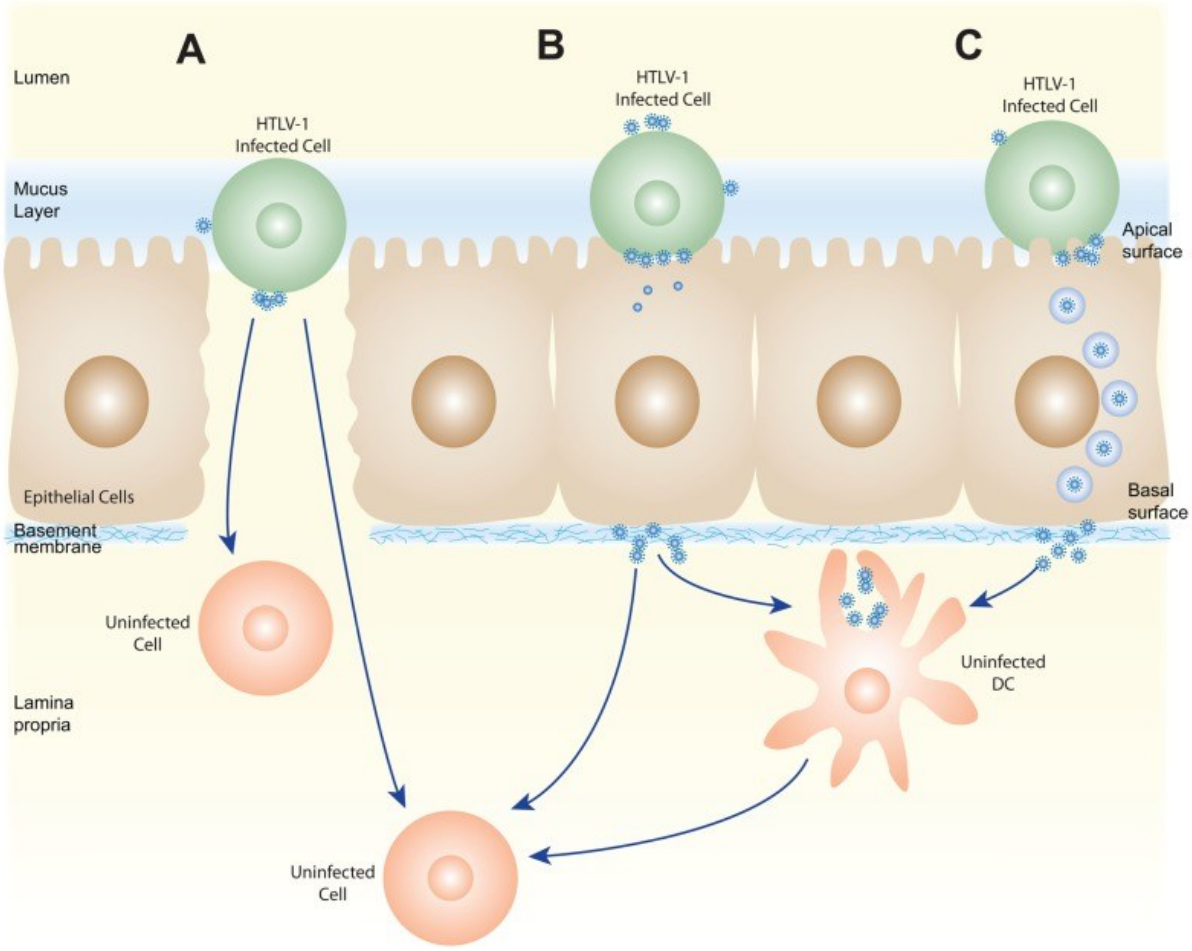

Figure 11: Schematic representing the various possible methods of HTLV-1 infection across the mucosa. In A, via damage to the epithelial barrier. In B, cell-to-cell infection to the cells of the epithelial barrier could then lead to transmission of the virus on the other side of the mucosa. In C, the virus is transported via transcytosis across the mucosa. In any event, cell-free or cell-associated HTLV-1 can then infect cells in the body and begin establishing a reservoir. Adapted from[274].

One historical observation regarding HTLV-1, is that its cell-free virions are poorly infectious of their main target, $CD4^+$ T-cells, especially compared to other retroviruses[275, 276]. It has been known for quite some time that cell-to-cell contact between infected and uninfected cells is required for efficient viral spread[277]. Myeloid cells on the other hand, are significantly easier to infect with cell-free HTLV-1, and are even more susceptible than $CD4^+$ T-cells to cell-to-cell infection[278].

DC that have been exposed to HTLV-1 can spread the infection to T-cell in cis or trans[56], but a more recent study has shown that productive infection of DC significantly enhances transmission[121]. Rizkallah *et al.* also demonstrated a difference in HTLV-1 infection based on the maturation status of the DC; immature DC were productively infected at a higher rate than mature DC. There was also an inverse correlation between productive infection and virus capture; immature DC captured less HTLV-1, but were more likely to become infected. Ultimately, productive infection was linked to the route of entry and the subsequent pH of the receiving vesicle; macropinocytosis in immature DC and via clathrin-mediated endocytosis in mature DC. This data was partially corroborated by a study using HIV-1[279], and may help future research in determining which modes of entry restrict retroviral replication.

One *in vitro* study demonstrated that cell-free HTLV-1 can cross an epithelial barrier through transcytosis, to then infect dendritic cells[280]. Another study on HIV-1 demonstrated similar results, but that cell-associated transcytosis was significantly more efficient than cell-free transcytosis[281]. Taken together, this seems to suggest that infected lymphocytes interact with epithelial cells to efficiently transfer HTLV-1 across the membrane. At this point, cell-free HTLV-1 can then infect dendritic cells which will transfer them to $CD4^+$ T-cells. It is also possible that HTLV-1 infected cells cross the epithelial barrier altogether, allowing for direct infection of the new hosts $CD4^+$ T-cells. A number of different molecular mechanisms of HTLV-1 transmission, other than cell-free infection, have been described and are outlined below in the following subsections. The consequences of these cell-to-cell mechanisms are likely that the virus is hidden from detection and inactivation by the immune system. They include virological synapses, conduits and biofilms, and are not mutually exclusive.

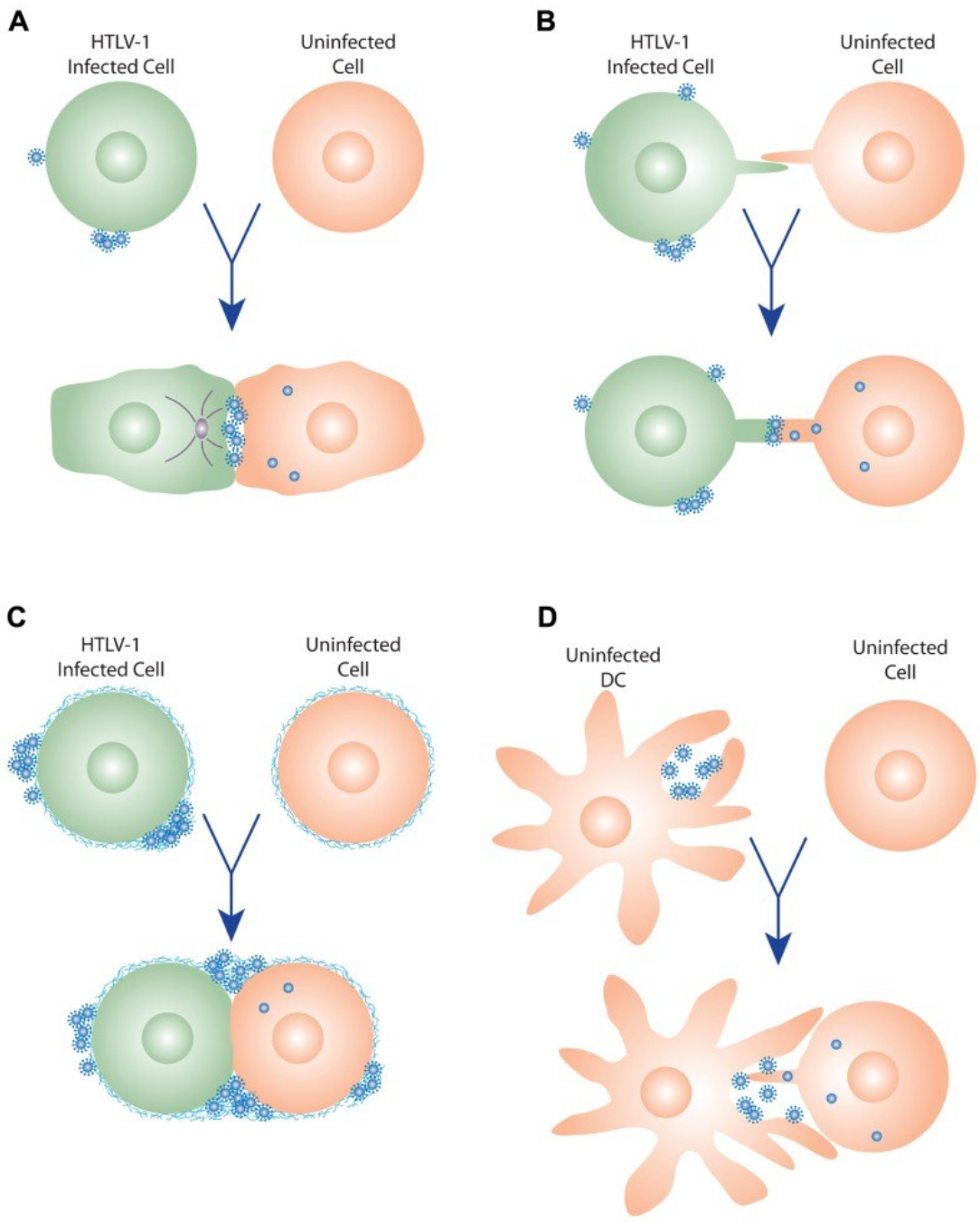

Figure 12: Representation of the various models of HTLV-1 cell-to-cell transmission. In A, the cells form a virological synapse to transmit HTLV-1. In B, the infected cell forms a viral conduit that can infect cell via a mini-virological synapse. In C, the infected cell accumulates HTLV-1 on its cell surface in the form of biofilms that can be easily transmitted via cell-to-cell contacts. In D, cell-free infection of DC is possible, which can then transmit HTLV-1 in a cell-to-cell manner in trans. Adapted from[274].

Transmission of HTLV-1 virions to uninfected cells seemed to play a rather small contribution to the proviral load. While many $CD4^+$ T-cells in infected individuals harbor a single provirus[76, 221], there are usually only a few hundred unique integration sites[282], suggesting that most of the infected cells and pathology of HTLV-1 are the result of clonal expansion rather than *de novo* infection. To support this is the fact that the HTLV-1 provirus displays remarkable genetic stability[27, 29], which cannot only be due to the relative fidelity of the HTLV-1 reverse transcriptase[176, 177], but likely to the stability of host DNA polymerases II. Furthermore, the addition of reverse transcriptase inhibitors, even early after infection, does not reduce the proviral load[283-285], suggesting that retroviral replication is not an important factor in HTLV-1 proviral load. Lastly, sense viral gene expression is often silenced in malignant HTLV-1 infected cells via methylation, 5' LTR deletion or nonsense mutations[286-288], indicating that retroviral gene expression was not required for HTLV-1 pathology. Overall it was hypothesized that HTLV-1 replicated primarily through oligoclonal replication and that disease progress was tied to this[289].

However this theory has been recently challenged. New techniques have identified 10 to 100 thousand unique integration sites[221, 290], and a high number of individual clones is associated with disease progression[208]. The number of unique clones is also associated with proviral load, and not mitotic replication of HTLV-1[290]. This would suggest that HTLV-1 maintains low level replication within an infected individual, and that clonal diversity drives disease progression[291]. Additionally, the silencing of transcription from the 5' LTR is mostly associated with high clonal abundance. In other words, the clones found at the highest frequency, which are not likely related to disease progression, do not express viral transcripts, but rare clones still expressed viral proteins such as Tax[211]. The Tax silencing is thought to confer a replicative advantage in vivo, as the CTL (cytotoxic T lymphocytes) response can strongly target Tax[292-295]. Thus silencing Tax would allow a specific clone to replicate quickly without affecting disease, while the Tax positive clones that are spreading infection and contributing to pathology are kept at in low numbers. This theory is further supported by the fact that the anti-Tax CTL response is chronically active[296], which supports the notion of continuous Tax expression[297].

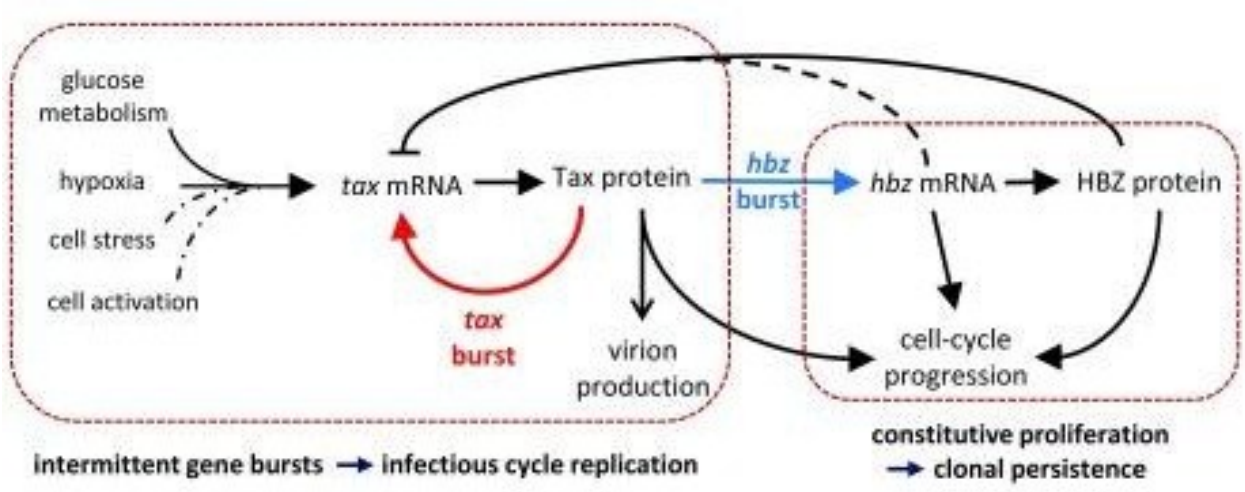

Figure 13: Current model for the regulation of HTLV-1 transcription and expression of viral proteins *in vivo* conditions. Various stimulants can lead to a small initial wave of Tax transcription that can then lead to a large burst of it. This will lead to virion production, but also HBZ and the eventual shutdown of any viral transmission. Adapted from[297].

1.1.5.1. Virological Synapse

The virological synapse, so called due to its similarities with immunological synapses, is an area of cell-to-cell contact initiated by the virus to transmit infection. An infected cell, upon engagement of its ICAM-1 (intercellular adhesion molecule 1) by LFA-1 (leukocyte function antigen 1) on an uninfected cell, will polarize its microtubule organizing center (MTOC) towards an uninfected cell in a Tax dependent manner[113, 298, 299]. Here, gRNA, MA, NC, Env and host proteins that stimulate cell adhesion will accumulate and then bud and transfer to the uninfected cell through small clefts between the cell membranes[112, 300]. It was recently shown that FSCN-1 (Fascin), that stabilizes actin and microtubules, and is present in cellular protrusions[301, 302], plays an important role in the release of HTLV-1 virions[115].

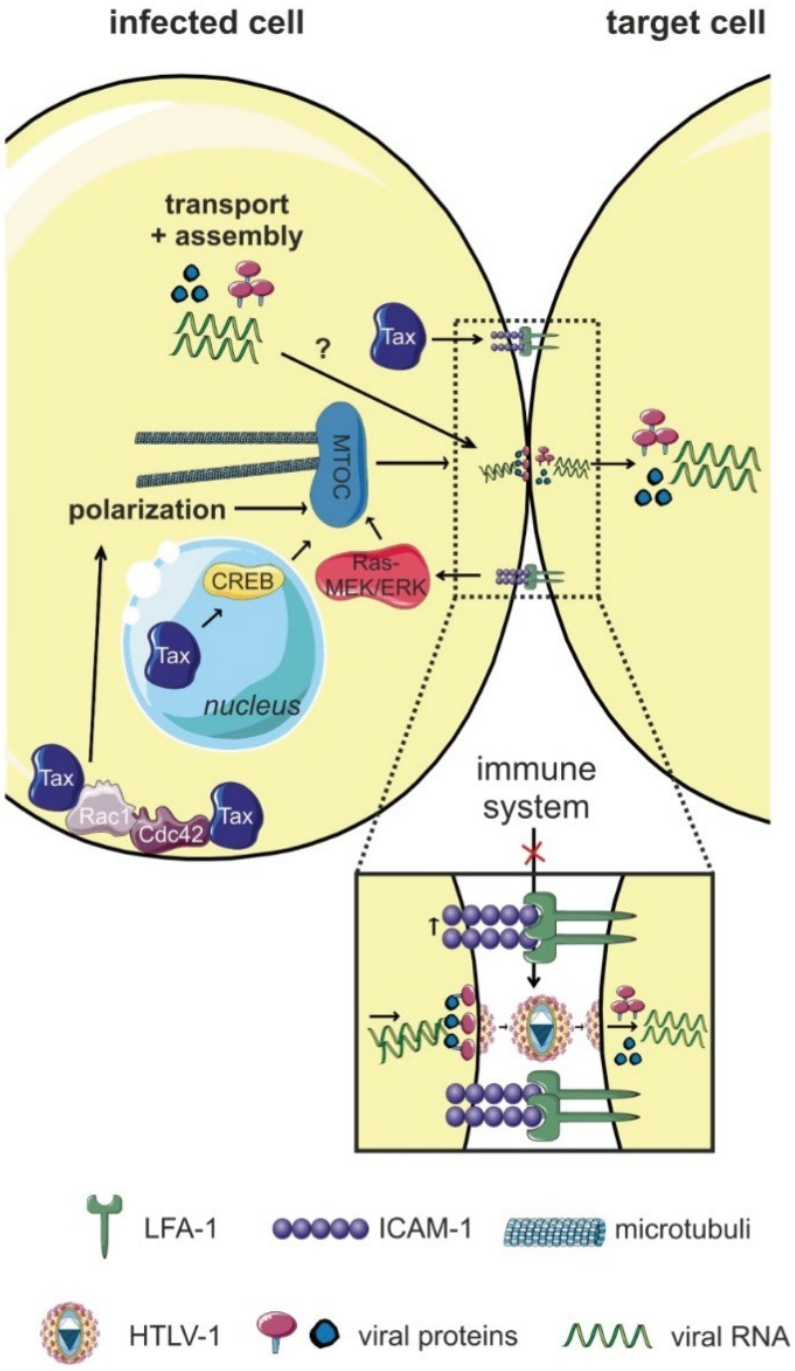

Figure 14: Representative model of the HTLV-1 virological synapse between an infected and uninfected cell. Several known factors are represented here, including important cell-to-cell contact mediators like LFA-1 and ICAM-1. Following stimulation, Tax will induce MTOC activation to cause the accumulation of viral products near the virological synapse Adapted from[303].

1.1.5.2. Viral Biofilms

Similarly to the naming convention of virological synapses, viral biofilms are so called due to their similarity with biofilms formed by bacteria. In the context of HTLV-1 however, the virus induces the accumulation of many cellular proteins on the cell surface, such as collagen, agrin, lectins, galactin-3, CD4, CD25, CD43, CD45, etc., to form large extracellular bodies on the cell surface[304, 305]. These bodies contain many HTLV-1 virions, and large parts of these biofilms can be transmitted to uninfected cells during cell-to-cell interaction mediated by a virological synapse[306]. Interestingly, the viral biofilms also contain tetherin, which normally acts as a viral restriction factor against HIV-1 by preventing its proper budding[307]. These biofilms may also disassociate from the cell surface of the producing cell, and are highly infectious of myeloid cells, and can infect $CD4^+$ T-cells more efficiently than cell-free HTLV-1[278].

1.1.5.3. Viral Conduits

Unlike the preceding two mechanisms of viral spread, HTLV-1 can also induce the formation of relatively long-ranged cellular conduits between uninfected and infected cells. These temporary conduits are mediated by the HTLV-1 accessory protein p8, which also induced T-cell conjugation via LFA-1 clustering. HTLV-1 virions were detected in the conduits, and could be transmitted to another cell via interaction with another conduit or the cell surface. It is assumed that HTLV-1 virions can bud from the tip of the conduit, and form a virological synapse-like structure at the point of contact. No viral surfing was observed on the outside of the cell along the conduits[83].

1.1.6. Regulatory and Accessory Protein Functions

HTLV-1 encodes numerous regulatory proteins that are vital for viral replication *in vivo*, and the induction of HTLV-1 associated diseases. The Tax protein is known to alter the host cell in a plethora of ways, including; cell-cycle regulation, apoptosis, proliferation, immunity, etc. Unlike Tax, HBZ is found in chronically HTLV-1 infected cells, and plays a vital role in disease progression. HTLV-1 also encodes numerous less well known proteins such as p30 and p12. The following sections briefly introduce the signaling pathways affected, and then detail the functions of the viral proteins.

1.1.6.1. Tax

HTLV-1 Tax modulates a multitude of viral and cellular processes due it its ability to interact with a very wide range of proteins due to its many important domains. While primarily found in the nucleus, it also localizes in the cytoplasm and can be excreted outside the cell. It is post-translationally modified to be sumoylated, ubiquitinated, phosphorylated and acetylated. Its role in the induction of ATL and HAM/TSP has been well documented, and it is the most well studied HTLV-1 protein. Its expression alone can immortalize cells and lead to cancers in animal models[308-311].

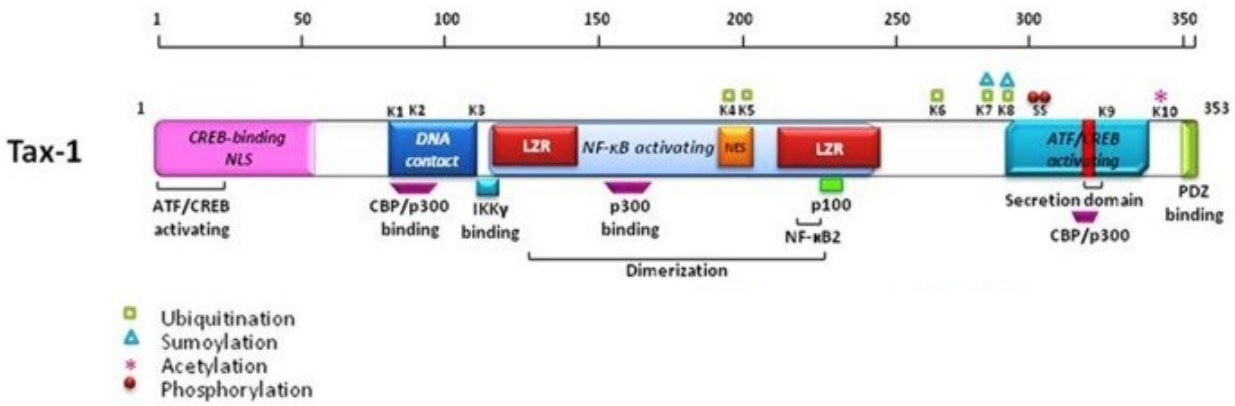

Figure 15: Schematic representing the HTLV-1 accessory protein Tax along with its functional, interaction, and domains. Adapted from[312].

1.1.6.1.1. Viral Transcription Induced by Tax

One of the most important functions of Tax is its activation of viral transcription[313]. Tax mediates this activity by the interaction of various cellular transcription factors (TF) such as CREB, and by enhancing their interaction with the viral analogues of the cAMP response element (CRE) found in the TRE-1 repeats. The vCRE found in the TRE-1 repeats are flanked by G/C rich regions. Mutations in the middle repeat reduced viral transcription to the greatest extent, suggesting that it is the most vital region[314]. Tax itself does not directly interact with host or integrated viral DNA[313], but stabilizes large TF complexes when dimerized[223, 315]. Tax does however, interact with DNA at the minor groove during CREB-mediated activation of transcription, at the G/C rich regions of the TRE-1 repeats[316-319].

Tax can directly interact with many TF of the CREB/ATF family, including; CREB, CREB2, CREM, ATF1, ATF2, ATF3, etc.[320-325]. These interactions enhance TF activation and leads to the stable formation of TRE-CREB/ATF-Tax complexes[326-328]. This ternary complex acts as a binding site for further recruitment of co-activators such as p300/CRB (CREB binding protein), and P/CAF (p300/CBP-associated factor) in a Tax dependent manner[228, 229, 329, 330] which further stabilizes the complex via histone modification and chromatin remodeling that will be explored in another section. Considering how Tax also interacts with the TRE-1, it is not surprising that transcriptional activation occurs at the viral LTR[223, 326, 327, 331-333].

All three of the CREB-regulated transcription coactivator (CRTC) proteins have also been implicated in LTR activation through Tax interaction[334, 335]. Indeed CRTC2 cytoplasmic translocation may be an important method of reducing HTLV-1 transcription in vivo[336]. CRTC normally augment CREB and TAF4 (TBP-associated factor 4), of the TFIID (general transcription factor II D) complex, interaction to induce RNA pol II transcription. Tax can also interact with other TFIID members like TAF11[337] and TBP[225], as well as TFIIA[338].

It was previously reported that the transcriptional complexes formed at each TRE-1 repeat were not identical[314]. The 5’ and middle repeat bind CREB through their CRE sequences, while the 3’ repeat bound the transcription factor Sp1 in a CRE independent manner to induce transcription. CREB and Sp1 LTR interaction were mutually exclusive.

HTLV-1 also contains a second Tax-response element; TRE-2, located between the middle and 3' TRE-1[339]. TRE-2 contains a CArG box, which is an important part of the serum response element (SRE)[340]. Similar to CRE and CREB/ATF, SRE attracts SRF (serum response factor) and Ets proteins[341], and subsequently CBP/p300 and P/CAF[342]. Tax furthermore interacts with both SRF[343-346] and Ets proteins such as Elk-1 and SAP-1[347, 348]. It follows then that SRF leads to LTR transcription in a TRE-2 dependent manner[340, 343].

Tax can also interact with ATFx, which represses LTR transcription[349]. ATFx is cell-cycle dependent, and is expressed during the G_1/S but not G_2/M phases of the cell cycle[350]. Tax induces G_2/M phase accumulation[351, 352], suggesting it can avoid ATFx mediated suppression, and adds another layer of complexity in terms of viral transcriptional control.

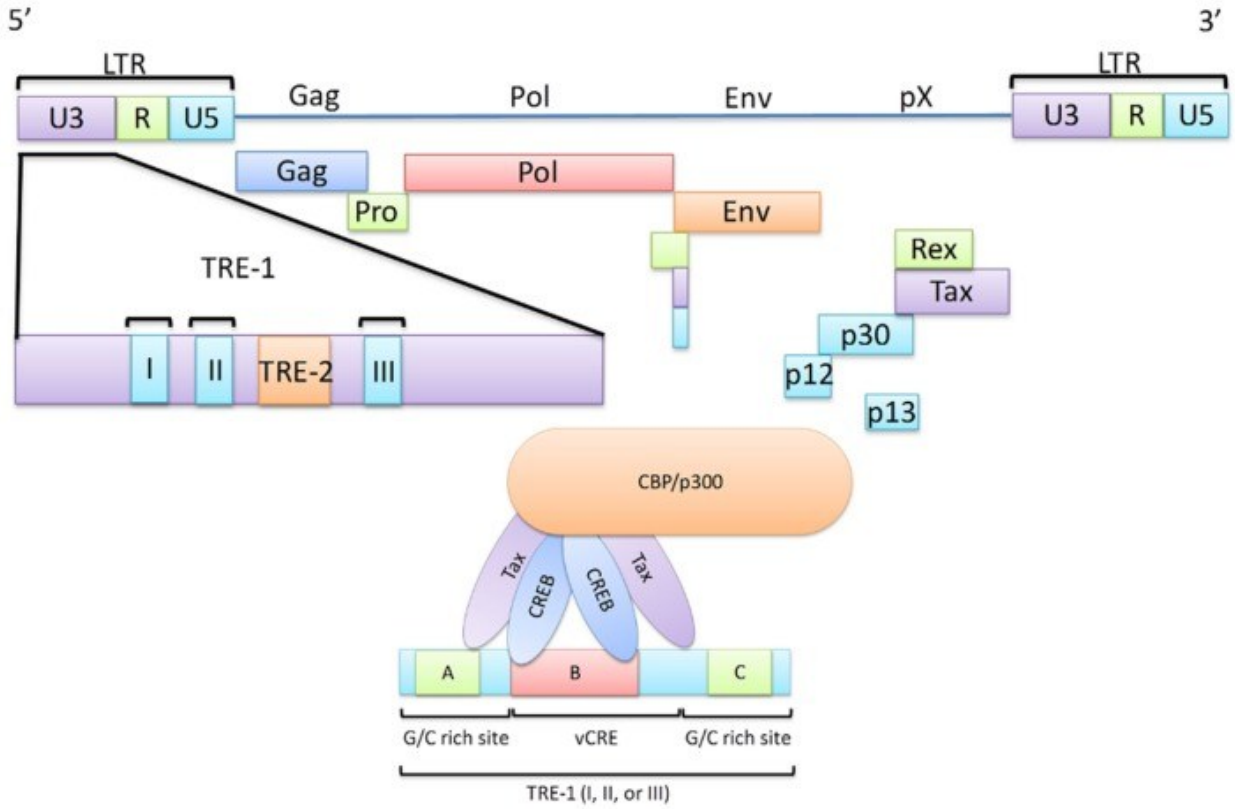

Figure 16: Detailed representation of the viral genome with special focus on its 5' LTR. Tax forms a complex with CBP/p300 and CREB, which interacts with viral TRE sequences. This causes significant transactivation of viral transcription, eventually leading to viral gene expression. Adapted from[389].

1.1.6.1.2. Cellular Transcription Mediated by Tax

Tax also activates genes such as IL-17 (interleukin-17)[353, 354] via CREB/ATF stimulation, similar to that of its own LTR. Furthermore, many CREB/ATF controlled genes are dysregulated during Tax-mediated immortalization and transformation[347, 354-357]. Many CREB/ATF genes have been implicated in cell growth, survival and apoptosis and likely play an important role in HTLV-1 associated diseases such as ATL[358].

SRF-mediated activation by Tax also induces cellular genes in addition to the viral LTR. Tax activates the transcription of several genes that contain SRE without directly interacting with DNA[359-361]. As with CREB/ATF, Tax interaction stabilizes the complex and alters the DNA selection of the complex[343]. SRF activation is further demonstrated by several of its downstream target genes such as *FOS* and *JUN*, whose gene products make up the AP-1 (activator protein 1) transcription factor, displaying increased expression in Tax positive cells[360, 362]. Both *FOS* and *JUN* are proto-oncogenes, whose dysregulation also likely plays an important role in HTLV-1 associated pathology.

Tax deactivates genes such as cyclin A and p53[363-365] through CREB/ATF. It is thought that Tax can inhibit the transcription of certain cellular genes by outcompeting cellular TF for important co-activators such as p300 and P/CAF[366, 367]. This is likely the case for p53 to which Tax has poor binding affinity, but is nonetheless inactivated during infection[368-370].

1.1.6.1.3. Chromatin Remodeling via Tax

In general terms, host DNA is often organized into chromatin, and forms dense structures called nucleosomes whose formation is mediated by histone and DNA interactions. Histones can be post-translationally modified by a number of different factors that either increase or decrease their association with DNA, and therefore silence or enhance transcription respectively. One modification is acetylation, which opens histone complexes and increases transcription. There are several important regulators of chromatin including the SWI/SNF complex. HTLV-1 infection is associated with reduced histone levels[371], and Tax expression by itself leads to histone depletion[372].

Tax can interact with many of the players in the SWI/SNF complex which lead to LTR activation and transcription[373], it can also induce the expression of some of the components, such as Baf170[374]. The mechanism is not clear, but it likely involves nucleosome remodeling to enhance transcription[329], which leads to decreased histone density at the provirus[375]. In addition, one of the main functions of the CBP/p300 complex that is recruited by Tax, is to modulate nucleosomes by acetylating histones. This complex is known to promote chromatin remodeling at the site of HTLV-1 integration[371]. Tax also accelerates DNA replication in the S phase of the cell cycle by p300 recruitment and histone acetylation, which generates genomic lesions[376].

1.1.6.1.4. Tax Mediated Interference of the Cell Cycle and DNA Repair

The cell cycle is a tightly controlled process divided into several phases. Cells in the G_0 phase are quiescent and have stopped replicating. The G_1 phase occurs after cell division or quiescence, and is a period of cellular growth. The S phase is where DNA replication occurs. G_2 is another period of cellular growth and stability. Cell division, whereby the cytoplasm and chromosomes are divided into daughter cells forms the M phase. The M phase is further divided into several other important steps, as the separation of chromosomes is not a simple process. Entry into each of the various stages is controlled by the expression of cyclins and CDK (cyclin-dependent kinase) proteins. In general, once the cyclin and CDK are activated, they promote the expression of genes, activate enzymes and/or degrade inhibitors required for the completion of that phase. One important example is the CyclinD/CDK4 complex in G_1 that phosphorylates Rb (retinoblastoma susceptibility protein). This causes the dissociation of the complex that inhibits E2F, which allows it to activate the transcription of various G_1 to S phase genes. The APC/C (Anaphase-promoting complex/cyclosome) is another example of a set of proteins. These control several key steps during the M phase, degrading key proteins once certain checkpoints are met allowing the process to continue.

Tax can induce the expression of several Cyclins and CDK proteins[351, 377], as well as directly interacting with them to modify their activity. It binds with CDK4 and CDK6, and enhances their interaction with Cyclin D[378-381]. Tax can also interfere with p15 and p16 mediated cell-cycle arrest by preventing their interaction with CDK4[382-384]. Tax directly binds to Rb to target it

for degradation[385]. These actions lead to an increased transition of infected cells towards the S phase. Tax also inactivates Chk1 and 2 (check point kinase 1/2) which activates Cdc25 (cell division cycle protein 25) and the CyclinB/CDK1 complex. This complex normally drives the cells through the G_2/M checkpoint towards mitosis, and is inhibited by Tax, resulting in an accumulation of G_2/M cells[351, 352, 386]. However, when Tax positive cells are treated with gamma radiation, Tax interaction with Chk1 and Chk2 prevents cell cycle arrest at the G_2/M phase[387, 388]. Overall Tax dysregulates the cell cycle, increasing the likelihood of replication errors such as aneuploidy and multinucleation[389].

The host DNA repair pathways are vital to ensure productive replication, and have intertwined signaling pathways with the cell cycle. It follows then, that HTLV-1 and Tax especially, can interfere with a myriad of DNA repair pathways such as; BER (base excision repair)[391], NER (nucleotide excision repair)[392], and MMR (mismatch repair)[393]. NHEJ (non-homologous end joining) is also prevented via KU80 downregulation, but Tax also directly interacts with the KU70. This leads to constitutive activation that oversaturates the machinery, disabling its ability to detect new DNA damage[394]. KU70 saturation also leads to Chk2 activation[351]. ATM (ataxia telangiectasia mutated) is an important DNA damage sensor that is inhibited by Tax[395]. Normally ATM binds to dsDNA breaks and becomes phosphorylated, a step that Tax inhibits, and thus prevents the downstream activation of this pathway.

Tax can also cause direct damage to the host DNA through a variety of mechanisms. One mechanism involves the upregulation of ROS (reactive oxygen species) in Tax positive cells that can directly damage DNA[396, 397]. Another involves dysregulation of centrosomes and the mitotic spindle assembly during replication, that causes aneuploidy[398] often found in ATL cells[399, 400]. AID (activation-induced cytidine deaminase) is a host protein that induces mutations in DNA during recombination of immunoglobulin genes in B-cell development. AID is highly expressed in ATL cells, and can be induced by Tax expression[401]. Aberrant AID expression due to Tax could induce mutations observed in HTLV-1 infected cells.

1.1.6.1.5. Downregulation of the Innate Immune Response by Tax

For an overview on innate immune signaling and detection, please refer to section 1.2.

HTLV-1 infection is associated with several diseases, most of which involve a dysregulation of the immune response[402]. At a molecular level, HTLV-1 Tax expression antagonizes normal innate immune signaling at multiple levels. Tax inhibits IRF7 activation by interacting with TRIF, while simultaneously increasing NF-κB activation[403]. Tax can also interfere with IRF3 by directly interacting with STING[404] and TBK1[405]. This binding inhibited STING and TBK1 complex formation, and led to a decrease in IFN production. Downstream IFN signaling is also downregulated during infection[406]. This is likely due to the ability of Tax to induce and stabilize SOCS1 expression[407]. The role of SOCS1 is further exemplified by its upregulation in HTLV-1 patient samples, where it was shown to have a positive effect on viral replication[408]. Here a novel method of SOCS1 immunosuppression was shown by targeting IRF3 for proteasomal degradation, in addition to interfering with JAK phosphorylation. Lastly, Tax also competes with the ISGF3 complex for co-activating molecules like p300 and CPB, which results in a poor response to IFN[368]. Overall, Tax seems to specifically target IFN signaling and IRF activation for downregulation while increasing NF-κB activity.

1.1.6.1.6. Activation of the NF-κB Pathway by Tax

For an overview on the NF-κB Pathway, please refer to section 1.3.

The NF-κB pathway if often deregulated in HTLV-1 infected cell lines and primary ATL cells, and exhibits a constitutively active state. This activation is not solely due to extracellular signals, but can be driven by intracellular molecules like Tax that stimulate both the canonical and non-canonical pathways[409-412].

The canonical NF-κB pathway is activated when Tax directly binds to IKKγ which results in the phosphorylation and degradation of IκB and the nuclear translocation of RelA[413-418]. Tax/IKKγ interaction alone is insufficient to phosphorylate the downstream inhibitory units of NF-κB[419]. Shibata *et al.* demonstrated that K63-linked ubiquitination was essential to Tax-mediated IKKγ activation, suggesting that ubiquitination plays an important role in Tax-mediated NF-κB activation. This is supported by other groups that showed that ubiquitination of TRAF6 and Tax[420] and K63-linked ubiquitination is vital for Tax-mediated NF-κB activity[421, 422]. Tax also seems to be able to mediate phosphorylation of other IKK subunits; IKKα and IKKβ[423-425]. Overall however, the precise mechanisms of Tax mediated IKK activation is unclear, and other mechanisms are possible[389, 426]. For example, Tax expression may also lead to the activation of upstream factors such as; MEKK1 (MAPK[mitogen-activated protein kinase]/ERK[extracellular signal–regulated kinases] kinase kinase 1), TAK1 or TRAF6 which can all stimulate the IKK complex[427-431], although silencing these factors does not reduce Tax-mediated NF-κB activation[432-434]. Tax may also directly target the inhibitory IκB subunits for degradation to bypass IKK activation altogether[435, 436]. Tax activation of the non-canonical pathway is mediated by directly activating IKKα to induce p100 processing to p52[437, 438].

Tax can directly interact with several NF-κB TF, such as RelA, p50 and p52 which leads their activation and transcription[346, 439-441]. It has also been reported that Tax can recruit RelA from the nucleus following cytoplasmic translocation[442, 443].

Several cytokines can induce NF-κB activation, and many are upregulated during HTLV-1 infection in a Tax-dependent manner. Specifically, IL-2 and its receptor IL-2Rα, important

regulators of T-cell proliferation, are highly expressed in Tax positive cells, and was one of the first markers of NF-κB deregulation noticed during HTLV-1 infection[444-448]. Other important NF-κB-regulated molecules that are upregulated by HTLV-1 and Tax are; IL-21[449], IL-13[450], IL-17[353, 451], FSCN-1[452], IL-6[453], IL-6R[454], and TNF-α [455].

1.1.6.1.7. Tax and Apoptosis

Apoptosis is a well-regulated pathway that involves specific controls over the process and products of cell death. Unregulated cell death is called necrosis, and releases inflammatory factors. Apoptosis is divided into two broad categories, intrinsic and extrinsic. In the latter case, death is activated by extracellular signals that bind to death receptors like FAS or TRAILR (TNF[tumor necrosis factor]-related apoptosis-inducing ligand receptor), that activate caspase 8. Intrinsic cell death is mediated by intracellular signals, such as DNA damage, which activate Bax and Bak, or deactivate their repressors; the anti-apoptotic BCL-2 proteins. Bax and Bak will induce MOMP (mitochondrial outer membrane permeabilization), an event that causes cytochrome C release from the mitochondria. This leads to apoptosome formation and caspase 9 activation. In both cases, the end stage is caspase 3 activation[456].

In one study, the primary ATL cells tested contained a mutated form of caspase 8 that contained a 136bp insert that contained a premature stop codon[457]. When Fas was triggered in these cells, they failed to undergo apoptosis. While this study was not indicative of direct Tax activity, the source of apoptosis resistance was genetic damage that is linked to Tax. Tax does increase the expression of c-FLIP (Cellular FLICE [FADD(FAS-associated death domain protein)-like IL-1β-converting enzyme]-inhibitory protein) via NF-κB activation in $CD4^+$ T-cells, which inhibits Fas-mediated cell death[458] by inhibiting downstream activation of caspase 8[459]. Also mediated through NF-κB activation is the expression of XIAP (X-chromosome-linked inhibitor of apoptosis)[460], which binds to and inhibits numerous caspases including 3, 7 and 9[461]. Interestingly, Tax-mediated resistance to Fas in $CD8^+$ T-cells seems to be due to cIAP-2 (cellular inhibitor of apoptosis 2) and not c-FLIP[462].

A20 is a protein that interferes with NF-κB signaling, that can be indirectly inhibited by Tax. Tax disrupts the complex of co-activating molecules required for A20 activation, including Tax1BP1

(Tax1 binding protein 1)[463]. Interestingly, it was recently shown that A20 is highly expressed in HTLV-1 infected cells, and protected them from apoptosis[464]. This was mediated by interactions between caspase 8 with A20, which prevented the activation of the former by preventing its interacting with FADD. Overall, Saitoh *et al.* hypothesized that A20 is unable to repress NF-κB signaling in HTLV-1 infected cells, as its silencing did not increase NF-κB activity in those cells.

Tax can also directly interfere with the intrinsic pathway by upregulating the expression of anti-apoptotic BCL-2 proteins like Bcl-xL, through NF-κB activation[465, 466]. MCL-1, another member of the BCL-2 family, is stabilized by Tax expression, in a IKK and TRAF6 dependent manner[427]. In brief, Tax activated TRAF6 through IKK, which then translocated to the mitochondria to ubiquitinate MCL-1, which stabilizes and protects it from proteasomal degradation during DNA damage. Bax, the pro-apoptotic molecule that is repressed by BCL-2, is downregulated by Tax[467].

1.1.6.1.8. Regulation and Subcellular Localization of Tax

There are multiple types of post translational modifications that alter the functions of proteins. They include: ubiquitination, SUMOylation, phosphorylation, acetylation, etc. Tax is no exception, and these potential modifications alter not only its function, but also its subcellular localization. Indeed Tax is predominantly found in the nucleus[468] in structures called TSS (Tax speckled structures)[469] or Tax NB (nuclear bodies)[470]. Tax dimerization is required for nuclear localization[471].

Tax can be ubiquitinated at a number of sites by multiple proteins. RNF4 (Really Interesting New Gene Finger Protein 4) interacts with SUMOylated Tax when it is present in the nucleus, ubiquitinates it, and causes it to relocate to the cytoplasm[472, 473]. This translocation was associated with a decrease in CREB-mediated, and an increase in NF-κB-mediated Tax activity. SUMOylated Tax is primarily found in NB, and it is believed that this post-translational modification retains it in the nucleus[442]. Ubiquitination and SUMOylation sites in Tax often overlap since they determine localization. Tax nuclear/cytoplasmic shuttling seemingly occurs very easily via p62, a NUP, without the need for energy or carrier proteins[474]. K48-linked

polyubiquitination of Tax by PDLIM2 targets it for proteasomal degradation[475]. Ubc13 is also capable of mediating ubiquitination of Tax, and seems to be required for Tax:IKKγ interaction and activation[428, 476].

Tax interacts with IKK and translocates it to the Golgi, a process that requires Tax ubiquitination[418, 477]. Both Tax and IKK were required for this translocation. Journo *et al.* also demonstrated that NRP (NEMO-related protein) interacted with Tax1BP1 to modulate Tax ubiquitination and activation of NF-κB. It was recently shown that CADM1 (cell adhesion molecule 1), which is upregulated by Tax, recruits Tax and Ubc13 together[478]. This interaction led to Tax K63-linked ubiquitination and IKK recruitment and activation. CADM1 was also required for Tax and Tax1BP1 interaction and inhibition of the A20 complex. Intriguingly, CADM1 was found at the plasma membrane and the Golgi.

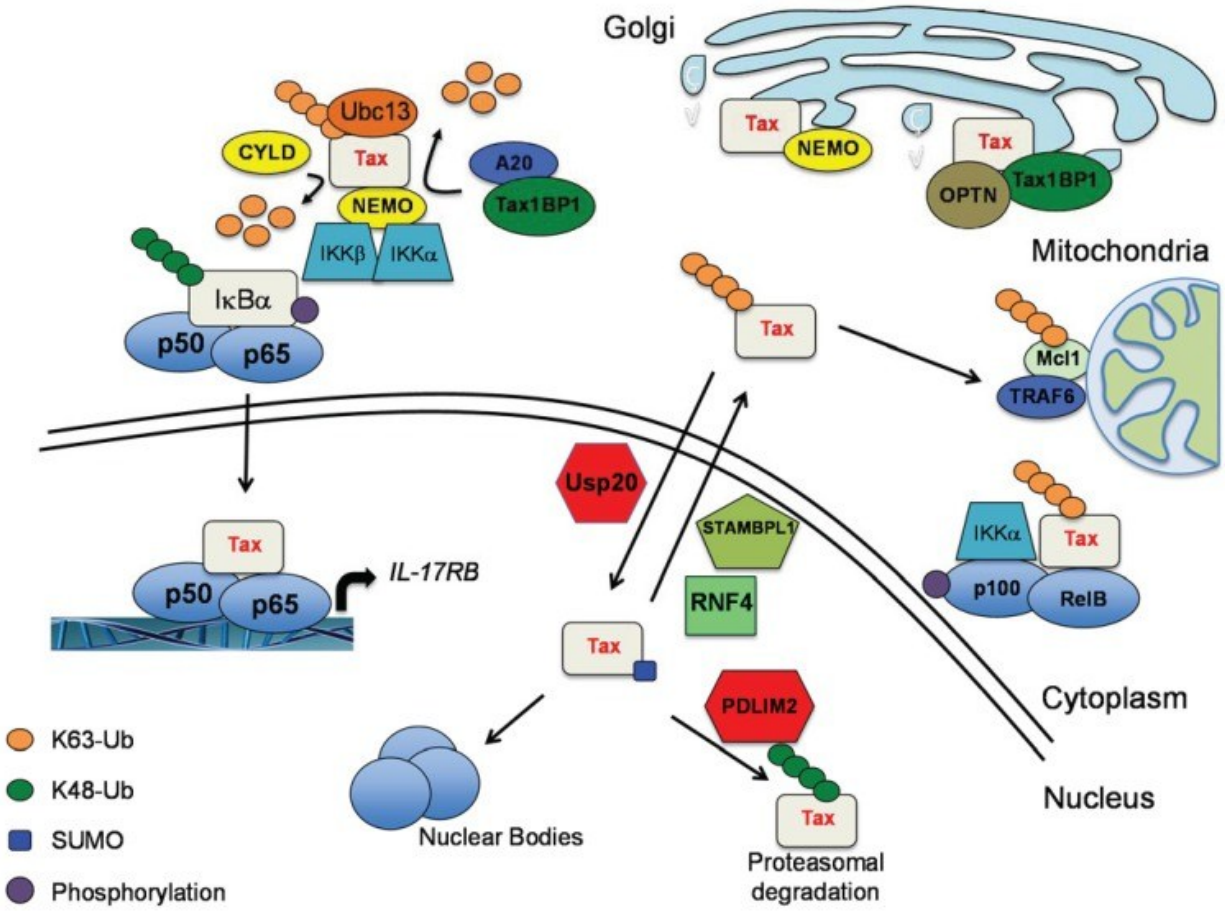

Figure 17: Schematic representation of Tax subcellular localizations and interactions with various NF-κB proteins depending on the post-translational modifications on Tax. Ubiquitinated Tax in the cytoplasm can interact with several proteins involved in NF-κB signaling, including the IKK complex. Adapted from[479].

Tax can be acetylated in the nucleus by p300, which enhances NF-κB signaling[480] and Rb phosphorylation[481]. Phosphorylation of Tax at Thr-215 is associated with inhibition of CREB and NF-κB promoter activity, while Thr-48 phosphorylation is only associated with NF-κB inhibition. Phospho-protein analysis revealed that these sites were phosphorylated in the majority of Tax proteins expressed[482]. Ser-300/301 phosphorylation is associated with nuclear body association and activation of CREB and NF-κB[483].

1.1.6.1.9. Tax downregulation

Tax expression is usually somewhat inhibited in patient samples, especially ATL ones, where Tax is only transcribed in a third of infected cells[286]. This is likely a strong method of immune evasion, as Tax is highly immunogenic and is aggressively targeted by the CTL response[292-295]. The mechanism by which Tax is silenced can occur by methylating or deleting the 5' LTR, or mutating the provirus in such a way that prevents Tax expression, like nonsense mutations[286-288, 484-486]. It is thought that prevalent Tax expression occurs early during the lifecycle of the virus, but is later silenced and intermittently expressed during the chronic phase of infection. This is not to say that Tax is not vital for disease progression. At the individual cell level, there is a positive correlation between spontaneous *ex vivo* Tax expression and *in vivo* cell division[487].

1.1.6.2. HBZ

In 2002 a group discovered that the 3' LTR of the HTLV-1 provirus functions as a promoter for another HTLV-1 accessory protein located on the negative strand[235]. The protein is called HBZ (HTLV-1 basic leucine zipper factor) and serves a vital role in the HTLV-1 lifecycle and disease progression.

1.1.6.2.1. HBZ Regulation and Activity

The 3' LTR promoter is usually intact and unmethylated[287], and while Tax and 5' LTR transcription is inactivated, most patient samples express HBZ[488]. In accordance with this, HBZ is poorly immunogenic[489-491]. In several animal models of HTLV-1 in addition to patient samples, a virus without HBZ or a knockdown of the protein, led to reduced viral load

and improved disease outcomes compared to wild type HTLV-1, suggesting an important role in disease progression[492-494]. Indeed, while it is poorly immunogenic, the CTL response to HBZ strongly correlates with disease progression[495, 496].

HBZ can be produced from two different types of mRNA, one spliced (sHBZ) and one unspliced (usHBZ)[236, 237], although sHBZ has the stronger promoter[497] and its mRNA, protein and stability are more prevalent and superior[497, 498]. Transcriptional activation from the 3' LTR is somewhat different than from the 5' LTR. Here Sp1 plays a vital role in the induction of sHBZ transcription, while Tax greatly enhances usHBZ[497]. The presence of Tax will lead to HBZ expression[499], however HBZ downregulates Tax mediated transcriptional activation[235, 497]. HBZ heterodimerizes with several CREB/ATF proteins and inhibits their ability to bind DNA[235, 500], in addition to interfering with the interaction between Tax and p300/CBP[501, 502]. Another method that HBZ employs to inhibit viral replication, is by countering Rex-mediated export of HTLV-1 structural mRNA[503]. Overall Tax and HBZ seem to have opposite functions in many of the cellular pathways they interfere with.

1.1.6.2.2. The Role of HBZ Protein and mRNA

The functions of HBZ seem to be split between its mRNA and protein. It was reported that the HBZ protein represses Tax-mediated transcription, while the mRNA can induce proliferation via TF E2F1 upregulation[494]. This was demonstrated by mutating all coding regions of HBZ with silent mutations(smHBZ), drastically altering the secondary structure of the mRNA without changing the translated protein. smHBZ or usHBZ cannot induce proliferation[497], but smHBZ can still repress Tax transactivation[494].

HBZ mRNA induces the promoter of survivin[504], despite it being a Tax/NF-κB target gene[505], which is an anti-apoptotic protein that inhibits caspase activation, and dysregulated in HTLV-1 infected cells[506]. It was demonstrated that survivin was important to prevent growth inhibition in HTLV-1 infected cells[504]. Mitobe *et al.* also demonstrated that both the mRNA and protein of HBZ could induce cell cycle entry into the S-phase, but that smHBZ induced cell death. Taken together with the pro-survival function of HBZ mRNA, wtHBZ (wild type HBZ) did not greatly alter cell survival. A recent publication has revealed the mechanism of smHBZ

induced apoptosis through the activation of the Rb/E2F complex[507]. HBZ interacts with Rb, which leads to the transcription of E2F-controlled genes and drives the cells towards the G_1/S phase. Apoptosis was likely due to E2F-induced expression of pro-apoptotic genes such as p53.

1.1.6.2.3. HBZ, Tax-Induced Senescence, and the NF-κB Pathway

Tax was previously shown to highly stimulate the SRF and NF-κB pathway, but it seems that HBZ targets NF-κB for downregulation[508]. Specifically, sHBZ protein downregulated p65-mediated, but not p52/p50-mediated, transcription by two methods; preventing the interaction between p65 and DNA, and by targeting p65 for proteasomal degradation via PDLIM2, an HBZ-inducible gene. usHBZ behaved similarly, but to a weaker extent, and HBZ mRNA had no effect. Conversely, another paper demonstrated that HBZ may also interfere with non-classical NF-κB activation, and did not degrade p65, suggesting the DNA binding interference may be the more important mechanism of NF-κB inactivation[509].

The physiological significance of HBZ downregulation of NF-κB was made clear when it was contextualized with Tax expression. As previously discussed, Tax deregulates the cell cycle, and one viral mechanism is through direct interaction and activation of APC/C during the S phase, before normal activation[510, 511]. This premature activation leads to an increase in CKI (cyclin-dependent kinase inhibitors), p21 and p27, during G_2/M leading to G_1 arrest or senescence[512, 513]. It was believed that escaping this outcome would be essential for cell transformation during HTLV-1 infection.

It was subsequently shown that HBZ possessed functions that could counter senescence [509]. Zhi *et al.* demonstrated that p21/p27 induction was mediated by Tax, via constitutive activation of the NF-κB pathway. The addition of HBZ protein inhibited NF-κB activation, and delayed Tax induced senescence. This was confirmed in a model of *de novo* infection, whereby most of the newly infected cells underwent NF-κB/Tax-mediated senescence, but produced viral particles. Cells that highly expressed HBZ on the other hand, had low NF-κB activation and proliferated[503]. Overall for Tax to promote cell growth via NF-κB, a process that occurs in many cancers, the induction of senescence must be disabled. Indeed, p21/p27 are impaired in HTLV-1 transformed cells[512, 514].

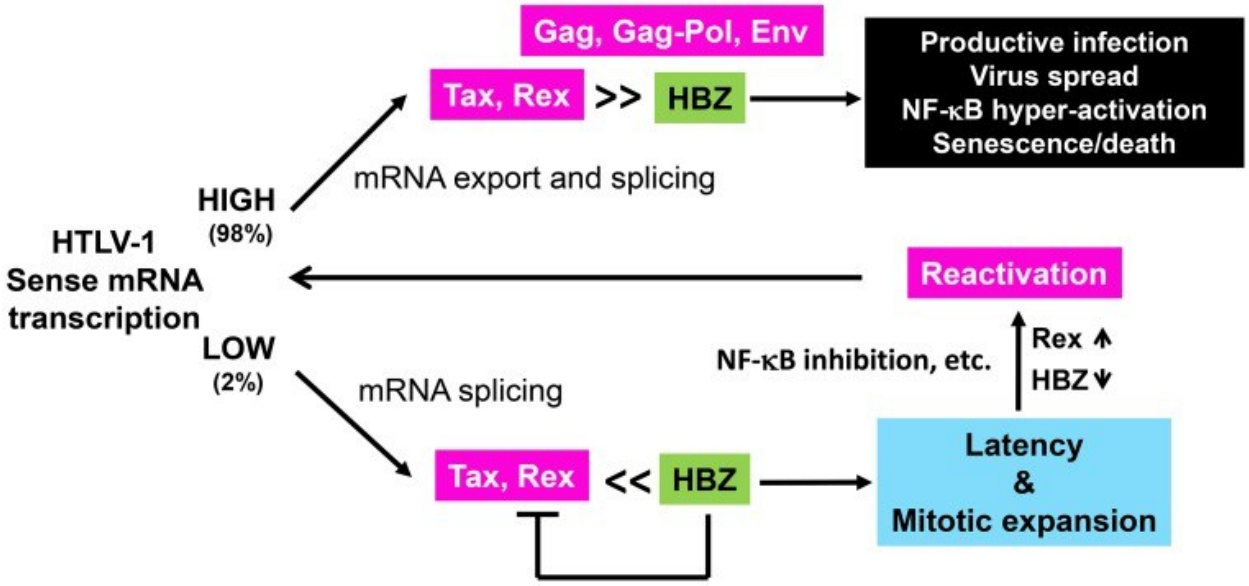

Figure 18: A model that characterizes Tax-induced senescence during NF-κB activation. HBZ can inhibit Tax-mediated senescence following overactivation of the NF-κB pathway. Ultimately however, Tax activation will occur cyclically, allowing for brief periods of viral gene expression and vulnerability to the host CTL response. Adapted from[503].

1.1.6.2.4. HBZ and Immune Dysregulation

HBZ expression in T-cells led to the expression of several inhibitory receptors on the cell surface such as TIGIT (T cell immunoglobulin and ITIM [immunoreceptor tyrosine-based inhibition motif] domain), PD-1 (programmed cell death 1) in addition to the T_{reg} TF Foxp3 (forkhead box P3)[515, 516]. TIGIT is not affected by Foxp3 expression, but is related to IL-10 production, an anti-inflammatory pro-survival cytokine. Additionally, HBZ downregulates the TIGIT competitive receptor CD226. Importantly, TIGIT induction seemed to reduce anti-Tax CTL activity, and could protect HTLV-1 infected cells *in vivo*. Indeed, TIGIT, as well as PD-1, was found upregulated in samples from ATL and HAM/TSP patients[517]. Yet TIGIT and PD-1 are inhibitory receptors that should reduce HTLV-1 infected cell proliferation following TCR (T-cell receptor) engagement, but this is not observed[518]. An escape is mediated by HBZ, which interferes with downstream activation of TIGIT/PD-1 inhibitory signals, preventing them from deactivating the TCR complex. While not completely elucidated, it appears that HBZ enhances TIGIT/PD-1 in terms of receptor-mediated cytokine production[517] while simultaneously shutting down TIGIT/PD-1-mediated inhibition of TCR signaling[518].

The HTLV-1 provirus is mainly detected in $CD4^+CD25^+$ cells in ATL and HAM/TSP patients[519-521], however the expression of the TF Foxp3 has recently expanded our understanding of the *in vivo* reservoir. Foxp3 is a vital factor in the development of T_{reg} cells, a subset of T-cells that is generally inhibitory of the immune system, and serves as an important brake or control[522-524]. Foxp3 requires cytokine signaling for its expression: a TGF-β trigger for downstream activation of Smad proteins[525]. T_{reg} cells are not terminally differentiated, and depending on the cellular environment can become iT_{reg} (induced T_{reg}) that do not express Foxp3 and are pro-inflammatory[526, 527].

HAM/TSP patients demonstrated reduced Foxp3 expression and activity, likely mediated by Tax[528-530], and displayed overactive pro-inflammatory cytokine production[531]. On the other hand, more than two out of three ATL cells express Foxp3[532, 533], although it remains unclear if HTLV-1 specifically infects and induces the replication of T_{reg} cells, as HBZ can induce Foxp3 expression[534] through Smad3 and TGF-β signaling[535]. Intriguingly, T_{reg} cells express Nrp-1, suggesting that HTLV-1 could preferentially infect this cell population[536].

Regardless, these $HBZ^{+}CD4^{+}CD25^{+}Foxp3^{+}$ cells are functionally weak and do not suppress T-cell proliferation as wtT_{reg} (wild type T_{reg}) cells could, likely due to transcriptional inhibition mediated by HBZ/Foxp3 interaction[534]. Indeed HBZ expression in mice leads to the production of pro-inflammatory invasive T-cells[537]. These cells lost or diminished their Foxp3 expression, and displayed markers of iT_{reg} cells. *In vivo* data has shown that the HTLV-1 provirus is located in activated $Foxp3^{low}$ cells that have poor suppressive ability[538]. However another group recently demonstrated that a certain population of $Foxp3^{high}$ ATL cells displayed suppressive function, while $Foxp3^{low}$ ATL cells did not[539]. This suggests the existence of multiple HTLV-1 infected $Foxp3^{+}$ cell subtypes that may have different functions in terms of disease development. This may explain why some groups have reported that ATL cells have suppressive activity[540, 541] while others have not[542].

HTLV-1 infected cells produce CCL22 in a Tax-mediated manner, that attracts and enhances the survival of functional T_{reg} cells[543, 544], which could simultaneously lead to their infection and suppress local anti-HTLV-1 responses when Tax expression is high. Then once infected, HBZ can deregulate Foxp3 function and its anti-proliferative capacity. Indeed there is a positive correlation between $Tax^{-}Foxp3^{+}$ cells and both proviral load, and Tax expression[545]. Similarly, there is a negative correlation between CTL activity and $Tax^{-}Foxp3^{+}$ cells.

1.1.6.2.5. Future Studies and Tax/HBZ Interplay

Ultimately, HBZ interacts with many cellular processes, including enhancing the Wnt pathway, telomerase activity and cellular migration, modulating IRF activity, and deactivating p53[546-550]. Additionally, HBZ can indirectly or directly interact with numerous cellular TF, such as members of the CREB/ATF, C/EBPα (CCAAT-enhancer-binding proteins) and Jun families[551], indicating that it may alter numerous cellular pathways to benefit the virus[500, 552-557].

A recent manuscript has shed light on the Tax and HBZ interplay, and has proposed the following concept[297]. Tax and HBZ transcription varies greatly, even among the same clones. Cells that are not actively expressing HTLV-1 genes are more likely to have a sudden burst of massive Tax transcription (and general positive strand transcription as well), than if they express

low levels of HBZ. Following this surge, a smaller burst of HBZ (negative strand transcription) is very likely to follow, which then suppresses Tax/positive-strand mediated transcription. Eventually, all HTLV-1 transcription is slowly silenced. A burst of HBZ/negative-strand transcription is not likely in Tax deficient cells. Overall these data are in accordance with most of the current theories of Tax and HBZ interaction, but suggest that this occurs cyclically. Intriguingly, positive strand mRNA is more likely to be exported into the nucleus versus HBZ transcripts, suggesting the HBZ mRNA has a more physiologically relevant role in disease progression than its protein.

1.1.6.3. Other regulatory and accessory proteins

The roles of Rex and p8 have been described in viral RNA export and viral transmission, respectively. Other HTLV-1 regulatory proteins include p30, p13, and p12, and are understudied. Many studies have indicated that they were disposable for cellular immortalization[558-560], are difficult to detect *in vivo*, and are encoded in the least conserved region of HTLV-1[561]. However one group did show that, individually, these proteins conferred significant replicative advantage to HTLV-1 replication, over viruses that did not contain them[562-566].

A recent study of p12 and p8 demonstrated that HTLV-1 required both in order to productively infect macaques[567] while the same group previously demonstrated this was not the case in rabbits[568]. Expressing p12 alone resulted in neither infection nor seroconversion, while p8 alone weakly stimulated antigen reactivity and infection. Co-expression of both proteins was associated with evasion of CTL-mediated lysis[567]. This could be due to a p12-mediated decrease in MHC-1 (major histocompatibility complex class I)[569], which are normally used for targeting by CTL. A decrease in MHC-1 could however, cause NK (natural killer) cell mediated cytotoxicity. However, this does not seem to occur, due to p12 which downregulates adhesion molecules such as ICAM-1 and 2, along with a general decrease in NK activating receptors on the infected cells[570]. Taken together, this suggests that p12 and p8 have important roles in immune evasion.

p13, when co-expressed with Tax, seems to interact with Tax in the nucleus, and interferes with its ability to interact with CBP/p300, and reduces Tax-mediated transcription[571].

Overexpression models have also shown that p13 interacts with the mitochondria, but the physiological relevance of this is not known[572]

p30 inhibits Tax and p300 interaction, preventing 5' LTR transcription[573, 574]. It also binds to the retroviral mRNA that encodes Tax and Rex and keeps it sequestered in the nucleus[244]. This seems to be mediated by a p30RE (p30 response element) present in mRNA that has spliced out the *env* region, including *tax/rex*, but not *env* or *gag/pol* mRNA. Additionally, p30 can interact with Rex, which likely modulates their activity[245]. Overall these papers suggest that p30 is an inhibitor of HTLV-1 expression. p30 appears to have other functions as well, such as deregulating the cell cycle[575-577], and interfering with TRL4 signaling and enhancing IL10 production[578].

1.1.7. HTLV-1 Diseases

In addition to ATL and HAM/TSP, HTLV-1 is the causal agent or associated with several diseases. HTLV-1 can cause inflammatory diseases in many different organs, including; the central nervous system, skin, muscles, and lungs[579-583]. Over 90% of individuals infected with HTLV-1 will remain asymptomatic throughout their lives. In contrast, only 0.3% of HIV-1 infected individuals can naturally control its replication continuously[584]. Around 5% of HTLV-1 positive individuals will develop ATL[291], while 2% will develop HAM/TSP[585]. Infection is also associated with a general weakening of the immune system, which causes a severe increase in opportunistic infection of *Mycobacterium tuberculosis* and *Staphylococcus aureus*[586, 587]. The diseases caused by HTLV-1, ATL and HAM/TSP, do not seem to be the result of genetic differences of the virus in those patients, which has led to the hypothesis that the disease state is mostly mediated by the host and his/her interactions with the virus. Indeed the efficiency of the CTL response seems to account for a significant portion of the control of proviral load[588, 589]. Currently, the best indicator of disease risk for both ATL and HAM/TSP is the proviral load, in terms of number of unique clones[290, 590]

1.1.7.1. Adult T-cell Leukemia

The molecular mechanisms by which HTLV-1 regulatory proteins cause immortalization and malignancy have been established, but it remains unclear how the progression actually occurs *in vivo*. Tax and HBZ can cooperate to promote the proliferation and immune evasion of a single clone for over 50 years, the average age of ATL onset, all the while accumulating various mutations. These may eventually result in permanent defects in tumor suppressor expression and/or function, cellular activation, proliferation, etc.[589]. Another non-exclusive possibility is that through integration, HTLV-1 could activate or deactivate a key malignancy-related gene, known as insertional mutagenesis. This is not a highly supported theory, as few ATL cases display insertion near genes that could be implicated in cancer development[221]. Another possibility is that the provirus can form long range chromatin loops that bring important genes much closer to the viral promoter than the integration site[591].

Many symptoms of ATL are vague and similar to other cancers such as non-Hodgkin's lymphoma; malaise, fever, drowsiness, weight loss, etc.[592], and so misdiagnosis is problematic in regions where the virus is not endemic[593]. ATL is divided into 4 subtypes, depending on the features of the disease: smoldering, chronic, lymphoma, and acute[592]. The delineation between an asymptomatic carrier and ATL is not clear, but a border state exists called pre-ATL, where the presence of cells with ATL morphology appear and disappear spontaneously[594].

Smoldering and chronic ATL are characterized by few serious clinical features, such as skin infiltration or high leukocyte numbers, but only have a median survival time of about 55 and 31.5 months respectively, with treatment. The other two forms of ATL, lymphoma and acute, are more aggressive and have a 10.6 and 8.3 month survival respectively, also with treatment. The aggressive forms of ATL, lymphoma and acute, account for 20% and 60% of all ATL cases respectively. The aggressive stages of ATL have severe symptoms including lymphadenopathy, lytic bone lesions, lung infiltrations, hypercalcemia, immune deficiency, etc.[595, 596]. Overall, ATL is a rapidly progressing disease with a low survival time following its onset.

Few reliable treatment options have been successfully developed in the last 35 years of ATL and HTLV-1 research[592, 596]. Chemotherapy is often used but largely ineffective at prolonging overall survival. Allogenic hematopoietic stem cell transplantation has demonstrated that it can lead to complete remission in some cases, however there are significant risks in undergoing this procedure[597-599]. AZT (azidothymidine) and IFNα is a promising co-treatment for non-lymphoma type ATL. It is unclear what the mechanism of action is for this co-treatment, as it does not appear to be related to inhibiting reverse transcriptase[600]. Encouraging results with the addition of Arsenic to AZT/IFNα have also been reported[601, 602].

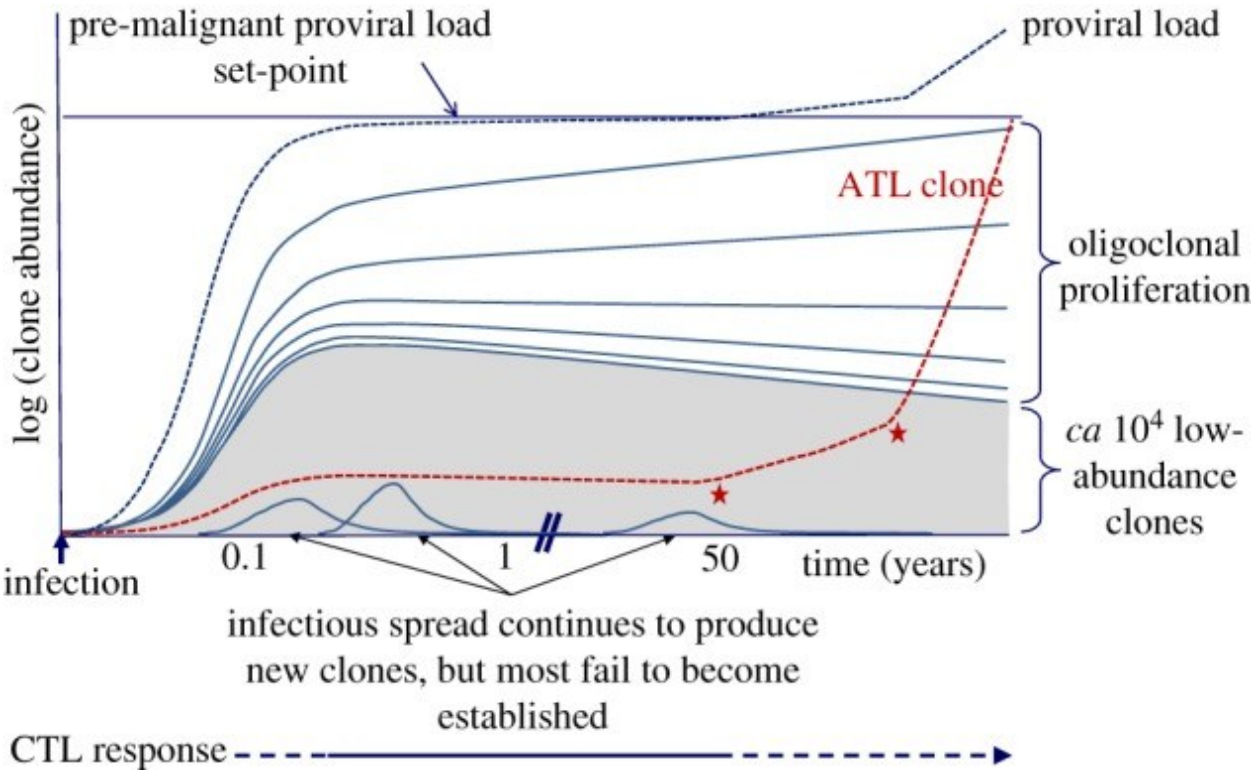

Figure 19: A model of the establishment of the proviral load. Many high number clones exist, but it is unlikely that ATL will develop from those. Instead, new clones that are constantly being produced in the background in an infected individual will likely be the cause of ATL. Adapted from[291].

1.1.7.2. HTLV-1-Associated Myelopathy or Tropical Spastic Paraparesis

HAM/TSP is characterized by inflammation whereby lesion-causing lymphocytes invade the central nervous system, especially in the upper thoracic spinal cord[603]. The infiltrating cells are predominantly composed of $CD4^+$ T-cells, although the proportion of $CD8^+$ T-cells rises as the number of lesions increases[604, 605]. In addition, resident astrocytes can also contribute to CNS damage, by further recruiting cells for infiltration[606]. It is unclear what the initial trigger of infiltration is, but it is thought that Tax-induced activation is a likely cause[607]. CTL activity against infiltrated HTLV-1 infected $CD4^+$ T-cells is one possible method of damage[608]. In addition, several groups have shown that ATL cells have T_{reg} immunosuppressive functions[539-541], while others have shown HTLV-1 infection may also lead to the development of iT_{reg} cells with inflammatory properties[534, 537, 538]. Indeed this pro-inflammatory phenotype was seen in HAM/TSP cells[609], and may be a driving mechanism of inflammatory disease during HTLV-1 infection. Overall, the prevalent theory is that bystander damage due to the infiltrating cells is the primary driver of spinal cord neurodegeneration and atrophy[589, 610-612].

The symptoms of HAM/TSP usually increase in severity over time, beginning with pain, and spastic weakness of the lower body and urinary problems. These can manifest at a variety of ages, but usually occur at a much earlier age than ATL. The median time before requiring aid to walk was 6 years, and wheelchair use was 21 years[613]. The development risk of the disease increases with age, and seems to vary by region and ethnicity[258, 614, 615]. HAM/TSP itself is not lethal, however it is associated with a reduction in life expectancy[613]. The pathology is divided into two phases; an inflammatory period characterized by lymphocyte infiltration, followed by spinal cord damage and neurodegeneration[610].

Here as well it can be misdiagnosed as an autoimmunity disorder, such as multiple sclerosis in non-endemic regions. Currently the treatment options for HAM/TSP are severely limited, and focus on management of the clinical symptoms, such as pain and muscle spasms. Clinical trials have been performed that use immunosuppressive drugs, but no large scale conclusive study has yet been performed[616].

1.2. Innate Immune Signaling and Detection

There exists many receptors expressed in different cell types and positioned throughout various subcellular locations that can detect foreign compounds to initiate an immune response. In the case of viruses, they do not possess many unique PAMPS (pathogen-associated molecular patterns) like bacterial cell walls, so the PRR (pathogen recognition receptors) most relevant to their detection sense intracellular nucleic acids. These receptors can be categorized as RNA or DNA specific, and broadly activate numerous cellular defenses through the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and IRF (IFN [interferon] regulatory factor) transcription factors 3, 5 and 7[617]. The NF-κB pathway will be explored in the subsequent section, while IRF3 activation will be introduced here.

The detection of DNA or 5'ppp dsRNA (double stranded) RNA with a triphosphate motif on its 5'-end) in the cytoplasm are clear signs for the cell that a virus is present. DNA is usually sequestered to the nucleus or mitochondria, and 5'ppp dsRNA is not produced by human cells. DNA detected here is usually via cGAS or IFI16, the former produces cGAMP (dinucleotide cyclic GMP–AMP) as a secondary messenger that activates STING (stimulator of IFN genes), while the latter can directly bind to STING. STING activation involves K63-linked ubiquitination by TRIM32 and TRIM56 (tripartite motif protein), which recruits and activates TBK1 (TANK [TRAF (TNF [tumor necrosis factor] receptor associated factor) family member-associated NF-κB activator]-binding kinase 1). TBK1 has been shown to activate the NF-κB pathway through the IKK (IκB kinases) complex and TRAF6[618], and phosphorylates IRF3 which induces its dimerization[619-621].

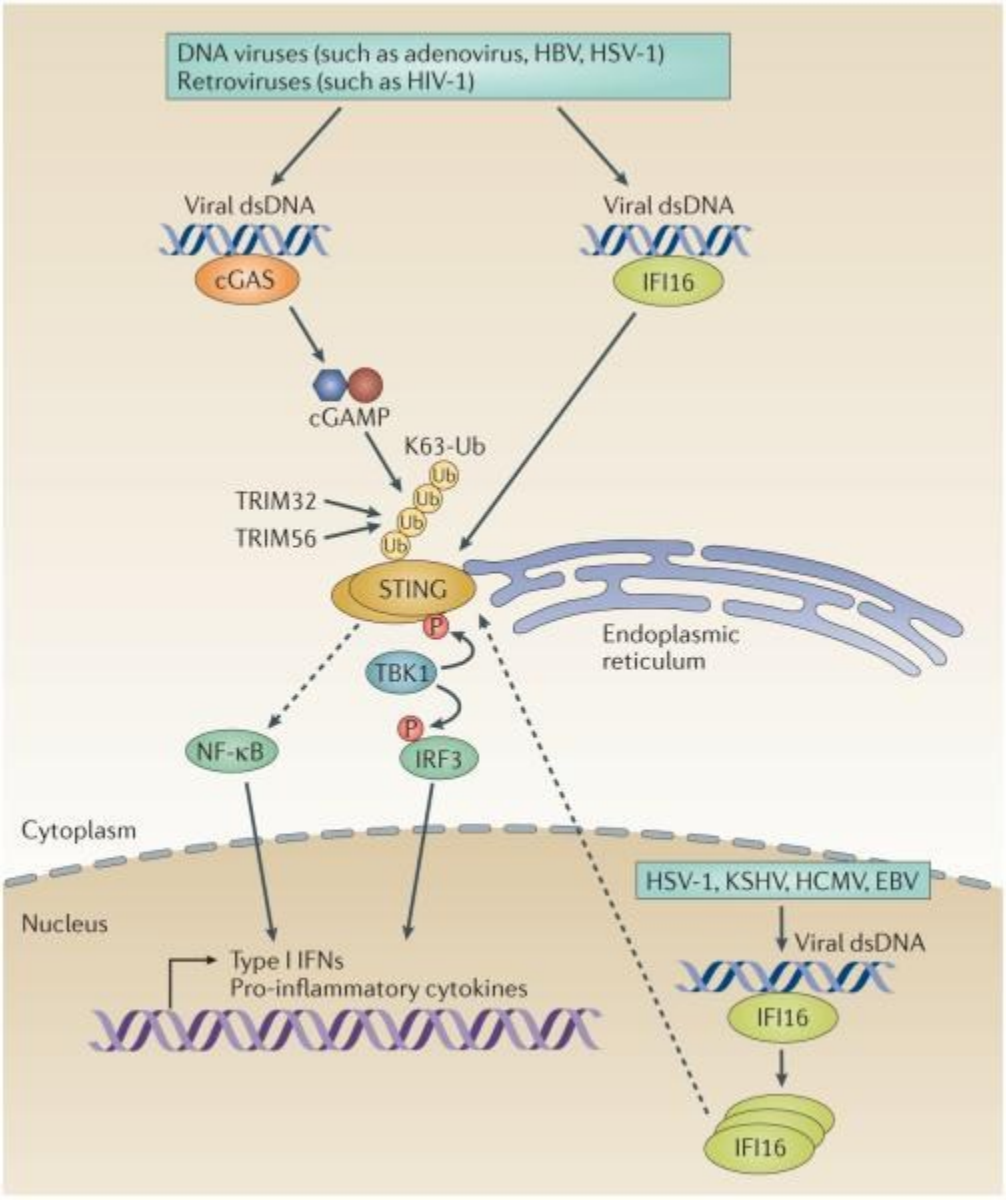

Figure 20: Schematic representative of the DNA detection pathway during the induction of the immune response. Following DNA detection, DNA PRR will activate STING to initiate TBK1/IRF3 and NF-κB activation. These transcription factors control the expression of many genes that will generate a defensive response. Adapted from[622].

The RIG (retinoic acid-inducible gene) family of RNA receptors is referred to as RLR (RIG-like receptors) that contains three members with very similar signaling pathways. These receptors interact with RNA via their DExD/H-box helicase domains. RIG-I detects relatively short uncapped 5'ppp dsRNA, while MDA-5, another RLR, detects longer dsRNA that does not possess 2'-O-methylation in the 5'-cap structure[623]. The RLR LGP2 does not contain a CARD (caspase activation and recruitment domain) domain, and inhibits RIG-I while enhancing MDA-5 signaling. Interaction with RNA stimulates the phosphorylated CARD domains of RIG-I and MDA-5, which recruits the phosphatase PP1 to de-phosphorylate them. TRIM25 is then recruited to ubiquitinate the CARD domains in a K63-linked manner, inducing tetramerization of the receptor and interaction with MAVS (mitochondrial antiviral-signaling protein) at the mitochondria. Activated MAVS forms a large structure that includes many other MAVS and TRAF proteins. It activates the NF-κB pathway through the IKK (IκB kinases) complex, and IRF3 through TBK1 and IKKε[622].

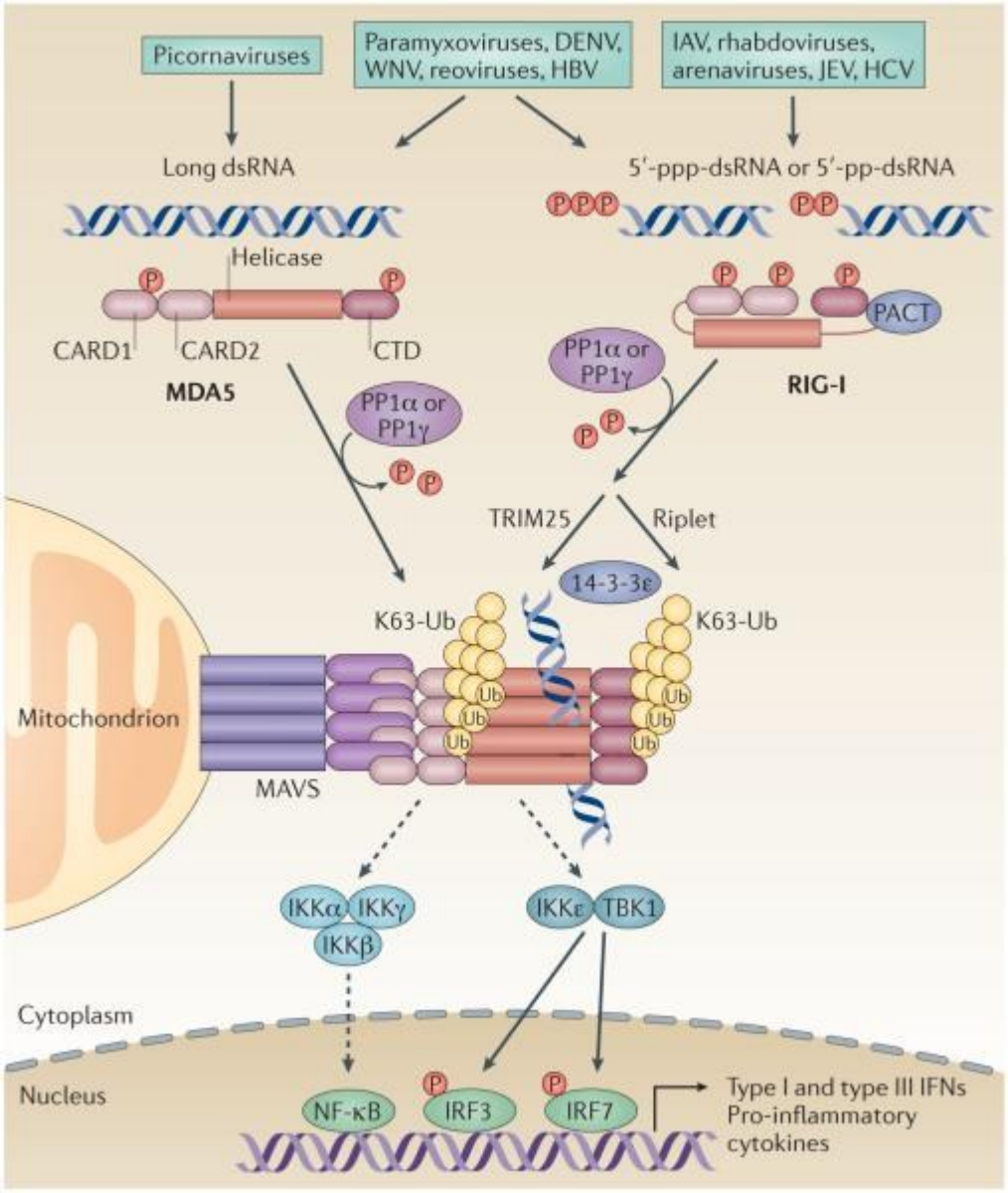

Figure 21: Schematic representative of the RNA detection pathway for the induction of the immune response. RIG receptor activation via 5' ppp RNA interaction will lead to oligomerization along with MAVS. This will activate the IKK complex and NF-κB activation along with the TBK1/IRF3 signaling pathway. Adapted from[622].

TLR (toll-like receptor) are another family of immune receptors that can recognize a variety of PAMPS like LPS (lipopolysaccharide) on bacterial cell walls via TLR4 or flagellin via TLR5. The ones that detect RNA are TLR3, 7, and 8, while TLR9 responds to DNA in humans. Interestingly, structural HIV-1 proteins like Env and Gag can be detected by TLR1, 2 and 4[624-626]. Apart from TLR3, these receptors signal through MyD88 (myeloid differentiation factor 88), which recruits several different IRAK (IL-1R1-associated protein kinases) proteins and trigger TRAF6. This leads to TAK1 (TGF-β [transforming growth factor-β] activating kinases 1 or MAP3K7[mitogen-activated protein kinase kinase kinase 7]) activation, which includes TAB (TAK1-binding protein) 1 and 2, that stimulate IKK. Several IRF TF can directly bind to MyD88, but the main induction of IFN stimulation in pDC (plasmacytoid dendritic cells) is done through a complex that includes MyD88, IRAK proteins, and TRAF proteins. TLR3 signaling is very similar, except that it utilizes TRIF (TIR [toll IL-1 receptor] domain–containing adaptor–inducing IFN-β) instead of MyD88[627, 628].

Although human cells express and use different PRR to bind to various PAMP, the downstream molecules are often shared among different immunological cascades, such as RNA versus DNA sensing pathways. STING and MAVS for instance, interact and support the activation of one another[629]. Similarly, the IRF and NF-κB proteins cooperate to induce the expression of many genes, including the production of type-1 IFNα/β[630], often considered to be the “first wave” of an immune response. Exposure to this type of IFN alone can induce a potent immune state, as it activates the transcription of the “second wave” inflammatory and anti-pathogen genes that induce signaling cascades and interfere with pathogen replication.

Type-1 IFN is detected by heterodimer IFNAR (IFNα/β receptor) 1 and 2. In the cytoplasm, two members of the Janus kinase protein family, JAK1 and TYK2 (tyrosine kinase 2) bound to the IFNAR complex become activated and phosphorylate each other following IFN stimulation. This recruits STAT (signal transducer and activator of transcription) 1 and 2, which are also phosphorylated here. This STAT dimer also binds to IRF9 to form the ISGF3 (IFN-stimulated gene factor 3) TF complex, which translocates into the nucleus to activate genes that have an ISRE (IFN-stimulated response element). STAT1 homodimers are also produced, and similarly interact with GAS (gamma activation sequence) elements[631, 632].

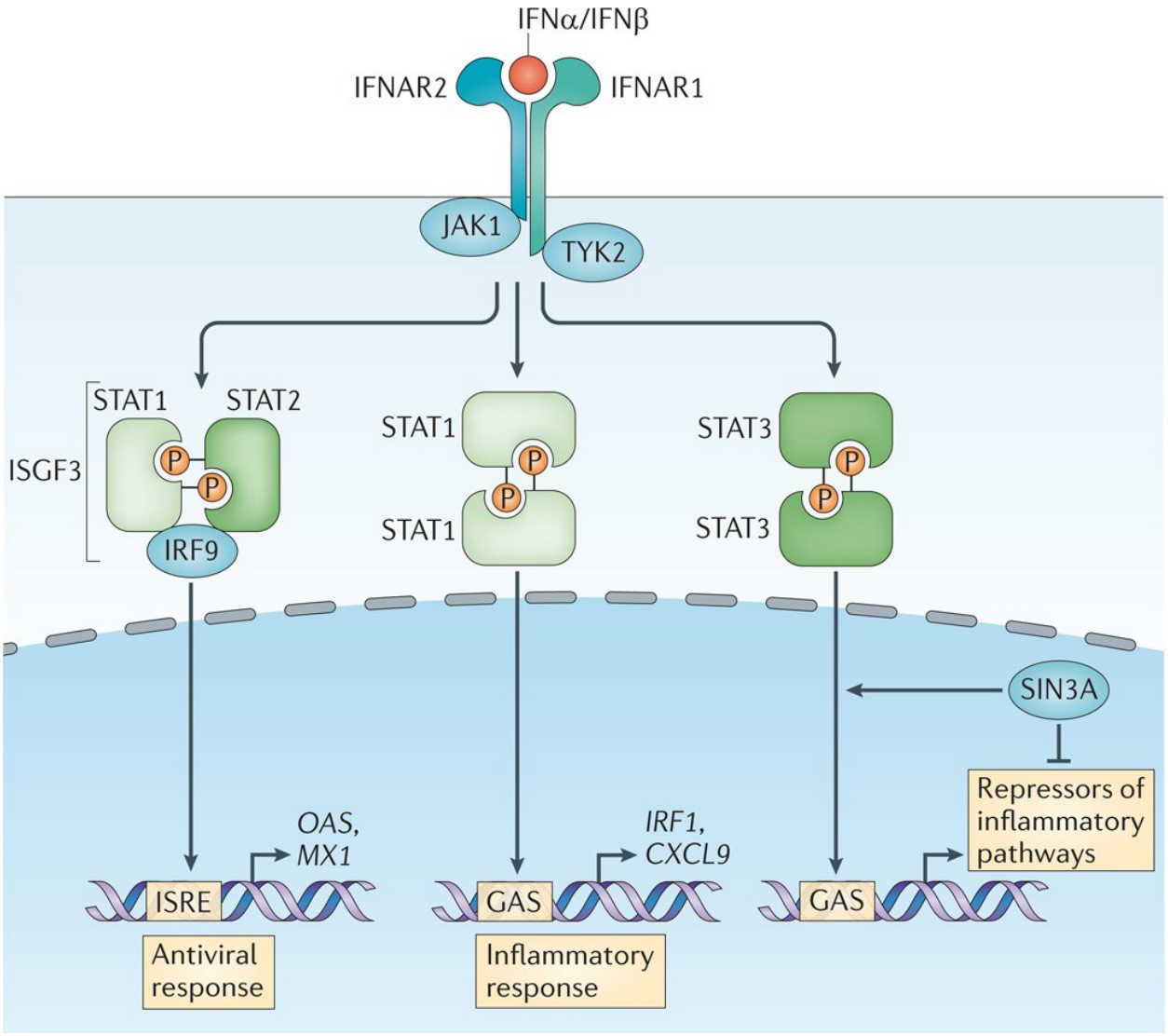

Figure 22: Model that represents the canonical type 1 IFN signaling pathway. SIN3A is a specific inhibitor of the negative feedback loop initiated by STAT3 dimerization during IFN signaling. Under most circumstances, type 1 IFN will interact with IFNAR to initiate STAT1 dimerization and activation. This transcription factor can then induce many anti-viral genes with ISRE promoters. Adapted from[632].

There are several different combinations of STAT dimers induced by type-1 IFN treatment, including the immunosuppressive STAT3 homodimer. IFN-induced STAT3 activation inhibits STAT1 homodimer interaction with DNA and thus reduces GAS-mediated gene activation[633], and ISRE activity [634]. Overall however, the details on the role STAT3 plays and its mechanisms remain unclear[635]. Another inhibitory protein family, SOCS (suppressor of cytokine signaling) 1 and 3, provide a negative feedback loop during type-1 IFN signaling[636]. SOCS1 interacts with the IFNAR1 and JAK complex, and prevents its interaction with STAT proteins[637, 638] as well as inducing its degradation[639].

1.3. The NF-κB Pathway

The NF-κB pathway affects several cellular pathways including inflammation, cytokine production, cell survival, etc. It can be activated by a number of stimuli such as cytokines, or intracellular signals to name a few. Following stimulation, the IKK complex that is composed of the catalytic subunits IKKα and IKKβ, and the regulatory subunit IKKγ (also called NEMO), phosphorylates IκB. This latter unit is a repressor, and can be one of many different proteins such as IκBα, IκBβ, IκBε, p150, p100, etc. Normally these inhibitors trap NF-κB proteins in the cytoplasm, and mask their NLS. Once phosphorylated, the IκB is ubiquitinated and targeted for proteasomal degradation. This releases NF-κB transcription factor homo or heterodimers from repression in the cytoplasm, where they then translocate into the nucleus to activate a variety of genes. The NF-κB proteins form a family of five different TF; RelA, RelB, c-Rel, p50 and p52[640, 641]. Different NF-κB heterodimers have distinct gene activation profiles, which is influenced by NF-κB binding partners and post-translational modifications[426, 642, 643].

Two distinct methods of NF-κB activation exist, the canonical pathway which regulates inflammation and apoptosis is stimulated by TNFR (TNF-α receptor), TCR (T-cell receptor), and PRR activity for example. Downstream of many of these receptors is a TRAF (TNF receptor associated factor) protein that becomes activated. TRAF6, for example, is an E3 ubiquitin ligase and auto-polyubiquitinates itself to form K63 chains. Activated TRAF6 ubiquitinates TAK1 and IKKγ with K63 chains, which leads to their activation. This complex then phosphorylates the IKKβ subunit that will remove the inhibitory IκB, activating the NF-κB heterodimer, predominantly RelA/p50[644].

The non-canonical pathway is involved in cellular maturation and differentiation, and is activated by CD40 among others. Following non-canonical stimulation, NIK (NF-κB-inducing kinase) is stabilized, and oligomerizes to auto-phosphorylate. This specifically activates IKKα and does not require IKKγ or IKKβ. The inhibitory subunit found here is p100, which is actually the unprocessed form of p52, and is already bound to RelB. This pathway primarily leads to the activation of a p52/RelB complex[438, 645].

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Chapter 2: SAMHD1 Host Restriction Factor: A Link with Innate Immune Sensing of Retrovirus Infection

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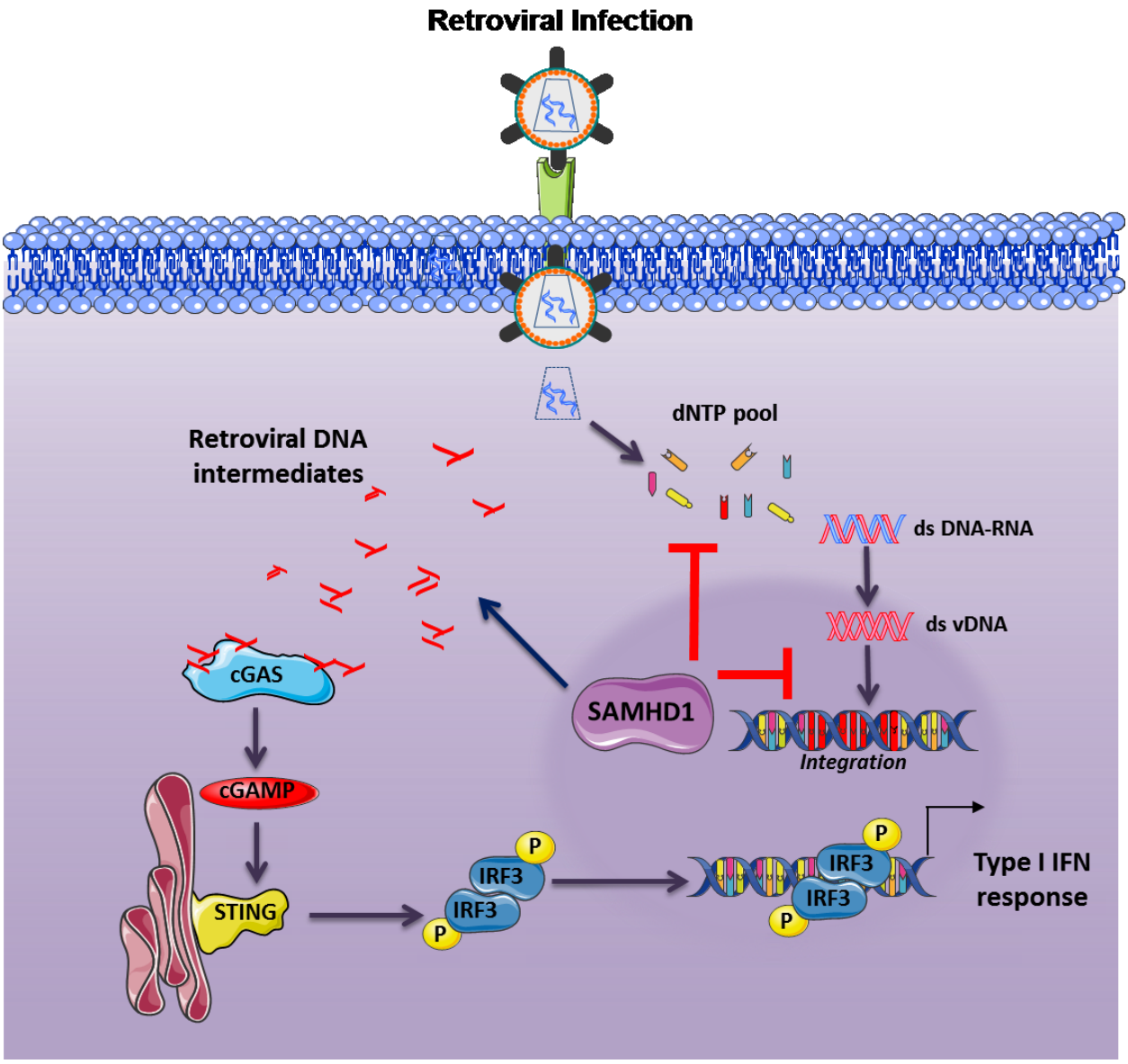
1. Abstract

SAMHD1 [sterile alpha motif and histidine-aspartic domain (HD) containing protein 1] is the most recent addition to a unique group of host restriction factors that limit retroviral replication at distinct stages of the viral life cycle. SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that degrades the intracellular pool of deoxynucleoside triphosphates available during early reverse transcription. SAMHD1 activity is blocked by the Vpx accessory function present in human immunodeficiency virus type 2 and SIVsm. Mutations in SAMHD1 are associated with the autoimmune disorder Aicardi–Goutières syndrome, thus emphasizing its role in regulation of the immune response. SAMHD1 antiretroviral activity is modulated by post-translational modifications, cell-cycle-dependent functions and cytokine-mediated changes. Innate receptors that sense retroviral DNA intermediates are the focus of intense study, and recent studies have established a link among SAMHD1 restriction, innate sensing of DNA and protective immune responses. Cell-cycle-dependent regulation of SAMHD1 by phosphorylation and the increasingly broad range of viruses inhibited by SAMHD1 further emphasize the importance of these mechanisms of host restriction. This review highlights current knowledge regarding SAMHD1 regulation and its impact on innate immune signaling and retroviral restriction.



Graphical Abstract

Abbreviations:

MDM, monocyte-derived macrophage; pDC, plasmacytoid dendritic cell; RTI, reverse-transcribed intermediate; AGS, Aicardi–Goutières syndrome; HIV-1, human immunodeficiency virus type 1; HTLV-1, human T cell leukemia virus type 1; CDK1, cyclin-dependent kinase 1; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA

Highlights

- SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase that degrades the dNTP pool required for reverse transcription.
- SAMHD1 activity is regulated by the Vpx accessory protein, post-translational modifications and cell-cycle-dependent functions.
- SAMHD1 restricts early retroviral reverse transcription, resulting in the formation of DNA replicative intermediates.
- Replicative intermediates are sensed by cGAS-STING, leading to induction of antiviral immunity and apoptosis.

1. Introduction

Inhibition of retrovirus replication by different host restriction factors has emerged as an important component of antiviral innate immunity [1, 2]. The essential role of the interferon (IFN) signaling pathways in eliciting and modulating these restriction factors—triggered by pattern recognition receptor (PRR) signaling—remains an under-appreciated aspect of host protection against infection. To trigger IFN expression, host cells recognize infection through several families of pattern recognition receptor that sense evolutionarily conserved structures known as pathogen-associated molecular patterns, including Toll-like receptors (TLR), RIG-I-like receptors (RLR), NOD-like receptors (NLR), as well as several recently identified cytoplasmic sensors of DNA virus infection [3-5]. Classically, viral nucleic acids are the predominant PAMPs detected by these receptors during infection. Recognition of cytosolic viral RNA by RLRs has been extensively studied [6], and recently, a number of DNA receptors have been identified, including AIM2, DNA-PK, IFI16 and cGAS [7-16]. The endoplasmic reticulum resident protein STING (stimulator of interferon genes) (also called MITA or MPYS) is central to intracellular signaling in response to cytosolic DNA [17, 18], and its role as an adaptor facilitating immune signaling in response to viral, bacterial and self DNA has been well described [19-21]. The newly described DNA sensor cGAS [22, 23] (cGAMP synthetase) produces cGAMP (cyclic guanosine monophosphate–adenosine monophosphate) following interaction with DNA and functions as a second messenger to activate a STING-dependent immune response via TBK1 and IRF3. Interestingly, cGAMP also acts in a paracrine manner to stimulate bystander cells through STING, revealing a novel mechanism that rapidly conducts antiviral immunity horizontally to adjacent cells in a manner independent of type I IFN [24].

The innate immune system employs intrinsic host restriction factors to limit replication at various steps of the retroviral life cycle. Particularly relevant for this review and for understanding the host response to infection are two human retroviruses human immunodeficiency virus type 1 (HIV-1) and human T cell leukemia virus type 1 (HTLV-1), both of which lead to serious human pathology. Physiological activities associated with APOBEC, TRIM, BST2 and SAMHD1 are listed in Table 1 and are reviewed elsewhere in this issue. There are several APOBEC-A3 (apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3) proteins that inhibit HIV-1 [25-29] and HTLV-1 [30, 31] infection. APOBEC activity

deaminates cytidine to uracil, resulting in G-to-A hypermutations during reverse transcription [32]. The HIV-1 accessory protein Vif and the HTLV-1 nuclear capsid interfere with APOBEC activity [33, 34]. The TRIM (tripartite motif) proteins interfere with viral replication by targeting different steps in the retroviral life cycle, such as uncoating and promoter activity [35]. The best-characterized member is TRIM5α, which binds to the capsid of incoming retroviral particles and targets capsid for proteosomal degradation [2]. Tetherin (also known as BST2) is named for its anchoring role in retroviral restriction. Tetherin exerts its antiretroviral function by cross-linking new HIV-1 viral particles to the cell surface and prevents nascent particle release [36]. The HIV-1 accessory protein Vpu targets tetherin for degradation [37, 38]. Conversely, HTLV-1 has evolved cell-to-cell transmission strategies that co-opt BST2 [39]. In this review, we provide a comprehensive overview of the activity and regulation of SAMHD1 and summarize the emerging role of SAMHD1 in modulation of the innate immune sensing of retroviral infection (Table 1).

Restriction Factor	Function	Retroviral Countermeasure
APOBEC-A3	Deaminates cytidine to uracil, leading to G-to-A hypermutations	HIV-1: Vif HTLV: nuclear capsid
TRIM5α	Targets retroviral capsids for proteosomal degradation	Capsid proteins cannot be bound by human TRIM5α
Tetherin	Cross-links new HIV-1 particles to the cell surface, preventing release	HIV-1: Vpu HTLV-1: cell-to-cell transmission strategy
SAMHD1	Hydrolyzes host dNTP levels, required for reverse transcription	HIV-2: Vpx

Table 1. Functions associated with HIV-1 host restriction factors.

2. SAMHD1 Structure and Function

The discovery that SAMHD1 [sterile alpha motif and histidine-aspartic domain (HD) containing protein 1] functions as a host restriction factor illustrates the importance of metabolic regulation as an additional mechanism to limit the replication of viral pathogens [40, 41]. SAMHD1 is a host deoxynucleoside triphosphate phosphohydrolase that cleaves deoxynucleoside triphosphates (dNTP) into their deoxynucleosides (dN) and inorganic triphosphates, thus depleting the cellular dNTP pool required for cellular DNA polymerase [42, 43]. SAMHD1 prevents efficient HIV-1 replication by degrading the dNTP pool and, in doing so, inhibits early steps of reverse transcription; blockade at this step prevents the synthesis of full-length double-strand DNA and disrupts later stages of the viral life cycle, including nuclear translocation and integration of proviral DNA [16, 44, 45]. This function has been recognized predominantly in non-cycling cell types including monocytes [46, 47], macrophages [16, 41, 44, 46], dendritic cells [40, 48, 49] and resting $CD4^+$ T cells [45, 50].

SAMHD1 is unusual as a phosphohydrolase in the sense that it does not generate monophosphates. SAMHD1 degrades deoxynucleoside triphosphates including those involved in DNA synthesis (dATP, dCTP, dTTP and dGTP), as well as dUTP, but is unable to hydrolyze nucleotide triphosphates (NTP) [42]. dNTP is used in the synthesis of host DNA, whereas NTP is a source of energy for the cell. SAMHD1 triphosphohydrolase activity was initially shown to be dependent on the presence of dGTP [42, 43], but it has since been demonstrated that a number of dGTP derivatives can similarly activate the enzyme: dGDP, ddGTP, GTP, GDP and GMP can all promote hydrolysis of dATP by SAMHD1 [51, 52]. Considering the physiological abundance of each molecule, it is likely that GTP is the main activator of SAMHD1 *in vivo*, given its higher intracellular concentration [53, 54]. SAMHD1 exists primarily as a dimer in solution [42], but it is unclear if oligomer formation is required for SAMHD1 activity; Yan *et al.* demonstrated that tetramerization was required for HIV-1 restriction [55], whereas other studies indicated that oligomerization was not required for activity [56, 57].

SAMHD1 contains two important domains (Fig. 1): the sterile alpha motif domain is involved in protein–protein interactions and contributes to SAMHD1 nucleic acid binding, although it is not absolutely required for its nuclease activity [58]; the HD domain of SAMHD1 contains the

enzymatic sites crucial for its triphosphohydrolase activity, RNA binding and nuclease activity. Overall, the expression of the HD domain alone is sufficient to restrict HIV-1 replication [57]. SAMHD1 is a predominantly nuclear protein, endowed with a nuclear localization signal located at its N-terminus (sequence: ^{11}KRPR^{14}) [46, 56, 57, 59-61], but a fraction of SAMHD1 is also found in the cytoplasm [45]. SAMHD1 may also contain other nuclear localization signals since deletions of the N-terminal region did not lead to exclusive cytoplasmic expression of SAMHD1 [59], and mutations in locations other than the nuclear localization signal caused relocalization of SAMHD1 to the cytosol [62, 63]. Interestingly, mutated versions of SAMHD1 that localized to the cytoplasm were still capable of restricting HIV-1 infection [56, 57, 61] (Fig. 1).

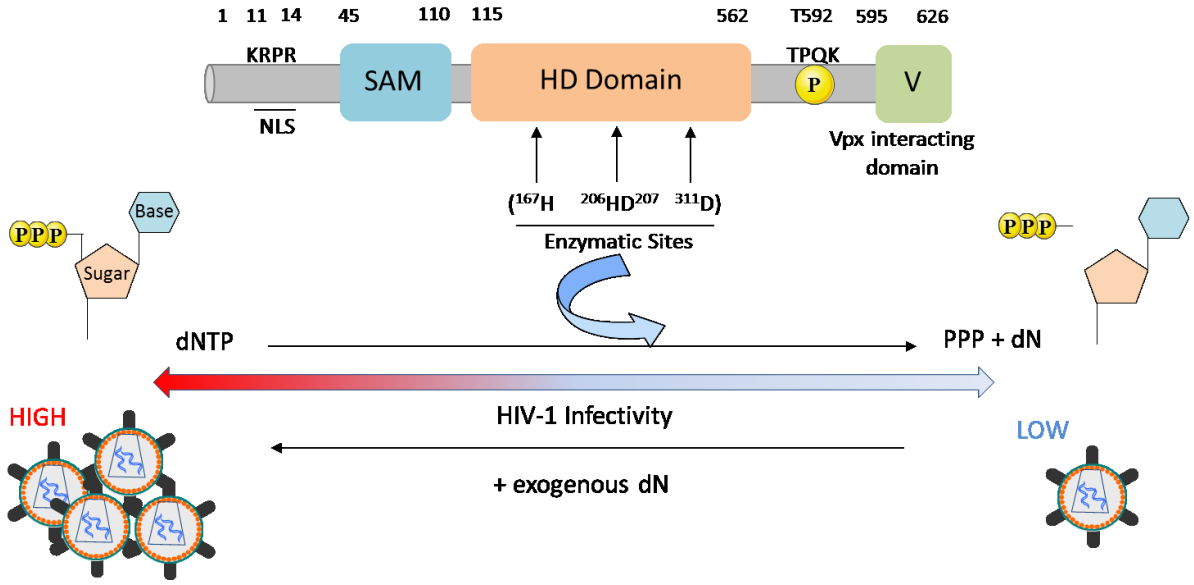


Figure 1. Structure and anti-retroviral activity of SAMHD1.

(Top) Representative schematic of the important domains of SAMHD1, including enzymatic sites, nuclear localization signals and activation/phosphorylation site. (Bottom) Overview of SAMHD1 current anti-retroviral activity mediated by dNTP depletion.

SAMHD1 also contains a domain at the C-terminus capable of interaction with the HIV-2/SIVsm Vpx accessory protein [64]. Prior to the discovery of the antiretroviral function of SAMHD1, it was unclear how Vpx contributed to productive HIV-1 infection of non-permissive myeloid cells such as monocytes, macrophages and dendritic cells [46, 65-69]. It is now recognized that Vpx disrupts SAMHD1 function in non-dividing cells and promotes its proteasomal degradation (Fig. 2) [40, 41]. Conversely, HIV-2 or SIV that is Vpx deficient is unable to productively infect these cells [66-68, 70]. Physical interaction between SAMHD1 and Vpx in the nucleus or the cytoplasm is required to induce SAMHD1 degradation [56, 59]. Cytoplasmic SAMHD1 mutants cannot be degraded by Vpx that localizes in the nucleus [61, 71]. Similarly, mutant Vpx that does not localize to the nucleus cannot inhibit wild-type SAMHD1 [65, 72-74]. Vpx interacts with the cullin-4-based E3 ubiquitin ligase CRL4/DCAF1 and tethers SAMHD1 to the Cull4/CRL4/DCAF1 complex, leading to ubiquitination proteosomal degradation via the C-terminus of SAMHD1, although it is unclear if degradation occurs in the cytoplasm or the nucleus [56, 61, 65-67, 75, 76]. The N-terminus of SAMHD1 can also interact with Vpx proteins from divergent lentiviruses [77]. Neddylation inhibitors that reversibly interfere with proper CRL4/DCAF1 function prevent Vpx-mediated degradation of SAMHD1 [78]. Following SAMHD1 degradation by Vpx, there is a concomitant increase in intracellular dNTP levels [48, 79]. Additional work has also shown that Vpx can inhibit SAMHD1 activity prior to its degradation [80] (Fig. 2).

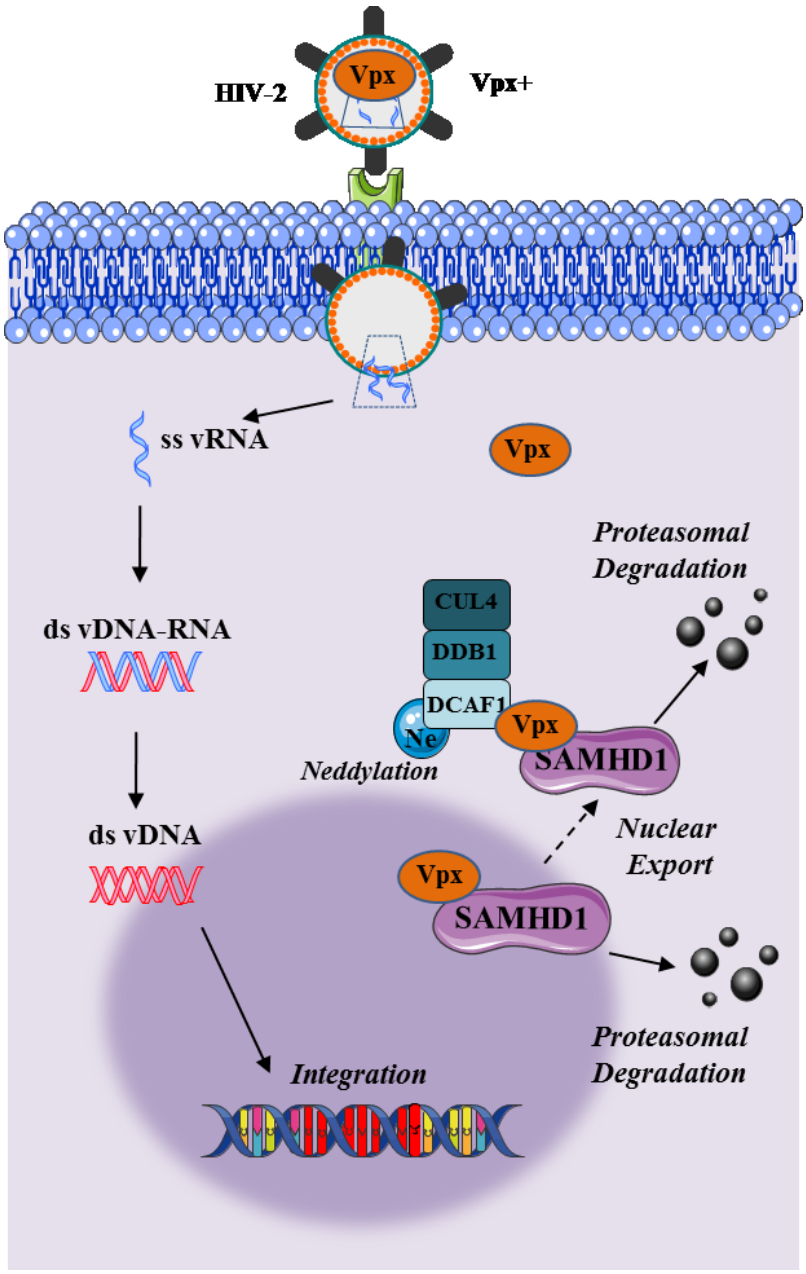


Figure 2. Abortive HIV-1 infection prevents activation of the innate immune response.

Infection of host cells with Vpx^{+} virus results in the degradation of SAMHD1 and productive infection. Vpx interacts with the cullin-4-based E3 ubiquitin ligase CRL4/DCAF1 and tethers SAMHD1 to the Cull4/CRL4/DCAF1 complex, leading to ubiquitination proteosomal degradation via the C-terminus of SAMHD1. With SAMHD1 degradation, retroviral reverse transcription is completed and proviral DNA integrates into the host genome.

3. Regulation of SAMHD1

SAMHD1 activity can be regulated by a number of factors, including phosphorylation state and the production of type 1 IFN. SAMHD1 restriction and its antiretroviral function, as well as low dNTP levels, are not solely correlated with its expression level; for example, SAMHD1 is expressed in cycling and non-cycling $CD4^+$ T cells, but only in non-cycling cells is SAMHD1 restrictive to HIV-1 replication [45, 50]. SAMHD1 was initially discovered as the homologue of a mouse IFN-inducible gene termed interferon-γ-inducible protein (*Mg11*). Human SAMHD1 protein expression does not appear to be up-regulated by IFN-α or IFN-γ in human dendritic cells, macrophages or $CD4^+$ T cells [46, 48], although a transient increase in mRNA levels is observed [48]. Expression can be induced in certain cell lines, such as U87-MG [16], HEK 293T or HeLa cells [48]. One report indicated that IFN-α can increase SAMHD1 expression in primary monocytes [47], whereas another study did not demonstrate any increase [81]. A combination of IL-12 and IL-18 can lead to an increase in SAMHD1 expression in monocyte-derived macrophage (MDM), resulting in the generation of cells that are more resistant to HIV-1 infection [82]. Why certain cytokine combinations will increase SAMHD1 expression in some cell types is unknown, although changes in methylation status of the SAMHD1 promoter may be involved [83]. Interestingly, IFN-α or IFN-γ treatment of PMA-matured THP-1 cells prevented Vpx from degrading SAMHD1 [46]. IFN-α-mediated protection of SAMHD1 also applies to primary plasmacytoid dendritic cell (pDC) and myeloid dendritic cell, both of which are highly resistant to HIV-1 infection [84]. Treating pDC with an anti-IFN antibody cocktail partially alleviated restriction and permitted SAMHD1 degradation [84].

SAMHD1 expression is significantly influenced by cell cycle status. Franzolin *et al.* demonstrated that the highest levels of SAMHD1 occurred during quiescence, while SAMHD1 expression was barely detectable in cycling cells [60]. The size of the dNTP pool was also affected by fluctuations in SAMHD1 abundance. SAMHD1 was essential to prevent accumulation of G_1 cells and arrest of the cell cycle, indicating that SAMHD1 impacts the regulation of the cell cycle [60]. The myeloid cell line THP-1, which is routinely used as representative myeloid cell model, expresses SAMHD1 constitutively at high levels. THP-1 are permissive to HIV-1 infection but can be made resistant following PMA-mediated differentiation

into non-cycling macrophage-like cells [40, 57, 65, 81, 85]. In this context, PMA appears to stimulate SAMHD1 activity but does not influence its expression [81, 85] (Fig. 3).

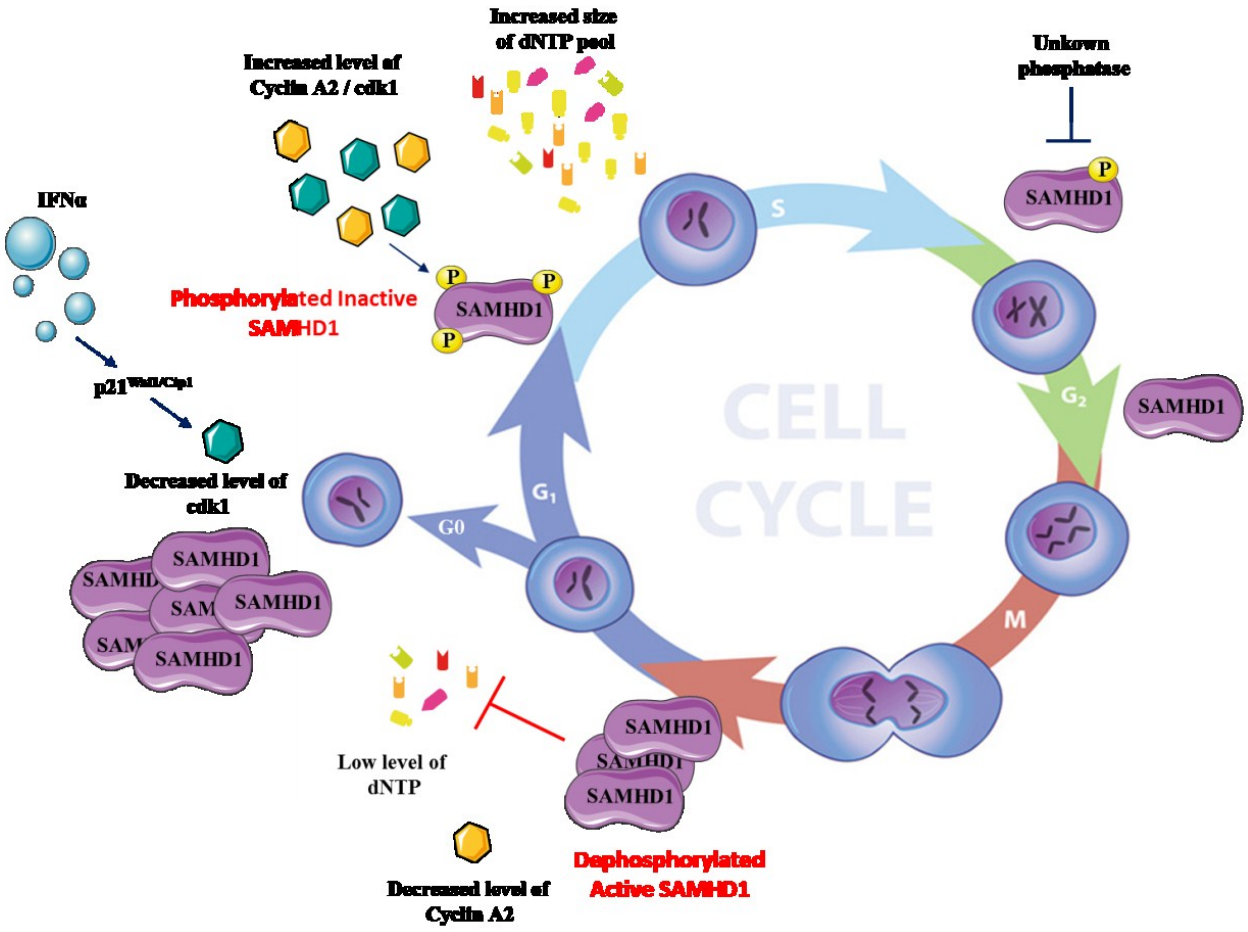


Figure 3: Levels of SAMHD1 expression and activation status during the cell-cycle.

SAMHD1 expression is modulated by the cell cycle. SAMHD1 is highly expressed during quiescence (G_0 phase), but minimally expressed during replication (S phase). Its activation status is dependent on phosphorylation by Cyclin A2 and CDK1; T592 phosphorylation indicates inactivation. Cyclin A2 is highly expressed during S phase, when high intracellular dNTP levels are found. IFN-α treatment leads to the de-phosphorylation of SAMHD1, mediated by the induction of $p21^{Waf1/Cip1}$ that decreases the levels of CDK1. The phosphatase that de-phosphorylates SAMHD1 is currently unknown.

Three recent studies demonstrate that SAMHD1 antiretroviral activity is regulated by phosphorylation in a cell-cycle-dependent manner (Fig. 3) [81, 85, 86]. SAMHD1 is phosphorylated at threonine 592 (T592), by the cyclin-dependent kinase 1 (CDK1) and cyclin A2 *in vivo* [81] or by cyclin B *in vitro* [85]. T592 phosphorylation of SAMHD1 was detected in permissive cells such as activated $CD4^+$ T cells and untreated THP-1 cells, while HIV-resistant cell types such as myeloid cells, PMA-treated THP-1 cells and resting T cells expressed predominantly non-phosphorylated SAMHD1. This observation implies an inverse correlation between T592 phosphorylation residue and the antiretroviral state [81, 85, 86]. A number of other phosphorylation sites have also been identified, although their association with cell cycle status and retroviral restriction has not been established [81, 85, 86].

Inactivation of SAMHD1 by cyclin A2 likely occurs during the S phase of the cell cycle, when cyclin A2 is highly expressed [87], and there is a requirement for dNTP during chromosomal DNA replication. Furthermore, differentiation of cells to an HIV-1-resistant phenotype is associated with a loss of CDK1 expression [88, 89]. Interestingly, Cribier *et al.* demonstrated a decrease in T592 phosphorylation in cells treated with IFN-α, thus implying a link between type 1 IFN signaling and SAMHD1 activation [81] that may involve $p21^{Waf1/Cip1}$, a cell-cycle-regulated inhibitor of HIV-1 replication [90], and CDK1, a cell-cycle-regulated kinase activity that is induced by IFN-α [91, 92].

Regulation of SAMHD1 by CDK1-mediated phosphorylation at T592 is complicated by the observation that non-phosphorylated SAMHD1 (at the T592 residue) correlated with HIV-1 restriction, but not with modulation of its triphosphohydrolase activity [85, 86]. Indeed, while the current paradigm is that SAMHD1 restricts HIV-1 by hydrolyzing the dNTP pool [44, 48], White *et al.* and Welbourn *et al.* suggest that additional mechanisms are involved in SAMHD1-mediated restriction. Regardless of phosphorylation status, SAMHD1 could still hydrolyze dNTP *in vitro* and T592 phosphorylation had no effect on oligomerization, Vpx-mediated degradation, RNA binding or nuclear localization [85, 86]. It is likely that additional factors and mechanisms contribute to the modulation of SAMHD1 activity.

4. The Role of SAMHD1 in the Autoimmune Disorder Aicardi–Goutières Syndrome

Hereditary and loss-of-function studies have demonstrated that mutations in SAMHD1 dramatically impact host immunity; SAMHD1 was first associated with Aicardi–Goutières syndrome (AGS) [93], a rare autoimmune disorder characterized by chronic inflammation and high levels of IFN-α in the cerebrospinal fluid [94], reminiscent of chronic viral infection [95]. It is hypothesized that the improper metabolism of host nucleic acids triggers chronic stimulation of the innate immune response in autoimmune diseases such as systemic lupus erythematosus and AGS[95-97]. SAMHD1 likely prevents the generation of stimulatory host DNA by hydrolyzing the dNTP pool; mutations of SAMHD1 found in AGS patients are located in the enzymatically important HD domain, thus supporting this idea (Fig. 1) [93]. AGS cells that harbor a defective SAMHD1 are highly permissive to HIV-1 replication [47], further implicating the triphosphohydrolase activity of SAMHD1 in AGS. It was also recently reported that the absence of SAMHD1 led to the spontaneous expression and release of type 1 IFN [98]; hence, SAMHD1 is described as a negative regulator of IFN signaling in this context [93].

AGS is also associated with mutations in the exonuclease TREX1 and endonuclease RNase H2 [99, 100], both of which have DNA editing properties that are diminished during disease [95]. TREX1 functions to digest DNA—reverse-transcribed intermediates (RTIs)—produced by endogenous and exogenous retroviruses [101, 102]. Depletion of TREX1 leads to immune activation and IFN production through the endoplasmic reticulum resident DNA sensor STING [102], which is a central regulator of TBK1-IRF3 signaling cascades following stimulatory DNA accumulation [103]. STING regulation likely also plays a vital role in autoimmune disorders such as AGS.

Mutations in RNase H2 cause increased rNTP incorporation into DNA, spontaneous DNA breaks and the initiation of the DNA damage response [104]. Unlike SAMHD1 activity that inhibits retroviral replication, TREX1 and RNase H2 appear to be vital for HIV-1 replication [102, 105]. RNase H2 prevents rNTP incorporation into HIV-1 DNA and, therefore, prevents mutation [53, 106]. Overall, it is less clear how mutations in RNase H2 contribute to AGS.

5. SAMHD1 and Innate Immune Sensing of Retroviral Infection

One of the most exciting developments in viral immunology in the past 5 years has been the identification and characterization of the molecular basis of DNA sensing by the innate immune system. More than 10 cytosolic receptors of DNA have been identified in recent years, including the PYHIN family proteins AIM2 (absent in melanoma) and IFI16 (IFN-inducible protein 16) [107, 108]. Furthermore, during the past year, a new second messenger system has been discovered that senses, modifies and transmits signals to the innate immune response via the adapter protein STING, a key adaptor protein for most DNA-sensing pathways. Two studies from the Chen group demonstrated that stimulation of cells with cytosolic DNA induced synthesis of cyclic-di-GMP-AMP (cGAMP) from ATP and GTP by a cyclase enzyme called cGAMP synthetase (cGAS), leading to STING-dependent induction of IFN [7, 109]. This cGAMP isomer, termed 2′3′-cGAMP, which contains both 2′–5′ and 3′–5′ phosphodiester linkages, functions as a second messenger that binds and activates the endoplasmic reticulum protein STING [22]. Based on structural analysis of the C-terminal domain, STING exists as a dimer in an autoinhibited state; cyclic dinucleotide binding, or activation of STING by upstream DNA sensors, relieves autoinhibition and provides a scaffold that permits association with TBK1 and IRF3, leading to phosphorylation-dependent activation of IRF3 (Fig. 4). Thus, STING directs TBK1 to activate IRF3 in response to DNA sensing and activation of antiviral innate responses; it remains unclear whether STING recruitment of TBK1 also controls NF-κB activation in response to cytosolic DNA. Cyclic dinucleotides can also activate the NLRP3 inflammasome through a unique pathway independent of STING but dependent on the mobilization of potassium and calcium ions [110], thus broadening the spectrum of cGAS activity in triggering both antiviral and inflammatory innate immune responses.

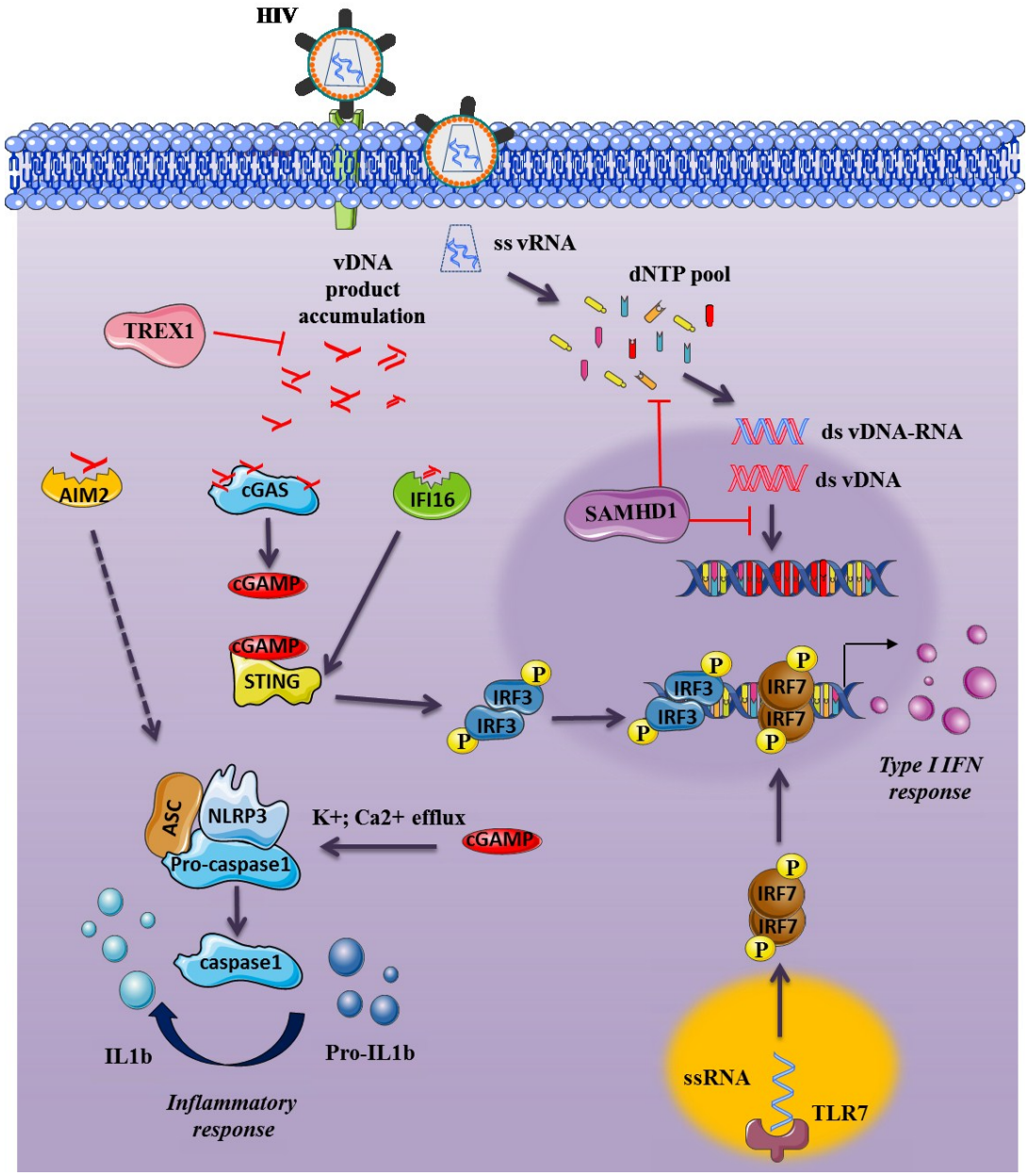


Figure 4. HIV Infection activates the innate immune response through RTI production.

Inhibition of HIV-1 reverse transcription mediated by SAMHD1 leads to the production of RTIs. RTI may be degraded by TREX1 but can also be detected by the DNA sensors cGAS and IFI16 that activate STING and the type 1 IFN response. RTI detection also leads to inflammasome activation, possibly through AIM2 activation, leading to the release of active IL-1β. Furthermore, activation of TLR7 by genomic RNA in pDCs can activate the IFN response.

Two human PYHIN proteins, absent in melanoma (AIM2) and IFN-γ-inducible (IFI16), have been characterized as DNA sensors that are essential for distinct DNA-activated innate responses. AIM2 localizes to the cytoplasm and binds DNA; AIM2 is essential for interleukin-1β production in response to double-stranded DNA (dsDNA) and functions by assembling an inflammasome complex with ASC and caspase 1 (Fig. 4). IFI16 has been shown to bind dsDNA and induce STING-dependent IFN-β responses [11] (Fig. 4).

Three recent studies have identified the interaction of retroviral DNA intermediates with innate DNA sensors [111-113]. In one study, Chen and colleagues demonstrated that HIV-1 infection activated cGAMP synthase (cGAS) to produce cGAMP, which bound to and activated the adaptor protein STING to induce type I IFN[111]. Inhibitors of HIV reverse transcriptase, but not integrase, eliminated IFN induction by HIV-1, suggesting that reverse-transcribed HIV-1 DNA triggered the innate immune response. When cGAS was depleted, cytokine induction by HIV-1, murine leukemia virus and simian immunodeficiency virus was abrogated, thus identifying cGAS as an innate immune sensor of HIV-1 and other retroviruses. Interestingly, this second messenger cGAMP can also act *in trans* and be delivered to neighboring cells through gap junctions to stimulate innate immunity through STING, in a manner independent of type I IFN [24]. A second study from the Paludan group in Denmark introduced single-stranded DNAs (ssDNAs) derived from HIV-1 proviral DNA into primary human macrophages and demonstrated that HIV-1 DNA induced IFN expression, dependent on interferon-inducible protein 16 (IFI16) [112]. Immunostimulatory DNA directly bound to IFI16 and activated the STING-TBK1-IRF3/7 pathway. This response was stimulated by stem regions in the HIV-1 DNA structure, and IFI16 knockdown augmented lentiviral transduction, as well as HIV-1 replication. Again, these results pinpoint IFI16 and the STING adapter as sensors for HIV-1 reverse transcriptase intermediates in macrophages. In the third study, we approached these questions using a primary infection model with HTLV-1 and primary monocytes [113]. Unlike most retroviruses, cell-free HTLV-1 virions are poorly infectious and do not stably infect their primary $CD4^+$ T lymphocyte target. However, HTLV-1 efficiently infects cells of the myeloid lineage, leading to productive infection of myeloid and pDCs or abortive infection of primary monocytic cells. *De novo* HTLV-1 infection of monocytes rapidly induced a type 1 IFN response, as well as apoptosis, in a SAMHD1-dependent manner. Silencing of SAMHD1 by

RNA interference or the addition of exogenous dNTPs to the infected monocyte culture blocked apoptosis and the antiviral response. The reverse transcriptase inhibitor azidothymidine also inhibited apoptosis, suggesting a role for reverse transcription intermediates (RTI) in triggering the antiviral and apoptotic response. A 90-nucleotide RTI from the U5 region of HTLV-1, corresponding to strong-stop DNA, was introduced into monocytes and induced apoptosis; the RTI bound to the endoplasmic reticulum resident DNA sensor STING, which mediates activation of the host antiviral response via IRF3. Using siRNA knockdown and co-immunoprecipitation, we confirmed that STING-mediated apoptosis in HTLV-1-infected monocytes required the generation of pro-apoptotic complex between IRF3 and the Bcl-2 family member Bax. A similar result was obtained using an RTI corresponding to HIV-1 strong-stop DNA. Altogether, these studies provide a mechanistic link among SAMHD1 restriction of reverse transcription, sensing of retroviral reverse transcription intermediates by IFI16-cGAS-STING and the initiation of IRF3-dependent antiviral and apoptotic responses.

In earlier studies, Doitsh *et al.* demonstrated that HIV-1 abortive infection of resting $CD4^+$ T cells initiated a pro-inflammatory response and led to apoptosis [114]. Cell death was mediated by RTI production that elicited the formation of an inflammasome complex and the induction of caspase-1- and caspase-3-mediated apoptosis [114]. In light of the abovementioned recent experiments, it is likely that the observations relating to host restriction and triggering apoptotic response were dependent on DNA sensing of RTI by DNA sensors, with a contribution from SAMHD1 activity [45, 50]. The induction of apoptosis via IRF3-Bax interaction was previously characterized during paramyxovirus infection as a host mechanism to prevent persistent infection [115]. This mechanism of retroviral DNA-induced apoptosis involving IRF3-Bax mitochondrial depolymerization appears to be distinct from the mechanism recently described by Nabel's group; in that study, proviral DNA in the nucleus was detected by the DNA-dependent protein kinase (DNA-PK) during integration, leading to apoptosis [9]. Additionally, while STING-IRF3 axis has also been implicated in mitochondria-dependent cell death in hepatocytes [116], it may not be required for inflammasome activation [110]. These emerging studies suggest that multiple, non-redundant DNA sensors and mechanisms may be activated concurrently in retrovirus-infected cells to mediate innate immune and cell death responses.

It has also been proposed that HIV-1 may take advantage of the SAMHD1-TREX1 dichotomy to hide the DNA evidence of its presence and avoid activation of the innate immune system [44]. HIV-1 infection of MDM remains undetected, unless TREX1 is silenced to allow RTI accumulation and subsequent immune activation [102]. Interestingly, TREX1-mediated degradation of cytosolic DNA was recently shown to be inhibited by oxidative stress, which modified the DNA and prevented its degradation [117]. It is currently unknown if this pathway is active during retroviral infection; nevertheless, oxidative modifications of pathogen DNA during reactive oxygen species generation may be an intrinsic mechanism that prevents TREX-mediated degradation of RTI, thus facilitating virus detection by the DNA immune sensors.

6. Missing Pieces in the SAMHD1 Puzzle

As a retroviral restriction factor, SAMHD1 limits virus infectivity and replication in certain cell types and inactivation of SAMHD1 may ultimately shape the development of the retroviral reservoir; however, it is unclear to what extent this occurs *in vivo*. For instance, SAMHD1 expression limits the synthesis of HIV-1 provirus in macrophages *in vitro* [16, 41, 44, 46], yet this cell type can serve as a viral reservoir *in vivo* [118, 119]. One *in vitro* study reported that the removal of SAMHD1 significantly reduced azidothymidine-mediated restriction of HIV-1 replication in MDM [52], suggesting that SAMHD1 may play a role in enhancing the effects of antiretroviral therapy during HIV-1 infection. A recent *in vivo* study reported that SAMHD1-deficient mice were not more susceptible to HIV-1 infection but were susceptible to a mutant virus that had a lower affinity for dNTP [120].

Why HIV-1 accessory proteins have not evolved a specific function to counter SAMHD1 activity similar to HIV-2 or SIV Vpx is not fully understood. *Vpx* appears to have evolved from a duplication of the *vpr* gene [121]. The ability to degrade SAMHD1 was initially a function of the Vpr protein, but the two accessory proteins began evolving different roles [122]. It has been suggested that the SIV ancestor of HIV-1 lost the *vpx* gene [123] and that HIV-1 maintains an evolutionary advantage by not replicating in myeloid cells and by reducing the impact of IFN production in certain cell types [49, 111]. HIV-2, in contrast to HIV-1, inherited the SAMHD1-degrading *vpx* gene but is much less pathogenic than HIV-1. More than 70% of HIV-2-positive individuals are long-term non-progressors [124]. HIV-2 also does not replicate well in myeloid

cells [125], likely due to the induction of the IFN response. It should be noted, however, that SIV harboring a defective form of *vpx* was much less pathogenic than wild-type SIV [126], possibly due to functions additional to SAMHD1 degradation, such as nuclear import of cDNA [121]. It was also established that Vpx-mediated induction of the IFN response in myeloid cells was not associated with control of HIV-2 disease progression [127]. That study concluded that Vpx and infection of monocytic cells during HIV-2 infection were not the factors determining pathogenesis.

SAMHD1 may have additional antiretroviral functions, in addition to its triphosphohydrolase activity (Fig. 5) [57, 85, 86]. SAMHD1 also has a metal-dependent 3′-to-5′ exonuclease activity that degrades single-stranded RNA or ssDNA overhangs [58]. The exonuclease and triphosphohydrolase activities cannot be performed by SAMHD1 simultaneously, suggesting that both activities require the same active site [58]. SAMHD1 showed no exonuclease activity against circular ssDNA, blunt-ended dsDNA or double-stranded RNA/DNA hybrids. However, 3′ and 5′ RNA overhangs in double-stranded RNA/DNA hybrids were degraded, with 3′ exonuclease activity occurring more rapidly than 5′. SAMHD1 could bind to HIV-1 sequences *in vitro*, and strong binding occurred when the target sequence formed secondary structures [58]. Consistent with this, another group also showed that SAMHD1 could interact with single-stranded RNA and ssDNA *in situ* [63]. It is possible that SAMHD1 attacks retroviruses at this additional level, but it is unclear how SAMHD1, as an exclusively nuclear protein, could interact with retroviral genomic RNA, although Berger *et al.* have indicated that SAMHD1 may be exported to the cytoplasm during the early phases of infection [47] (Fig. 5).

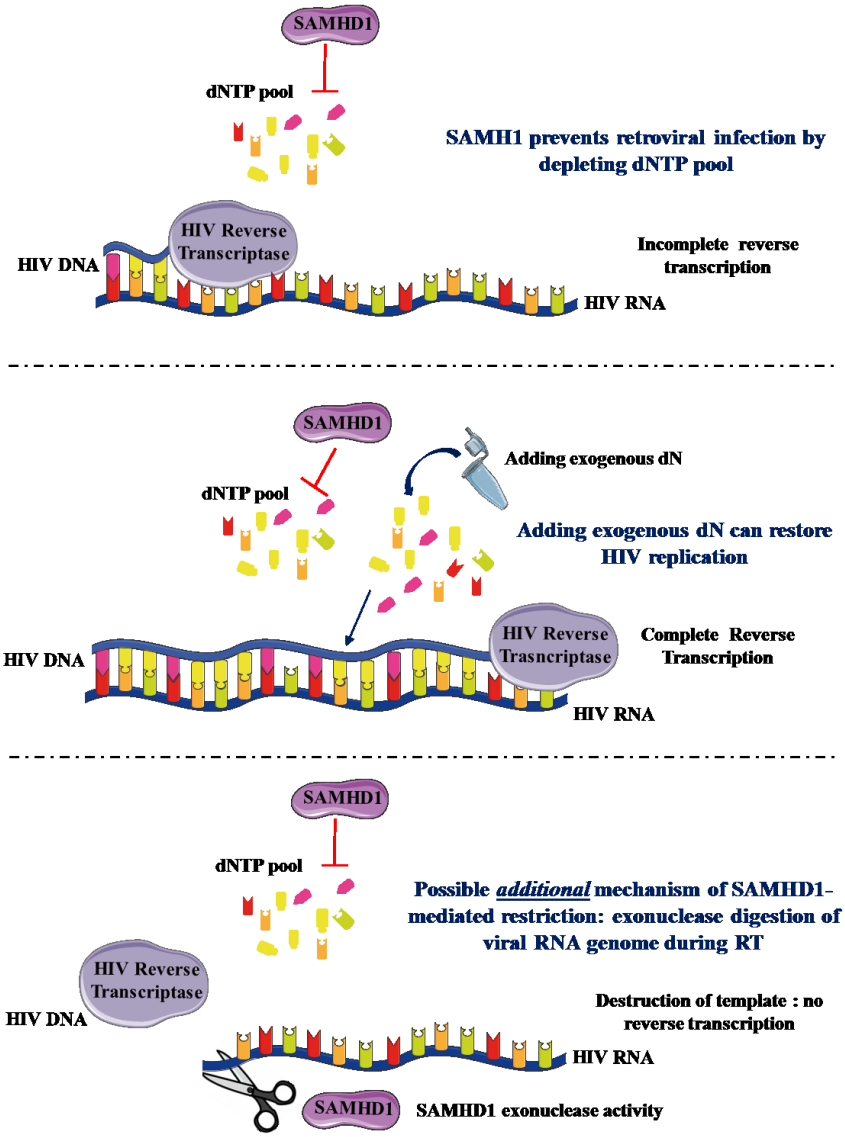


Figure 5: Additional Mechanism of SAMHD1 Mediated Restriction.

A) Current accepted mode of SAMHD1 restriction is dNTP depletion. Incoming viral genomes are unable to complete reverse transcription. B) Addition of exogenous dN can replenish the dNTP pool and counter SAMHD1 function, resulting in the completion of reverse transcription and productive infection. C) Additional mechanisms of SAMHD1 mediated restriction involves its exonuclease activity. SAMHD1 directly targets the RNA genome during slow reverse transcription and degrades it. The destruction of the RNA template prevents retroviral infection.

7. Broad Range Activity of SAMHD1

SAMHD1 also contributes to the restriction of a number of different retroviruses [57, 85, 128]; feline immunodeficiency virus, bovine immunodeficiency virus, murine leukemia virus, equine infectious anemia virus and mason pfizer monkey virus could also be inhibited by SAMHD1 [57, 85, 128]. Gramberg *et al.* showed that two retroviruses, the prototype foamy virus (PFV) and HTLV-1, were unrestricted by SAMHD1 in MDM and monocyte-derived dendritic cell models. In contrast, our data generated with primary monocytes demonstrated the ability of SAMHD1 to restrict HTLV-1 infection and generate cytoplasmic RTI [113]. PFV reverse transcription occurs prior to entry of targets cells [129], which explains how it is unaffected by SAMHD1.

A recent paper by Hollenbaugh *et al.* has expanded SAMHD1-mediated restriction to non-retroviruses, such as vaccinia and herpes simplex virus type 1. The replication of both viruses could be enhanced in infected cells by Vpx expression, resulting in the degradation of SAMHD1 [130]. Interestingly, vaccinia virus encodes dNTP biosynthetic enzymatic activities such as thymidine kinase and ribonucleotide reductase to increase dNTP levels in infected cells [131, 132]. Mutant vaccinia that did not contain thymidine kinase was more sensitive to SAMHD1-mediated restriction, and infected cells displayed lower dNTP levels than those infected with wild-type virus, indicating that SAMHD1 triphosphohydrolase activity mediated restriction [130]. Overall, these studies demonstrate that SAMHD1 host restriction extends beyond human retroviruses and includes the capacity to inhibit a broad range of viruses.

8. Perspectives

This review highlights the convergence of two rapidly evolving areas of viral immunology—SAMHD1 host restriction factor inhibition of virus multiplication and innate immune sensing of viral DNA. With the recognition that early retroviral reverse transcriptase intermediates—essentially strong-stop DNA—are recognized by emerging DNA sensors such as IFI16, cGAS and STING, many questions emerge. What are the precise associations between these signaling molecules leading to the antiviral response; how are antiviral responses linked to cell death programs in abortively infected cells; what role do other sensors play in a cell type or differentiation specific context. SAMHD1 has other non-triphosphohydrolase activities that

potentially restrict retroviral infection, but their modes of action are not understood. SAMHD1 and TREX1 likely function in tandem in normal physiology, but molecular mechanisms that regulate SAMHD1 and TREX1 activity are unknown. It is not yet clear how SAMHD1-mediated host restriction and innate sensing of retroviral DNA intermediates impact proviral DNA generation and integration, or if this restriction ultimately affects the generation of a latent HIV-1 reservoir. A considerable amount of work remains to clarify the mechanistic details that link SAMHD1 host restriction of HIV-1 and other retroviruses with downstream innate signaling and apoptotic pathways. These and other important insights will assist in the development of new antiviral therapies to boost the immune response to viral and bacterial pathogens.

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Rationale and Objectives

One of the major obstacles in the development of effective anti-HTLV-1 therapies is the lack of basic understanding regarding the factors that determine viral associated pathology. The early events of HTLV-1 infection are significantly understudied and may be key in discerning pathological outcomes. Specifically, viral interactions with the immune system likely play a major role in disease progression and are the focus of the following chapters. We set out to study the early events of HTLV-1 infection in two important immunologically relevant peripheral blood mononuclear cell populations, monocytes and $CD4^+$ T-cells.

Monocytes are one of the most common types of immune cell, and likely the first cells that interact with cell-free HTLV-1 once it enters the bloodstream. During studies with primary untouched human monocytes, we wished to investigate what the impact of *de novo* HTLV-1 infection was on survival and immune responses, and if SAMHD1 could restrict HTLV-1 like HIV-1. We had the following three specific aims:

1. Characterize *in vitro* HTLV-1 infection of human monocytes.
2. Investigate and analyze monocyte survival following HTLV-1 infection.
3. Identify underlying mechanism responsible for apoptosis within infected cells.

The normal function of $CD4^+$ T-cells is vital for proper immunological responses. They are also the main target of HTLV-1 infection, and their deregulation is associated with HTLV-1 diseases. Our investigations in activated primary human $CD4^+$ T-cells led us to investigate if HTLV-1 could repress FOXO3a signaling during the early stages of infection to influence viral persistence and spread. We had the following three specific aims for that study:

1. Characterize *de novo* HTLV-1 productive infection of primary human $CD4^+$T-cells in terms of cellular persistence.
2. Investigate and analyze Foxo3a activity following HTLV-1 infection.
3. Identify underlying mechanism responsible for enhanced persistence.

Chapter 3: Host Restriction Factor SAMHD1 Limits Human T Cell Leukemia Virus Type 1 Infection of Monocytes via Sting-Mediated Apoptosis

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1. SUMMARY

Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia and HTLV-1-associated myelopathies. In addition to T cells, HTLV-1 infects cells of the myeloid lineage, which play critical roles in the host innate response to viral infection. Investigating the monocyte depletion observed during HTLV-1 infection, we discovered that primary human monocytes infected with HTLV-1 undergo abortive infection accompanied by apoptosis dependent on SAMHD1, a host restriction factor that hydrolyzes endogenous dNTPs to below the levels required for productive reverse transcription. Reverse transcription intermediates (RTI) produced in the presence of SAMHD1 induced IRF3-mediated antiviral and apoptotic responses. Viral RTIs complexed with the DNA sensor STING to trigger formation of an IRF3-Bax complex leading to apoptosis. This study provides a mechanistic explanation for abortive HTLV-1 infection of monocytes and reports a link between SAMHD1 restriction, HTLV-1 RTI sensing by STING, and initiation of IRF3-Bax driven apoptosis.

HIGHLIGHTS

- HTLV-1 infection of primary monocytes is nonproductive and elicits an antiviral response
- Host restriction factor SAMHD1 aborts HTLV-1 reverse transcription
- HTLV-1 reverse transcription intermediates (RTI) form a complex with STING
- RTI sensing by STING generates an IRF3-Bax complex that triggers apoptosis to limit HTLV-1

Graphical Abstract

Figure S6

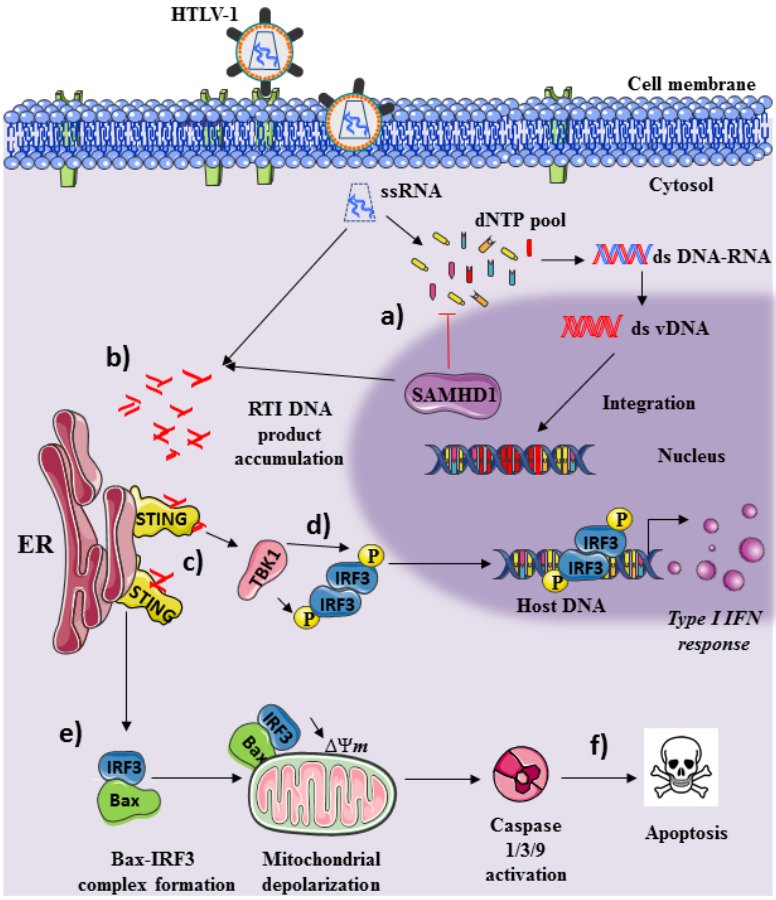


Figure S6. Schematic of SAMHD1-mediated inhibition of HTLV-1 infection in monocytes.

HTLV-1 elicits a non-productive infection in primary monocytes that culminates in the activation of an IRF-3 mediated antiviral response and cellular apoptosis. SAMHD1 hydrolyzes the host dNTP pool, thus limiting the reverse transcription of vRNA in monocytes a), leading to the production of reverse transcription intermediates (RTI) (b). The viral RTI complex with the endoplasmic reticulum DNA sensor STING (c) and mediate activation of the type I IFN response through IRF3 (d). STING signaling also leads to the formation of a pro-apoptotic IRF3-Bax complex (e), leading to apoptosis via mitochondrial depolarization and caspase 1/3/9 activation (f).

2. INTRODUCTION

Infection with human T-cell leukemia virus type 1 (HTLV-1) affects approximately 20 million people worldwide (Cook et al., 2013), and is a major cause of mortality and morbidity in endemic areas such as southern Japan, the Caribbean basin, Central/South America, and Western Africa (Ragin et al., 2008). Although most of the infected individuals are asymptomatic carriers of the virus, they are also at risk for opportunistic infections (Verdonck et al., 2007). In addition, chronic infection with HTLV-1 can lead to a number of severe pathologies associated with poor prognosis, including the aggressive and fatal adult T-cell leukemia (ATL), progressive HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP), uveitis and infective dermatitis in children (Cook et al., 2013; Yamano and Sato, 2012). HTLV-1 has a preferential tropism for $CD4^+$ T-cells in asymptomatic carriers and ATL patients, while both $CD4^+$ and $CD8^+$ T-cells constitute viral reservoirs in HAM/TSP patients (Cook et al., 2013). Unlike most retroviruses, cell-free HTLV-1 is poorly infectious and does not stably infect its primary $CD4^+$ T lymphocyte target. Rather, HTLV-1 utilizes various cell-to cell transmission strategies, including transfer of viral assemblies, formation of virological synapses, or formation of intracellular conduits (Igakura et al., 2003; Pais-Correia et al., 2010; Van Prooyen et al., 2010). HTLV-1 also infects cells of the myeloid lineage, which play critical roles in the host innate response to viral infection. For instance, previous studies have shown that cell-free HTLV-1 particles can productively infect DC, which then participate in viral transmission to and transformation of $CD4^+$ T-cells (Jones et al., 2008). HTLV-1 infection of DC elicits early antiviral responses that are mediated by the production of type I interferon (IFN) (Colisson et al., 2010), although the number of peripheral DC as well as the IFN response are markedly reduced in chronically infected asymptomatic and ATL subjects (Hishizawa et al., 2004). Monocyte precursors that would normally replenish the DC population are unable to properly differentiate during chronic HTLV-1 infection and recent evidence indicates monocyte depletion in HTLV-1 infected patients (Makino et al., 2000; Nascimento et al., 2011). The molecular consequences of *de novo* HTLV-1 infection on host innate immunity in monocytic cells have yet to be elucidated.

Human myeloid and bystander $CD4^+$ T-cells are refractory to HIV-1 infection, in part because the host restriction factor SAMHD1 prevents efficient viral DNA synthesis (Baldauf et al., 2012; Descours et al., 2012; Laguette and Benkirane, 2012; Laguette et al., 2011). SAMHD1 functions

within non-dividing cells as a deoxynucleoside triphosphate triphosphohydrolase, which hydrolyzes the endogenous pool of deoxynucleoside triphosphates (dNTP) to levels below the threshold required for reverse transcription (Ayinde et al., 2012; Goldstone et al., 2011; Lahouassa et al., 2012). SAMHD1 was initially characterized in the context of the autoimmune disorder Aicardi-Goutières syndrome, and genetic mutations that render SAMHD1 non-functional result in uncontrolled inflammatory and type I IFN responses against self DNA (Rice et al., 2009). Primary cells from these patients are highly susceptible to HIV-1 infection (Baldauf et al., 2012; Berger et al., 2011; Descours et al., 2012). The Vpx accessory protein of HIV-2 and its counterparts in certain strains of SIV antagonize SAMHD-1 by inducing proteasome-dependent degradation (Ayinde et al., 2012; Laguette et al., 2012). HIV-1 restriction mediated by SAMHD-1 is overcome by silencing its expression with Vpx, or by the addition of exogenous dN (deoxynucleosides) (Baldauf et al., 2012; Descours et al., 2012; Laguette et al., 2011; Lahouassa et al., 2012).

Recognition of evolutionarily conserved molecular structures shared by pathogens, known as pathogen-associated molecular patterns (PAMP), is critical for the initiation of innate immune responses (Kumar et al., 2011). Multiple surface and cytosolic pathogen recognition receptors (PRR) (Kawai and Akira, 2011) have now been identified that sense and respond to microbial infection. Toll-like receptors (TLR) detect distinct PAMP such as lipopolysaccharide (TLR4), single and double stranded RNA (TLR7/8 and TLR3, respectively) and CpG DNA (TLR9) (Blasius and Beutler, 2010; Kawai and Akira, 2011). The retinoic acid-inducible gene-I (RIG-I)-like receptors (RLR) - which include RIG-I and MDA5 - represent another PRR family that recognizes cytosolic viral RNA (Blasius and Beutler, 2010; Kawai and Akira, 2011). PRR responsible for the detection of cytosolic DNA include DAI, cGAS and IFI16 - all of which trigger IFN production - as well as AIM2 that stimulates an inflammasome dependent-secretion of the pro-inflammatory IL-1β cytokine (Barber, 2011; Sun et al., 2013). The endoplasmic reticulum resident adapter STING functions as a DNA sensor for bacterial cyclic GTP (Burdette and Vance, 2013) and mediates detection of viral DNA (Ishikawa and Barber, 2008; Ishikawa et al., 2009; Zhong et al., 2008). STING can also directly complex with single stranded (ss) and double stranded (ds) cytoplasmic viral DNA to initiate antiviral signalling (Abe et al., 2013).

In the present study, we characterized *de novo* infection of primary human monocytes by HTLV-1 and demonstrated that HTLV-1 infection induces SAMHD1-mediated apoptosis in monocytic cells. We further showed that production of HTLV-1 reverse transcription intermediates (RTI) in presence of SAMHD-1 complexed with the innate immune sensor STING and initiated IRF3-Bax directed apoptosis. These results elucidate for the first time the mechanism of HTLV-1 abortive infection of monocytes and link the reverse transcription restriction factor SAMHD1, the sensing of retroviral RTI by STING, and the initiation of IRF3-Bax-driven apoptosis.

3. RESULTS

i. Abortive infection of primary monocytes by HTLV-1 activates type I IFN response.

To characterize the impact of HTLV-1 infection on the activation of the host antiviral response in primary human monocytes, we first assessed the percentage of HTLV-1 infected monocytes at 3h post-infection (pi), using increasing concentrations of purified HTLV-1 (0-5 μg/mL). Virus binding to monocytes was dependent on the quantity of HTLV-1, as illustrated by surface staining with anti-Env (gp46) monoclonal antibodies (**Figure 1A**); for subsequent experiments, an HTLV-1 concentration of 2μg/ml was used, since >90% of the cells displayed virus binding (**Figure 1A**) (91.7 ± 4.8% $gp46^+$cells; $P > 0.05$; n = 5). HTLV-1 particles were efficiently internalized by monocytes, as demonstrated by the detection of intracellular HTLV-1 viral RNA (vRNA) starting at 3h pi, with the viral load gradually decreasing over the 120h time course (**Figure 1B**). HTLV-1 internalization in monocytes was further demonstrated by intracellular staining (ICS) for the viral matrix protein p19 at 24h pi (**Figure 1C**, blue histograms) (86.7 ± 6.2% of $p19^+$ monocytes; $P < 0.01$ compared to uninfected cells; n = 5). Importantly, viral binding and internalization was eliminated by pre-treating HTLV-1 with anti-gp46 neutralizing mAb or pre-treating monocytes with 0.1% trypsin, as previously reported (Jones et al., 2005) (**Figure 1C**) ($P < 0.01$ compared to infected cells). The absence of intracellular Tax protein and virion release at 24 and 48h pi indicated that monocytes were not productively infected by HTLV-1 (**Figure 1C** and **Figure S1** respectively). Monocyte-derived DC (MDDC) were productively infected with HTLV-1 (40.6 ± 5.7% Tax^+ cells at 24h pi) (data not shown).

Despite the non-productive infection by HTLV-1, monocytes generated a robust innate immune response, as illustrated by the induction of multiple parameters of the antiviral signaling pathway. An early increase in phosphorylated interferon regulatory factor 3 (P-IRF3) was detected at 3h pi (**Figure 1D**), while increased expression of STAT1, phosphorylated STAT1 (P-STAT1), interferon stimulated gene 56 (ISG56) and RIG-I were observed at 24 and 48h pi.

Figure 1

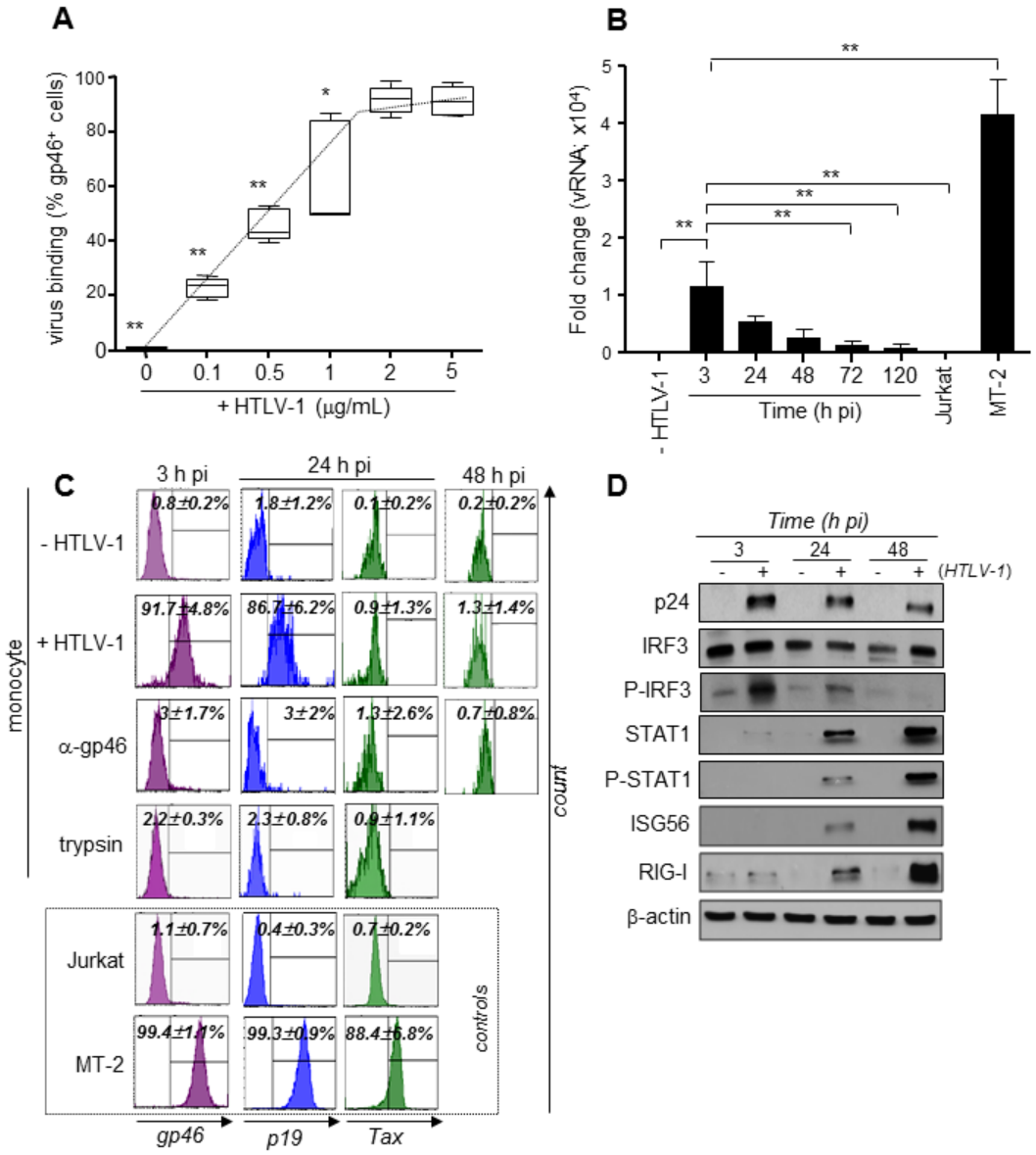


Figure 1. Early type I IFN response in monocytes following abortive HTLV-1 infection.

(A–C) Purified monocytes were infected with HTLV-1 for 3 hr and then cocultured with the autologous $CD14^{neg}$ PBMCs for 120 hr pi. (A) Virus binding to monocytes at 3 hpi in response

to various HTLV-1 concentrations, analyzed by flow cytometry with anti-gp46 Abs. P values were determined based on the comparison with cells infected with 5 μg/ml HTLV-1 (n = 5). (B) At 3–120 hpi, total RNA was extracted and analyzed for HTLV-1 viral load using primers located in the 5′ UTR of HTLV-1 genome (Table S1). Equivalent vRNA amounts were normalized to β-actin mRNA and calculated as fold change from the level of uninfected monocytes (arbitrary set as 1). Jurkat and MT-2 cells were used as negative and positive control, respectively (n = 5). (C) At 3, 24, and 48 hpi, levels of HTLV-1 binding, viral internalization, and de novo production of viral proteins were assessed by gp46 surface staining, p19, and Tax ICS, respectively. Histograms are representative of raw data from five independent experiments. The mean relative expressions ± SD for all conditions are indicated. MT-2 and Jurkat cells were used as positive and negative controls for all staining, respectively. (D) p24 expression and type I IFN responses were examined by immunoblotting (n = 3). See also related Figure S1.

Figure S1

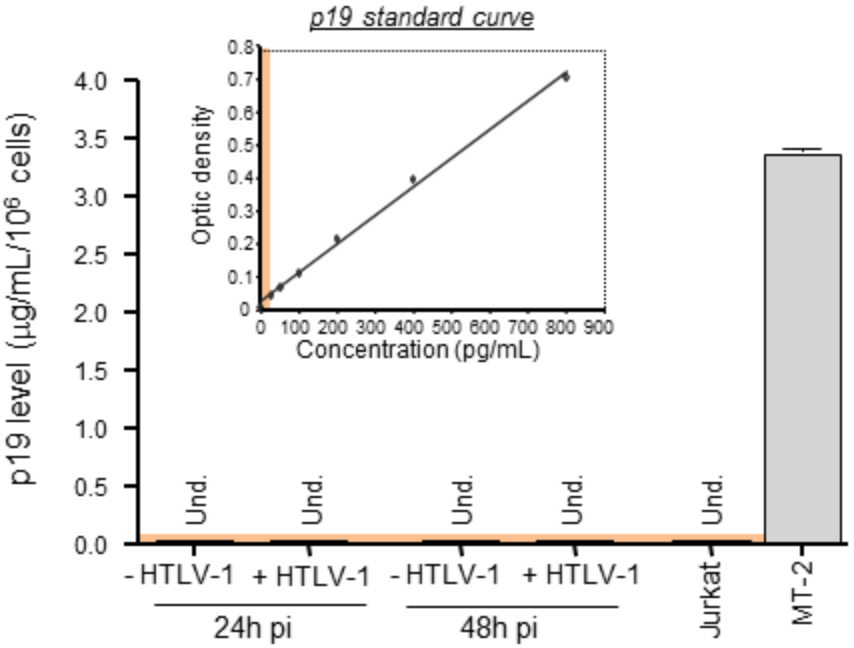
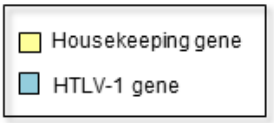


Figure S1. Abortive infection of primary monocytes by HTLV-1.

No *de novo* HTLV-1 release was detected in the supernatants of HTLV-1 infected primary monocytes (n = 5). Supernatants were collected at 24 and 48h pi to assess the status of HTLV-1 virion release using a p19 ELISA. Standard curve with the minimum detectable p19 concentration are shown above. Jurkat and MT-2 cells are used as negative and positive control respectively. Und. = undetectable level. Figure S1 relates to Figure 1.

Table S1



localisation	*Orientation*	Sequence
β- actin	*forward*	ACTGGGACGACATGGAGAAAA
	reverse	GCCACACGCAGCTC
erv-3	*forward*	CATGGGAAGCAAGGGAACTAATG
	reverse	CCCAGCGAGCAATACAGAATTT
alu	*forward*	GCCTCCCAAAGTGCTGGGATTACAG
5´utr	*forward*	TTCGTTTTCTGTTCTGCGCC
	reverse	GCTATAGAATGGGCTGTCGCT
gag	*forward*	CCCTCCAGTTACGATTTCCA
	reverse	GGCTTGGGTTTGGATGAGTA
env	*forward*	CATCCCGGTAAGCGCTAGTT
	reverse	AAAGTGGCGAGAAACTTACCC

Table S1. Primer sequences for HTLV-1 RNA and DNA detection and internal controls

ii. HTLV-1-infected monocytes undergo apoptosis.

We next evaluated the level of apoptosis in HTLV-1-infected $CD3^-CD14^+$ monocytes at 3-120h pi by Annexin-V staining. At 24h pi, infected monocytes displayed higher levels of apoptosis than uninfected cultures (**Figure 2A**) (22.9 ± 3.5% versus 8.6 ± 1.3%, respectively; $P < 0.01$; n = 5), and by 72h the percentage of HTLV-1 infected apoptotic cells increased to over 45%. Infection of monocytes with a low dose of HTLV-1 (0.1 μg/ml) also resulted in the induction of apoptosis in the $p19^+$population (**Figure S2A**). Apoptosis was accompanied by the generation of cleaved caspase-3 ($_{CL}$caspase-3) in infected cells (**Figure 2B** and **Figure S2B**) (33.4 ± 12.3% and 11.4 ± 2%, respectively for HTLV-1-infected and uninfected cells at 48h pi; $P < 0.01$; n = 5); infected cells also exhibited a loss of mitochondrial potential as shown by reduced $DiOC_6(3)$ intensity when compared to uninfected cells (**Figure 2C** and **Figure S2C**) ($P < 0.01$; n = 5). The pan-caspase inhibitor ZVAD or the blocking α-gp46 mAb prevented $_{CL}$caspase-3-mediated apoptosis (**Figure 2B**) and mitochondrial depolarization (**Figure 2C**). Treatment with ZVAD however did not interfere with the internalization of HTLV-1, as detected by measuring intracellular p19 (**Figure 2B**, blue histograms) ($P > 0.05$; n = 5). A strong statistical correlation between (*i*) caspase-3 activation, (*ii*) mitochondrial depolarization and apoptosis was confirmed by the non-parametric Spearman test (**Figure 2D**) ($r = 0.7845$ and 0.9036, respectively for (*i*) and (*ii*); $P < 0.0001$; n = 25). Several mitochondrial pro- and anti- apoptotic molecules were also analyzed; increased Bax expression and caspase-9 cleavage were observed in infected monocytes (**Figure 2E**) ($P < 0.05$ and $P < 0.01$ respectively; n = 3), while expression of Bcl-2 family members - Bim, Noxa, Bcl-2, and Mcl-1 - were unchanged. In the above experiments, we did not observe death by necrosis or an effect of necrostatin-1 on infected monocytes (**Figure S2D**). Cleaved caspase-1 and bioactive IL-1β were detected after HTLV-1 infection, indicating activation of the inflammasome (**Figure S2E**). To investigate caspase-1-associated pyroptosis, HTLV-1-infected cells were treated with Ac-YVAD-cmk (YVAD; the selective inhibitor of caspase-1), and a minor, even significant, improvement of cell survival was observed (**Figure S2F**).

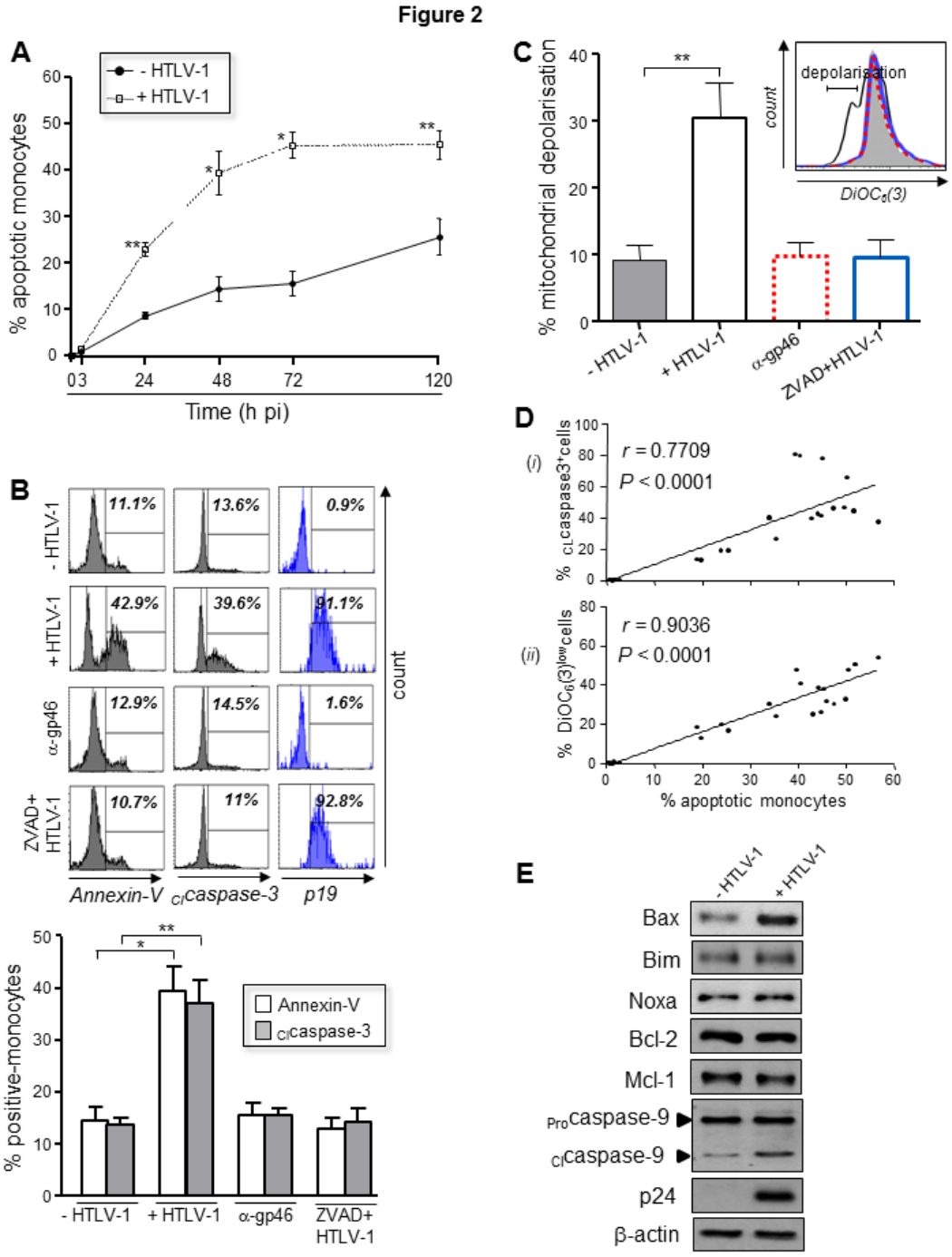


Figure 2. HTLV-1-infected monocytes undergo mitochondrial-dependent apoptosis.

(A) The percentage of apoptosis on gated $CD3^{-}CD14^{+}$monocytes was assessed by Annexin-V staining at 3-120h pi. *P* values were determined by the comparison with uninfected cells (n = 5).

(B) Levels of Annexin-V, $_{CL}$caspase-3 and p19 expression determined at 48h pi on monocytes, treated or not with ZVAD or with α-gp46 mAb. Representative histograms from five independent experiments are shown above. (C) At 48h pi, the loss of mitochondrial membrane potential on monocytes in the presence or absence of ZVAD or α-gp46 mAb was evaluated by flow cytometry using the fluorescent dye $DiOC_6(3)$. Results shown represent the percentage of $CD3^-CD14^+DiOC_6(3)^{low}$monocytes, as indicated above in the representative histograms (n = 5). (D) The correlations between the percentage of apoptotic monocytes and (*i*) $_{CL}$caspase-3 expression, or (*ii*) mitochondrial depolarisation were calculated in infected monocytes during the course of the co-culture (n = 25; Spearman test). (E) Expression levels of p24, Bcl-2 family members and caspase 9 cleavage were assessed by immunoblotting at 3h pi (n = 3). See related Figure S2.

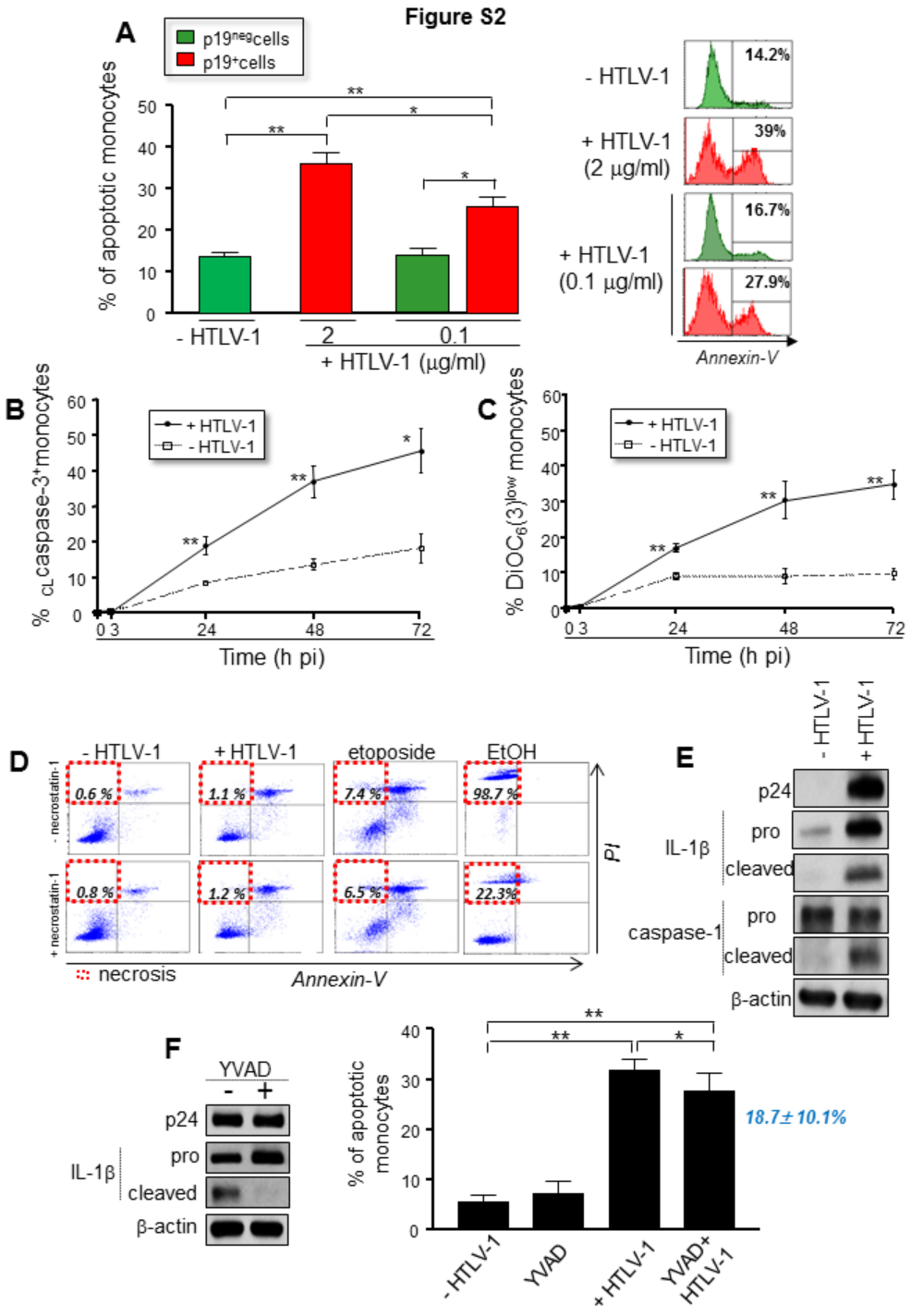


Figure S2. HTLV-1 infection induces apoptosis in infected monocytes.

(A) Monocytes were co-cultured for 48h in the presence of 2 or 0.1 µg/mL (low dose) HTLV-1 (n =5). At 48h pi, cells were stained with CD14-APC H7, p19-APC and Annexin-V-V450.

Results represent the levels of apoptosis in $p19_{neg}$ and $p19^{+}CD14^{+}$monocytes. >98% and 24.1 ± 6.1% of monocytes were $p19^{+}$cells following infection with 2 and 0.1 μg/ml HTLV-1, respectively. Representative flow cytometry histograms are also shown. (B, C) Increase of $_{CL}$caspase-3 expression and mitochondria depolarization following HTLV-1 infection. The levels of $_{CL}$caspase-3 expression (B) and mitochondria potential loss using $DiOC_6(3)$ dye (C) were determined in $CD14^{+}$monocytes at 3-72h pi using flow cytometry (n = 5). (D) Annexin-V/PI (propidium iodide) co-staining in infected $CD14^{+}$monocytes treated or not with 20nM necrostatin-1 (Sigma) at 48h pi. Levels of necrosis were determined by the percentage of PI^{+}Annexin-V_{neg}cells (red square). Treatment with etoposide and EtOH were used as positive controls for apoptosis and necrosis, respectively (n = 5). Treatment with necrostatin-1 did not impact Annexin-V/PI co-staining in monocytes, except for cells treated with EtOH. Data are represented as mean +/- SD. (E) Expression of p24, IL-1β, caspase 1 and β-actin were determined by immunoblotting in monocytes at 3h pi unless otherwise indicated (n = 3). Identical results were found for β-actin and p24 at 30 minutes pi. (F) Levels of Annexin-V expression determined at 48h pi on monocytes, treated or not with 100μM Ac-YVAD-cmk (YVAD). Results are expressed as mean ± SD for five independent experiments; included in blue is the % inhibition of apoptosis in the presence of YVAD treatment. YVAD activity was also confirmed in HTLV-1-infected monocytes at 3h pi, by its ability to prevent caspase-1activation of the pro-inflammatory IL-1β (n = 3). Data in all panels are represented as mean +/- SD. Figure S2 relates to Figure 2.

iii. A requirement for SAMHD1 in HTLV-1-driven apoptosis in infected monocytes.

The above observations, detailing abortive HTLV-1 infection of monocytes are reminiscent of the functional consequences of host restriction by SAMHD1 during HIV-1 infection (Baldauf et al., 2012; Descours et al., 2012; Doitsh et al., 2010; Laguette et al., 2011), prompting us to investigate whether SAMHD1 functioned similarly to restrict HTLV-1 infection. Small interfering RNA (siRNA) mediated silencing of SAMHD1 in primary monocytes resulted in a 72 ± 11.8% reduction in protein expression (**Figure 3A**). SAMHD1 silencing did not alter HTLV-1 internalization in monocytes 48h pi (95.5 ± 1% and 94.2 ± 3.1% [% of $p19^{+}$monocytes]; 10434.3 ± 5455.7 and 11634 ± 4381.6 [mean fluorescence intensity], respectively for control and SAMHD1-specific siRNA-transfected monocytes) (**Figure 3B**, **right histograms**). Following HTLV-1 infection, induced apoptosis was also reduced by ~70% in SAMHD1-silenced monocytes compared to control siRNA-treated monocytes (**Figure 3B**) ($P < 0.01$; n = 5). The correlation between inhibition of SAMHD1 expression and inhibition of HTLV-1-induced apoptosis in primary monocytes was significant (**Figure 3C**) ($r = 1$; $P = 0.0167$; n = 5, Spearman test).

The deoxynucleoside triphosphate triphosphohydrolase function of SAMHD1 has been shown to block reverse transcription of retroviral DNA by depleting the dNTP pool required for complete reverse transcription (Ayinde et al., 2012; Goldstone et al., 2011; Hollenbaugh et al., 2013; Kim et al., 2012; Lahouassa et al., 2012), an effect that is reversed by the addition of exogenous dN (Lahouassa et al., 2012). To explore the relationship between the triphosphohydrolase activity of SAMHD1 and induced apoptosis, primary monocytes were infected with HTLV-1 in the presence of increasing concentrations of exogenous dN (0-100nM). SAMHD1-mediated apoptosis was inhibited by the addition of exogenous dN in a dose-dependent manner (**Figure 3D**), with apoptosis reduced to control levels in monocytes treated with 100nM dN (> 80% inhibition of apoptosis). We did not observe *de novo* Tax expression following SAMHD1 knockdown or dN treatment in infected monocytes at 48h pi (n = 3; data not shown). These results demonstrate that nucleotide pool depletion, mediated by SAMHD1 function, correlates directly with HTLV-1-induced apoptosis.

Figure 3

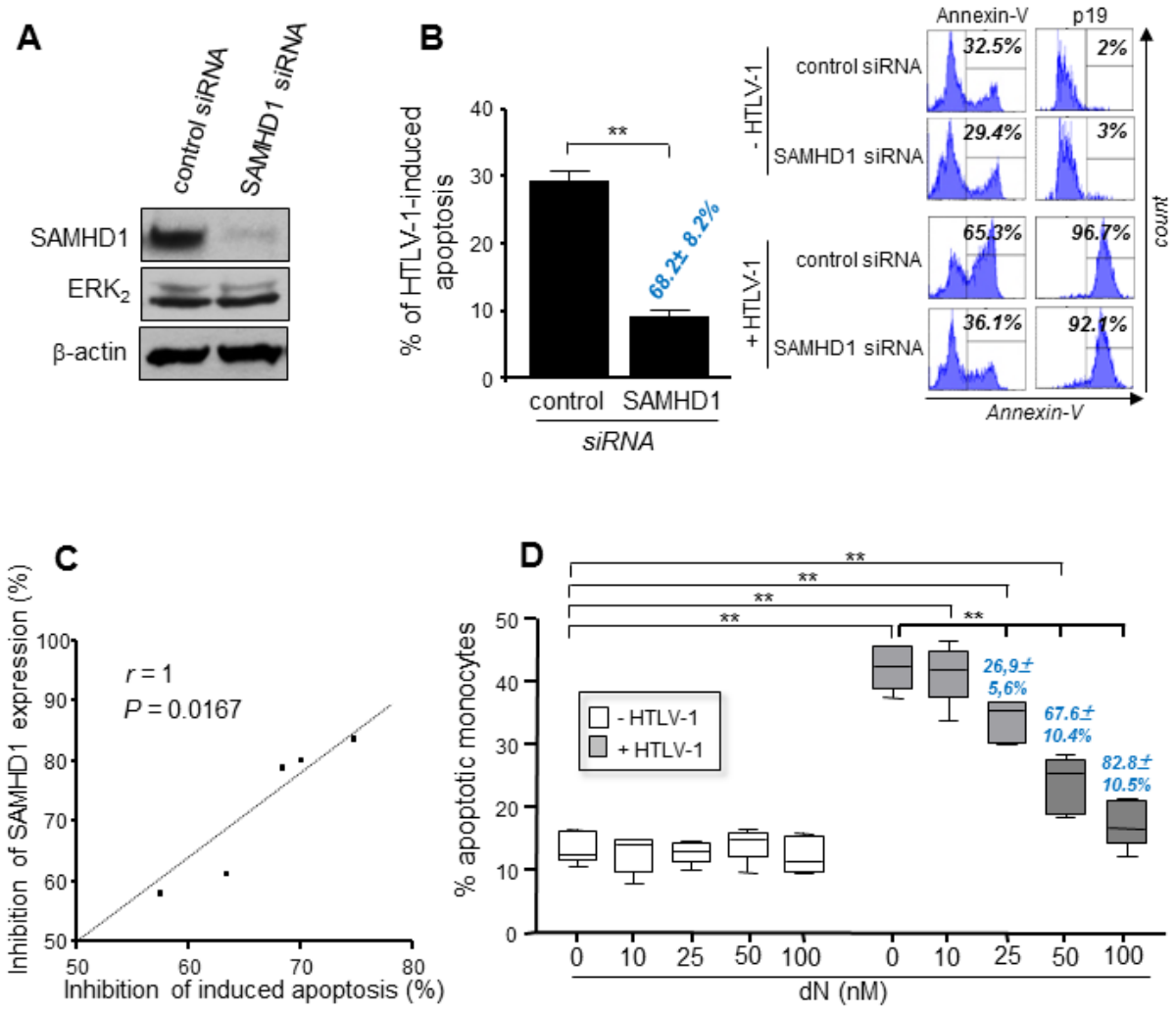


Figure 3. SAMHD1 drives apoptosis in HTLV-1-infected monocytes.

(A-C) Monocytes were transfected with control or SAMHD1 siRNA for 3d, and then subsequently infected with HTLV-1 for 48h. (A) Efficiency of SAMHD1 silencing was determined at day 3 by immunoblotting. (n = 5). No changes were observed in the levels of total ERK_2 expression, confirming the specificity of SAMHD1 silencing. (B) Levels of apoptosis and intracellular p19 on transfected $CD14^+$monocytes at 48h pi. Results represent the HTLV-1-induced apoptosis determined on transfected monocytes using the formula: % of apoptosis in

infected cells - % of apoptosis in uninfected cells. Data are expressed as mean ± SD for five independent experiments, including the inhibition of apoptosis (%) when SAMHD1 was silenced (indicated in blue). The flow histograms shown on the right side are representative of raw data. (C) Correlation between the inhibition of SAMHD1 expression and HTLV-1-induced apoptosis determined on transfected monocytes (n = 5 [values from Figs. 3A, B]; Spearman test). (D) Levels of apoptosis determined on monocytes in the presence of increasing doses of exogenous dN at 48h pi. Percentage of apoptosis inhibition determined on infected cells in the presence of dN are also indicated in blue. Statistical analyses are based on the comparison to untouched monocytes, except for those indicated in bold, that are calculated when compared to HTLV-1-infected, non-dN-treated monocytes (n = 5).

iv. HTLV-1-induced apoptosis correlates with the generation of cytosolic RTI.

Previous studies in the context of HIV-1 infection demonstrated that incomplete cytoplasmic DNA triggered a pro-apoptotic response in infected bystander $CD4^+$ T-cells (Doitsh et al., 2010). To assess the presence of reverse transcription intermediates (RTI) in HTLV-1-infected monocytes, viral DNA were measured in total versus nuclear fractions by qPCR at 3-120h pi; HTLV-1 RTI were detected only in the total fraction (**Figure 4A** and **Figures S3A and S3B**), indicating that viral DNA synthesis was initiated in the cytosol, but was not fully reverse transcribed and did not reach the nucleus. As previously reported, the presence of nuclear retroviral DNA implies the completion of reverse transcription and the formation of the pre-integration complex (Nisole and Saib, 2004). Silencing SAMHD1 expression or treatment with exogenous dN led to the detection of HTLV-1 DNA in the nucleus, as well as integrated provirus, in infected monocytes (**Figure 4B** and **Figure S3C**).

Several primer pairs were designed to monitor sequential steps in HTLV-1 reverse transcription, including the initial generation of strong-stop DNA (*5′utr*), and DNA elongation (*env* or *gag*) (**Figure 4C, upper schematic** and **Table S1**) (Doitsh et al., 2010). Primary monocytes were treated with 5μM azidothymidine (AZT), a nucleoside analog reverse transcriptase inhibitor that prevents elongation of reverse transcribed DNA after initiation (Doitsh et al., 2010) and then infected with HTLV-1. AZT treatment had no inhibitory effect on the initiation of HTLV-1 reverse transcription (**Figure 4C**), as detected with the *5'utr* primers (green), whereas DNA strand elongation was progressively inhibited, as detected using *env* (blue) and *gag* (orange) region specific probes (**Figure 4C**) (35.5 ± 31.3% and 65.5 ± 22.7% inhibition respectively; $P < 0.01$; n = 5). Following HTLV-1 infection, apoptosis in monocytes treated with AZT was reduced by >40% compared to infected cells without AZT (**Figure 4D**) (41.8 ± 12.3%; $P < 0.05$; n = 5). These results demonstrate that AZT blocked the generation of cytosolic RTI by interfering with HTLV-1 DNA elongation and suggested that cytosolic RTI generation was linked with virus-mediated apoptosis. These primers were also used in the context of SAMHD1 silencing, to investigate the production of early versus late stage RTI. SAMHD1 knockdown led to a significant decrease in the generation of total and early transcripts (green [*5′utr*]; 1.45 ± 0.41 fold decrease), concomitantly with an accumulation of late reverse transcription products (orange [*gag*]; 1.39 ± 0.27 fold increase) (**Figure 4E**).

To determine if HTLV-1 replicative intermediates directly mediated apoptosis, synthetic single (ss) or double stranded (ds) 90 nucleotide HTLV-1 DNA (vDNA) - conjugated with biotin - were introduced into primary monocytes. Monocytes harboring HTLV-1 ss or $dsDNA_{90}$ ($vDNA^{+}$cells; green histograms) underwent apoptosis (59.2 ± 3.5% and 48.9 ± 6.9% respectively), whereas vDNA-free monocytes from the same samples did not undergo apoptosis (purple histograms; ~30% apoptosis in cells electroporated only, or transfected with digested $ssDNA_{90}$ (data not shown) (**Figure 4E**). The transfection of monocytes with scrambled RTI (whose sequence was generated by randomizing the U5 sequence) did not result in the induction of apoptosis, confirming the specificity of HTLV-1 RTI to induce apoptosis (**Figure 4F**). Overall, these results provide evidence for the direct contribution of cytosolic RTI during HTLV-1-induced apoptosis.

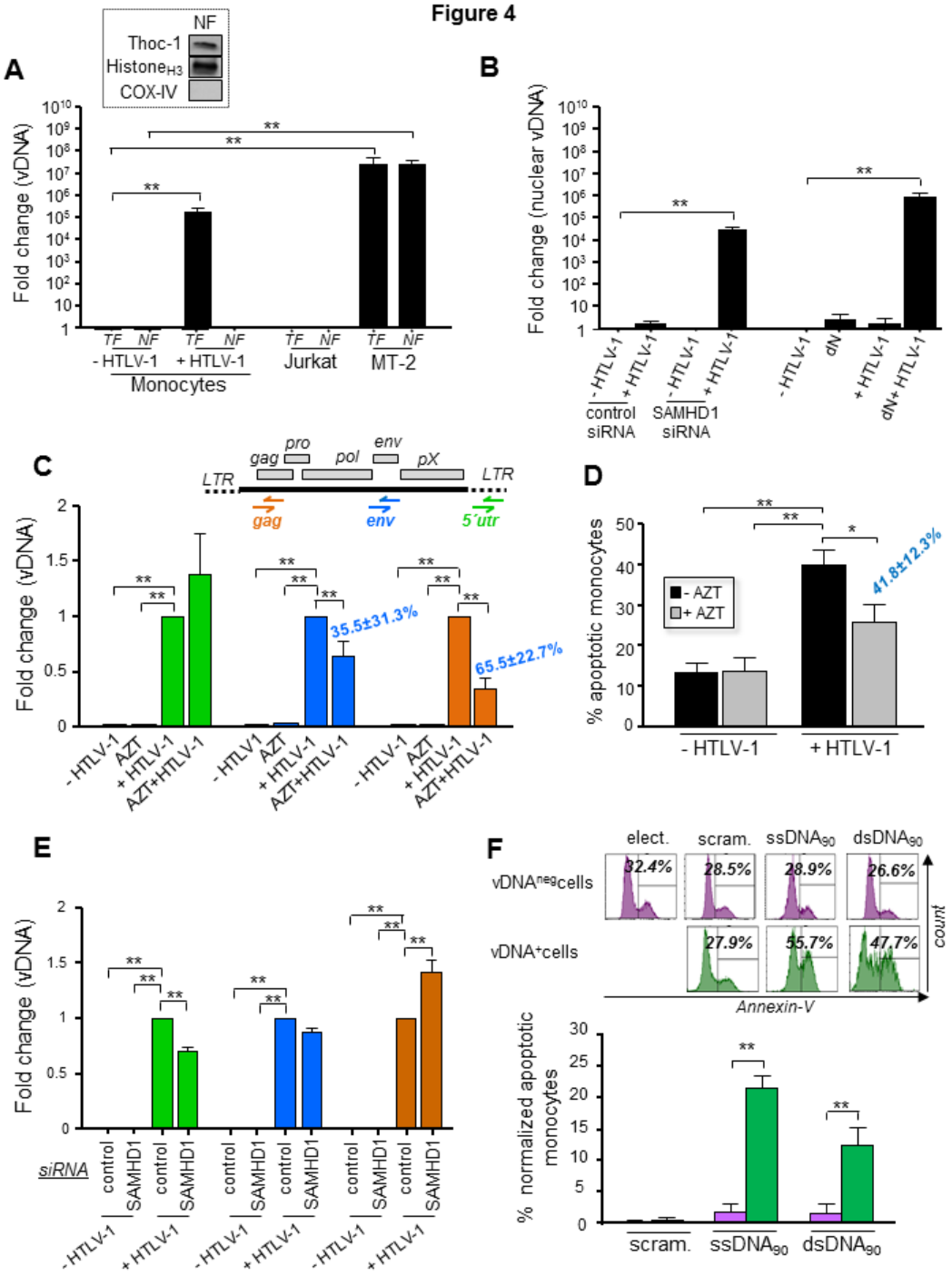


Figure 4. **Cytosolic RTI induces monocyte apoptosis following HTLV-1 infection**.

(A) At 24h pi, DNA was extracted from total (TF) and nuclear (NF) fractions in monocytes. Viral loads were determined by qPCR using 5´utr primers and normalized to erv-3. Results

shown represent the relative fold change and statistical analyses, compared to uninfected monocytes (n = 5). Purity of NF was determined by immunoblotting using antibodies against nuclear (Thoc-1 and $Histone_{H3}$) and cytosolic (COX-IV) proteins. (B) Monocytes were transfected with SAMHD1 siRNA, treated with exogenous 100nM dN, and then treated as in (A). Results represent relative fold change compared to uninfected cells treated with control siRNA (n = 5). (C) Fold change of vDNA determined in monocytes at 48h pi using several pairs of primers and relative to infected monocytes without AZT. (D) Levels of apoptosis on monocytes in the presence or absence of AZT at 48h pi. Inhibition of apoptosis mediated by AZT is indicated in blue. (E) Fold change of vDNA performed in monocytes expressing or not SAMHD1 at 24h pi using several primer pairs. (F) The addition of HTLV-1 RTI in monocytes initiates apoptosis. Purified monocytes were transfected with scrambled, with HTLV-1 ss or $dsDNA_{90}$. At 48h post-transfection, cells were labeled with Annexin-V-V450, CD14-APC H7, and streptavidin-APC. Streptavidin-APC ICS allowed the distinction between monocytes that were $vDNA^{neg}$ (purple) or $vDNA^{+}$cells (green) in the same sample. Elect. = electroporated only; scram. = scrambled $ssDNA_{90}$. Values represent the percentages of normalized apoptosis indicated by the formula: % of apoptosis in RTI-transfected monocytes with siRNA - % of apoptosis in electroporated-only cells. Apoptosis mediated on primary monocytes by the electroporation methods averaged between ~15-19%. Flow histograms shown above are representative of raw data from five independent experiments. See related Figure S3.

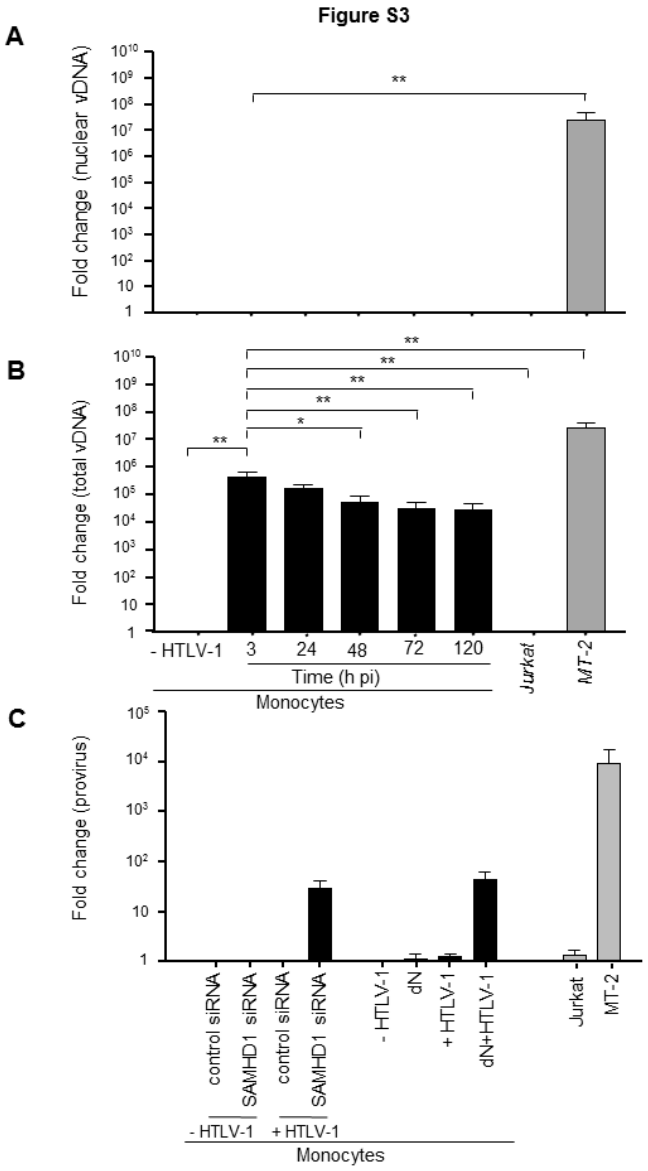


Figure S3. Quantification of total versus nuclear integrated vDNA after SAMHD1 silencing.

Quantitation of HTLV-1 vDNA on (A) nuclear versus (B) total fractions from monocytes at 3-120h pi. Jurkat and MT-2 cells were included as a negative and positive controls respectively (n = 5). (C) Quantitation of integrated HTLV-1 provirus in monocytes in the presence of SAMHD1 silencing and dN treatment. Results are depicted as fold change in proviral HTLV-1 DNA in three independent experiments. Data in all panels are represented as mean +/- SD. Figure S3 relates to Figure 4.

v. HTLV-1 RTI signals via STING to induce apoptosis and the IFN response.

The endoplasmic reticulum resident transmembrane sensor STING (stimulator of interferon genes) is central to the generation of an innate immune response, through its capacity to directly bind viral and bacterial DNA (Abe et al., 2013; Burdette and Vance, 2013; Ishikawa and Barber, 2008; Ishikawa et al., 2009). Given the importance of STING in initiating cytoplasmic DNA signaling events, we next examined whether STING could recognize and complex with HTLV-1 RTI. Using streptavidin beads to pull-down biotinylated HTLV-1 DNA, we demonstrated that STING was precipitated from primary monocytes via interactions with ss or $dsDNA_{90}$ (**Figure 5A).** Furthermore, the formation of the complex between STING and HTLV-1 DNA was associated with the induction of type I IFN signalling and apoptosis, as illustrated by increased expression of RIG-I, ISG56 and Bax (**Figure 5A**) ($P < 0.01$; n = 3). Transfection of monocytes with scrambled RTI resulted in STING pull-down and the induction of ISGs. However, no increase in Bax expression was observed in these cells. No complex formation was observed between HTLV-1 RTI and the RNA sensor RIG-I (**Figure 5A**).

To determine whether cytosolic detection of HTLV-1 RTI by STING was involved in triggering apoptosis, STING-specific siRNA was introduced into primary monocytes, resulting in ~65% inhibition of STING expression (**Figure 5B**) ($P < 0.01$; n = 5). Knockdown of STING in primary monocytes resulted in a significant improvement in cell survival. In multiple experiments, a 55-60% inhibition of apoptosis was observed (**Figure 5C**) ($P < 0.01$ compared to control siRNA-treated cells; n = 5), whereas silencing of RIG-I had no effect on HTLV-1-induced apoptosis (**Figure S4**). A positive correlation between the inhibition of STING expression and HTLV-1-induced apoptosis was established using the non-parametric Spearman test (**Figure 5D**) ($r = 1$; $P = 0.0167$; n = 5). Altogether, these results indicate that HTLV-1 RTI are generated in primary monocytes in a SAMHD1-dependent manner, and then complex with the DNA sensor STING to initiate apoptosis.

Figure 5

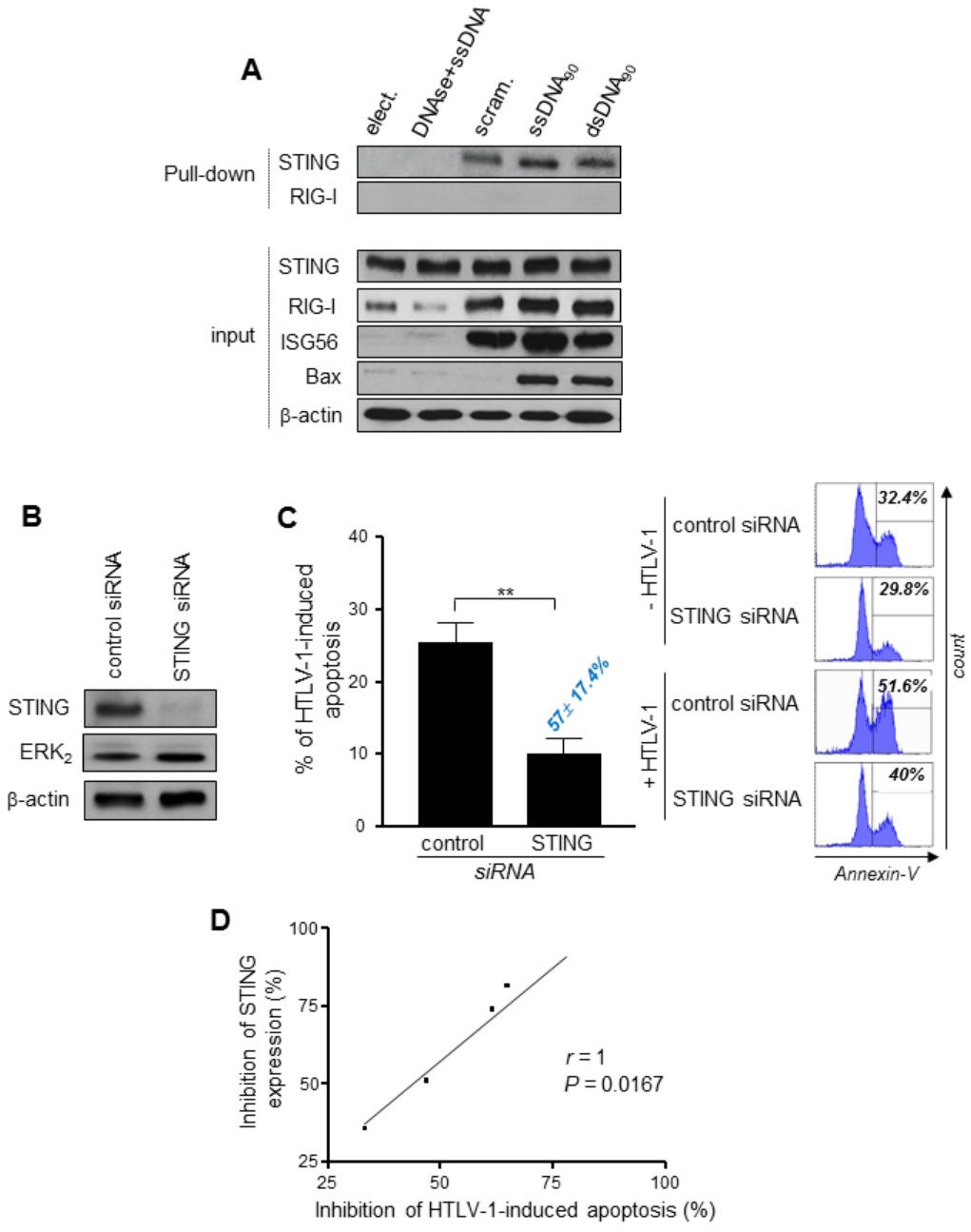


Figure 5. STING complex formation with HTLV-1 DNA induces apoptosis in monocytes.

(A) Monocytes were transfected with scrambled and HTLV-1 ss, or $dsDNA_{90}$ for 6h. As a negative control, 1 µg of HTLV-1 ssDNA was incubated with 1µl DNAse (Ambion; TX, USA)

for 1h at 37ºC. STING and RIG-I pull-down was performed from monocyte lysates via interactions with biotinylated DNA (*Methods* section). Input lysates were also analyzed by immunoblot for expression of several proteins including STING, Bax, β-actin and ISG (n = 3). (B-D) STING regulates the premature death of infected monocytes (n = 5). Purified monocytes were transfected with STING siRNA for 3 days and (B) the efficiency of STING silencing was determined via immunoblotting. (C) Annexin-V staining determined on transfected $CD14^{+}$monocytes at 48h pi. Values represent the % HTLV-1 induced apoptosis as determined by the formula: % apoptosis in infected cells - % apoptosis in uninfected cells. Inhibition of HTLV-1-mediated apoptosis following STING silencing is indicated in blue. Flow histograms shown on the right side are representative of raw data from five independent experiments. (D) Correlation between inhibition of STING expression and HTLV-1-mediated apoptosis on transfected monocytes (n = 5; Spearman test). See related Figure S4.

Figure S4

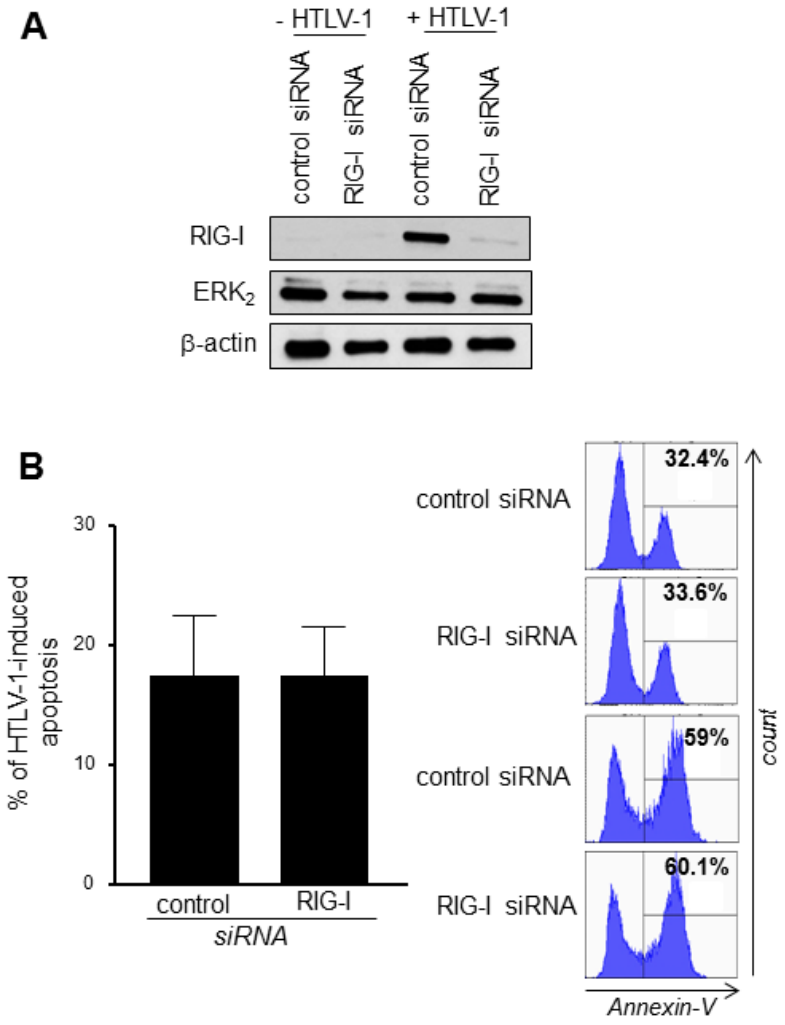


Figure S4. RIG-I silencing does not influence apoptosis in HTLV-1-infected monocytes.

(A, B) Monocytes were transfected for 3h with control or RIG-I-specific siRNA prior to HTLV-1 infection. Levels of apoptosis on transfected $CD14^{+}$monocytes were then investigated at 48h pi using Annexin-V staining. (A) The efficiency of RIG-I silencing using siRNA was determined by immunoblotting. (B) Results represent the percentage of HTLV-1-induced apoptosis on transfected monocytes and are expressed as mean ± SD for three independent experiments. Representative flow histograms are also shown above. Figure S4 relates to Figure 5.

vi. HTLV-1 RTI triggers mitochondrial apoptosis through IRF3-Bax signalling.

HTLV-1 infection of monocytes stimulated IRF3 activation and induction of pro-apoptotic Bax (**Figures 1D** and **2E**), suggesting that HTLV-1 may be triggering apoptosis via a mechanism involving the formation of a pro-apoptotic complex between IRF3 and Bax (Chattopadhyay et al., 2010; Chattopadhyay et al., 2011). To determine whether STING recognition of HTLV-1 RTI could trigger apoptosis through IRF3-Bax signalling, we measured Bax and ISG expression, as well as IRF3 phosphorylation in infected monocytes knocked down for STING expression. By immunoblot analysis, all parameters - Bax, P-IRF3, RIG-I and ISG56 expression - were respectively inhibited from 95.1 ± 8.5%, 72.1 ± 13.3%, 66.2 ± 48% and 64.5 ± 29.3% by STING knockdown ($P < 0.01$ compared to infected cells treated with control siRNA; n = 3) (**Figure 6A**), thus supporting a role for STING in regulating both the antiviral and apoptotic responses. Similarly, we observed a 73 ± 15.8% inhibition of Bax expression in infected monocytes when STING was silenced using a flow cytometry approach (**Figure S5**). As with *de novo* HTLV-1 infection, the addition of cytosolic ss or $dsDNA_{90}$ led to increased Bax expression (**Figure 6B**, **input blots**) ($P < 0.05$; n = 3). Both HTLV-1 infection and the addition of biotinylated ss or $dsDNA_{90}$ resulted in the generation of a complex between IRF3 and Bax (**Figure 6B**), whereas no IRF3-Bax complex was observed in monocytes transfected with scrambled RTI. The formation of this pro-apoptotic complex required both SAMHD1 and STING expression, since RNAi-mediated silencing of either target not only decreased Bax expression, but also abrogated physical association between IRF3 and Bax (**Figure 6C**).

Next, a multi-parametric antibody cocktail was generated to measure the levels of Annexin-V, Bax and $DiOC_6(3)$ co-staining on gated $CD14^+$monocytes. Infected monocytes expressing Bax (red subset) displayed higher levels of mitochondria depolarization and Annexin-V co-staining, compared to the green Bax^{neg} cells (**Figure 6D**) (74.3 ± 24.8% and 34.3 ± 5.9% $DiOC_6(3)^{low}$ Annexin-V^+ cells, respectively; $P < 0.01$; n = 5). Furthermore, siRNA-mediated silencing of Bax expression reduced the level of apoptosis by ~60%, thus confirming the involvement of Bax in HTLV-1 driven monocyte cell killing (**Figure 6E**).

Figure 6

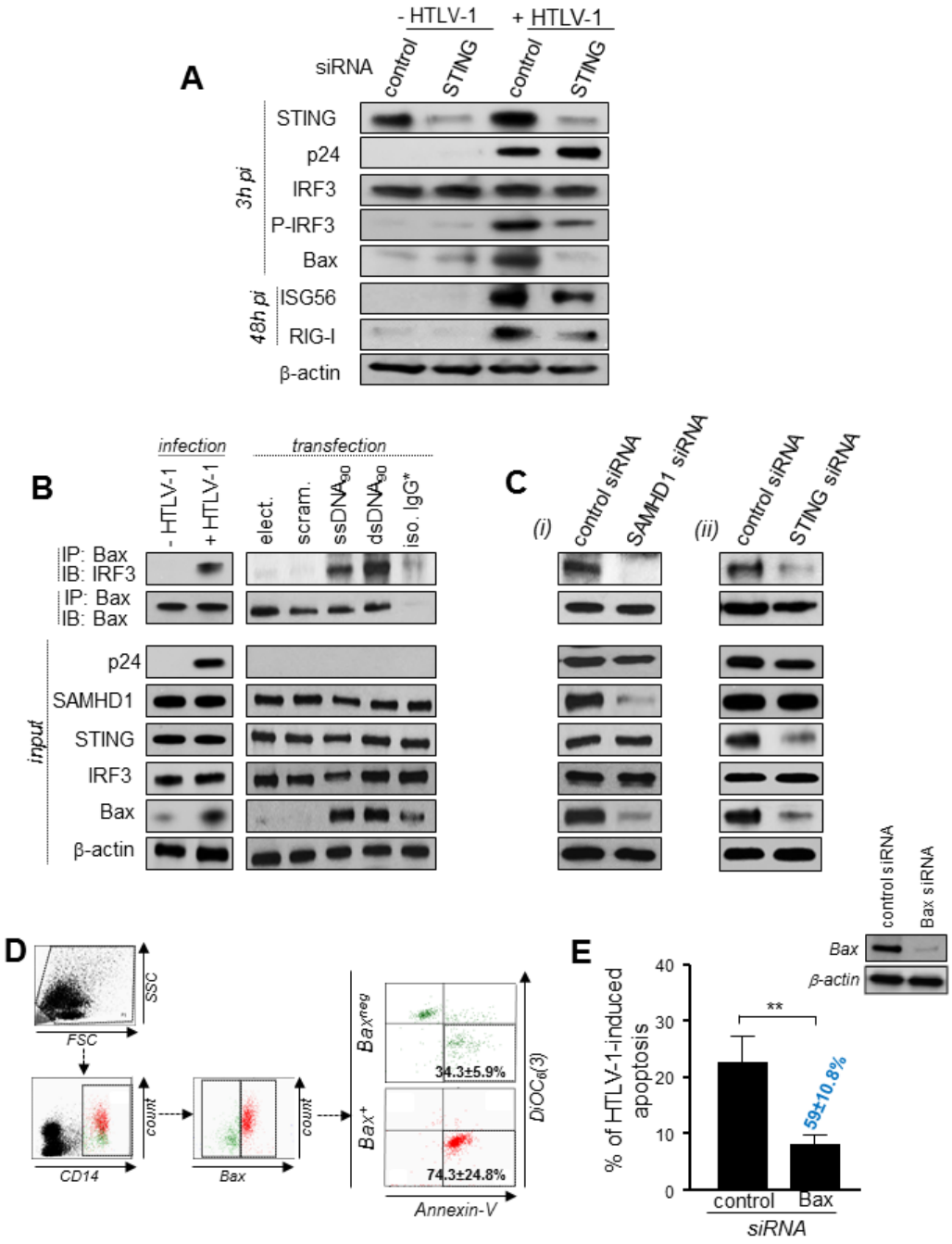


Figure 6. STING activation leads to the formation of the IRF3-Bax complex.

(A) Monocytes were transfected with STING siRNA. Expression of several viral and host and proteins (p24, STING, Bax, ISG and p-IRF3) were then determined by immunoblotting at 3 and

48h pi. Representative blots of three independent experiments are shown. (B, C) Anti-Bax co-immunoprecipitations (co-IP) were performed at 6h pi (B) following *in vitro* infection or transfection with HTLV-1 DNA, (C) or in the context of (*i*) SAMHD1 and (*ii*) STING silencing. Inputs were also analyzed for the expression of several proteins. * = cross-linked isotype IgG_1 antibody used during co-IP as a negative control; performed on monocytes transfected with $ssDNA_{90}$. (D) At 24h pi, a multi-parametric antibody cocktail with $DiOC_6(3)$ dye, Annexin-V-V450, anti-CD14-APC H7, and anti-Bax-APC antibodies was used on co-cultured monocytes (n = 5). Anti-Bax antibody was conjugated to Alexa647 dye using the Zenon^R mouse IgG_1 labeling kit. Isotype IgG_1 control was used to determine the positivity of Bax expression. Results shown represent flow cytometry gating strategy based on HTLV-1-infected monocytes transfected with control siRNA. Bax^{neg} (green) versus Bax^+monocytes (red) were analyzed for mitochondrial depolarisation and apoptosis. Percentages of $DiOC_6(3)^{low}$Annexin-V^+cells were indicated for both subsets. Data are represented as mean +/- SD. (E) Monocytes were transfected with Bax siRNA for 3h prior to HTLV-1 infection. Levels of HTLV-1-induced apoptosis were then investigated at 48h pi. Results are expressed as mean ± SD for three independent experiments, included in blue is the inhibition of apoptosis during Bax silencing. Bax knockdown was confirmed by immunoblotting 48h pi. Data are represented as mean +/- SD. See related Figure S5.

Figure S5

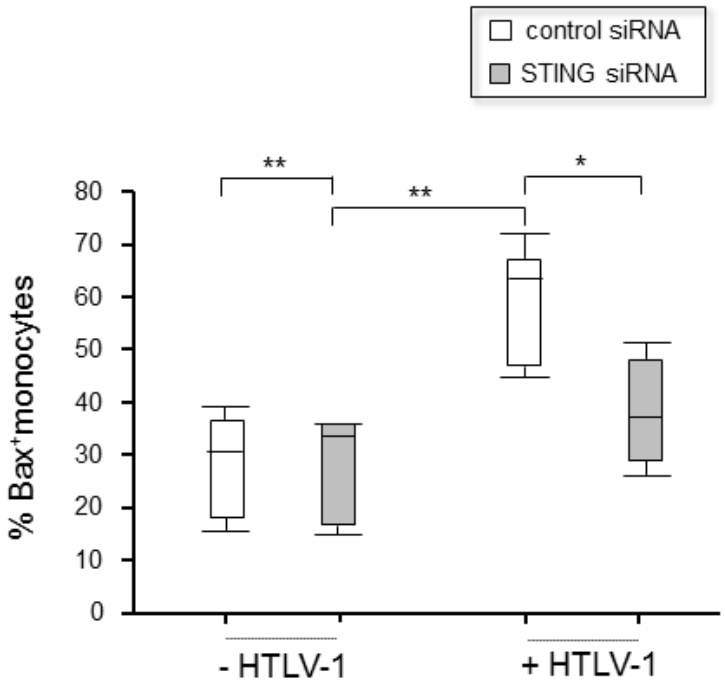


Figure S5. STING silencing decreases Bax expression in infected monocytes.

Monocytes were transfected with STING siRNA for 3 days and co-cultured in the presence of HTLV-1. At 24h pi, anti-Bax-APC antibodies were used to measure Bax expression using flow cytometry (n = 5). Results are expressed as the mean ± SD of $CD14^{+}Bax^{+}$monocytes. Figure S5 relates to Figure 6.

vii. HIV-1 DNA recognition by STING induces a pro-apoptotic response.

Finally, we next examined whether STING-mediated recognition of RTI corresponding to strong-stop HIV-1 DNA also led to monocyte depletion through IRF3-Bax driven apoptosis. HIV-1 $ssDNA_{90}$ RTI was introduced into primary monocytes for 3h; IRF3 phosphorylation and Bax expression were increased ~7-10 fold with the HIV-1 RTI (**Figure 7A**) ($P = 0.0053$ and $P = 0.0115$ respectively, compared to RTI-free monocytes; n = 3). Importantly, using streptavidin beads to precipitate biotinylated HIV-1 $ssDNA_{90}$, STING, but not RIG-I, was also readily pulled-down in association with HIV RTI (**Figure 7B**). As with HTLV-1, the addition of biotinylated HIV-1 $ssDNA_{90}$ resulted in the generation of a heterodimeric IRF3-Bax complex (**Figure 7C**). Furthermore, monocytes transfected 48h with HIV-1 RTI also displayed significantly higher levels of mitochondria-dependent apoptosis (**Figure 7D**). Overall, these data demonstrate that retroviral RTI recognition by STING induces mitochondria-dependent apoptosis via the formation of the pro-apoptotic IRF3-Bax heterodimer (**Figure S6**).

Figure 7

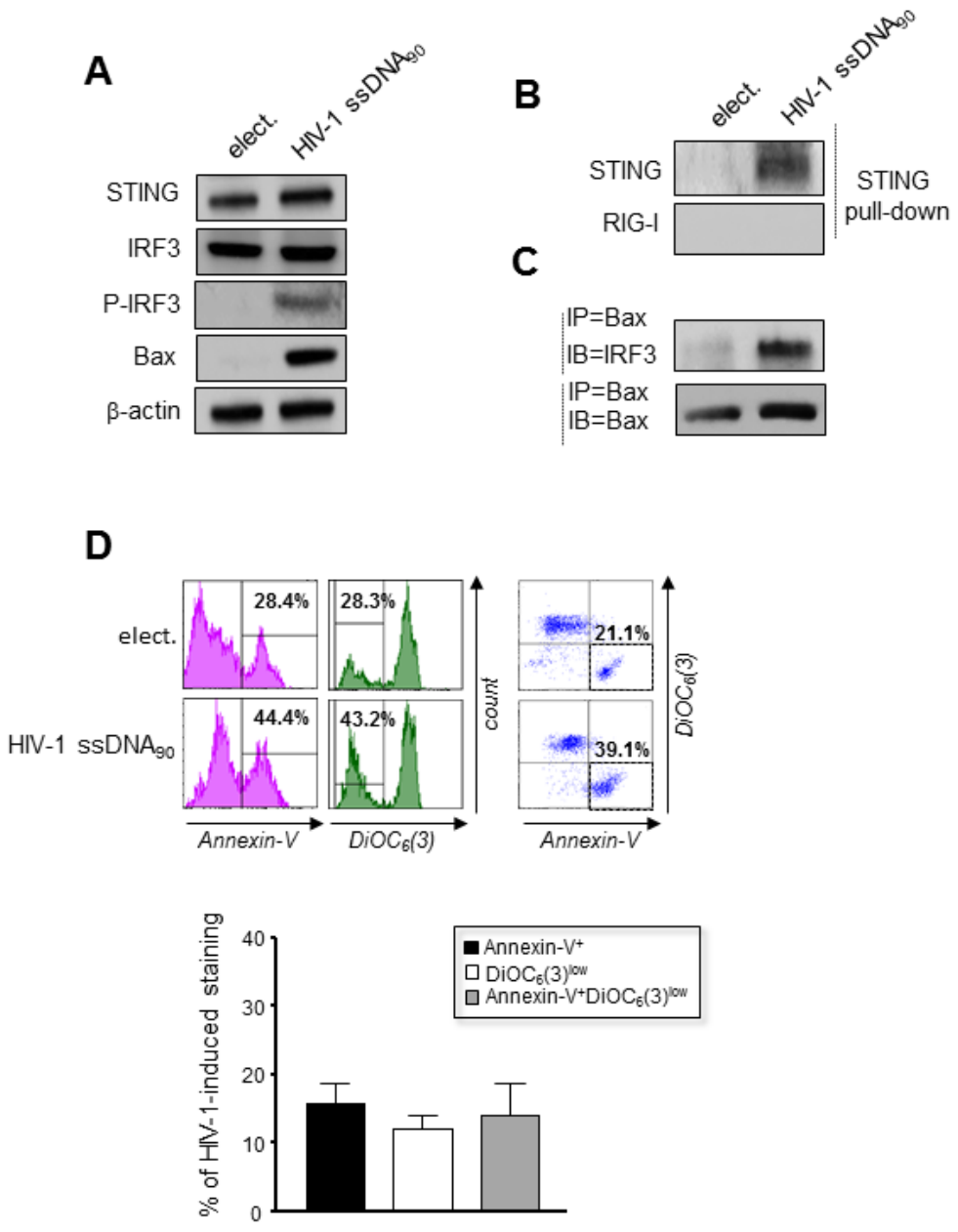


Figure 7. STING recognition of HIV-1 RTI leads to IRF3-Bax interaction and mitochondrial-dependent apoptosis.

(A) Expression levels of STING, IRF3, P-IRF3 and Bax were assessed by immunoblotting on monocytes transfected with HIV-1 ssDNA$_{90}$ for 3h. (B and C) STING pull-down (B) and anti-Bax co-IP (C) were performed on monocytes transfected with HIV-1 ssDNA$_{90}$ (n = 3). (D)

Monocytes treated with HIV-1 $ssDNA_{90}$ displayed higher levels of mitochondria-dependent apoptosis. At 48h post-transfection, levels of Annexin-V, mitochondria depolarisation (% of $DiOC_6(3)^{low}$cells) and co-staining were assessed on gated $CD14^+$monocytes in the presence of HIV-1 RTI. Results show the percentages of HIV-1-induced apoptosis and Bax expression, determined by the formula; % of staining in HIV-1 RTI-transfected monocytes - % of staining in monocytes electroporated alone. Flow histograms shown on the right side are representative of raw data from five independent experiments. See related Figure S6.

4. DISCUSSION

In this study, we demonstrate that *de novo* HTLV-1 infection initiates an abortive infection of primary monocytes and triggers mitochondrial depolarization and caspase-3 dependent cell death. HTLV-1 infection also stimulated induction of a type I IFN response, mediated through IRF3 activation and triggering of Jak-STAT1 signaling. HTLV-1-induced apoptosis in monocytes was dependent on the triphosphohydrolase activity of SAMHD1, since silencing its expression, as well as the introduction of exogenous dN in HTLV-1-infected monocytes significantly reduced apoptosis. HTLV-1 reverse transcription intermediates (RTI) were detected exclusively in the cytoplasm of infected monocytes from 3-120h post-infection, and it was only after inhibition of SAMHD1 expression that HTLV-1 DNA was detected in the nucleus compartment. The continued presence of RTI in the cytoplasm was sensed by the ER resident DNA sensor STING. Because of the involvement of STING in activation of the type I IFN response, we examined the fate of IRF3 and detected the formation of an IRF3-Bax complex, which was responsible for the mitochondrial depolarization and apoptosis. These experiments provide a mechanistic explanation for abortive retroviral infection of monocytes and establish a link between SAMHD1 restriction, sensing of retroviral RTI by STING, and the initiation of IRF3-Bax induced apoptosis. The scope of these observations was further extended by the demonstration that HIV-1 RTI also complexed with STING and triggered apoptosis in the same manner (summarized in **Figure S6**).

The analogy between the current results and restriction of HIV-1 by SAMHD1 is striking, and a number of recent studies have detailed the capacity of SAMHD1 to limit HIV-1 replication in quiescent $CD4^+$ T-cells and myeloid cells (Baldauf et al., 2012; Descours et al., 2012; Laguette and Benkirane, 2012; Laguette et al., 2011). Prior to the characterization of the role of SAMHD1, Doitsh *et al.* had demonstrated that abortive HIV-1 reverse transcription in resting tonsil $CD4^+$ T cells resulted in apoptotic cell death, triggered by the accumulation of incomplete HIV-1 reverse transcripts (Doitsh et al., 2010). It has since become clear that SAMHD1 is responsible for restricting HIV-1 reverse transcription through its dNTP triphosphohydrolase activity, which depletes the intracellular pool of dNTP (Ayinde et al., 2012; Goldstone et al., 2011; Lahouassa et al., 2012). The intracellular dNTP pool appears to be an important rate-limiting factor for retroviral reverse transcription, and SAMHD1 critically modulates this

process. SAMHD1 is highly active in monocytes that have low levels of intracellular dNTP (Kaushik et al., 2009; Triques and Stevenson, 2004). In the presence of SAMHD1, nuclear or integrated HTLV-1 DNA was never detected in infected monocytes, whereas SAMHD1 silencing or restoration of the dNTP pool resulted in the inhibition of virus-induced apoptosis, as well as the completion of vDNA synthesis and translocation into the nucleus. Similarly, reduction of cytoplasmic HTLV-1 RTI levels by administration of high doses of AZT also improved cell viability. To investigate directly a role for RTI in triggering apoptosis, a biotinylated 90 nucleotide single or double stranded RTI from the U5 region of HTLV-1 was introduced into monocytes and it triggered apoptosis in a manner similar to infection.

One of the outstanding questions raised by these cumulative studies is: how does SAMHD1-mediated restriction of retroviral replication initiate apoptosis and impact the host innate response to retroviral infection? Our experiments provide evidence that the endoplasmic reticulum resident adapter STING triggers both processes through IRF3 activation. STING is known to regulate type I IFN immunity following recognition of pathogen DNA (Ishikawa et al., 2009) and its ability to directly interact with vDNA has been recently characterized (Abe et al., 2013). Although STING was readily pulled down in biotin-streptavidin complexes with RTI, we cannot formally exclude the involvement of other DNA sensors such as MRE11 (Kondo et al., 2013), DDX41 (Zhang et al., 2011), IFI16 (Unterholzner et al., 2010), or cGAS (Sun et al., 2013) in the recognition of retroviral RTI. The release of type 1 IFN and/or pro-inflammatory cytokines during HTLV-1 infection could also contribute to the induction of apoptosis in monocytes,

Complex formation between STING and HTLV-1 RTI initiated apoptosis through the formation of an IRF3-Bax complex, a novel apoptotic mechanism involved in the clearance of virus-infected cells via activation of effector caspase-3 (Chattopadhyay et al., 2013; Chattopadhyay et al., 2010); furthermore, IRF3-Bax complex formation was dependent on RIG-I signalling (Chattopadhyay et al., 2010). We did not observe a requirement for the RIG-I pathway during HTLV-1-induced apoptosis of primary monocytes; no interaction was observed between RTI and RIG-I, and silencing RIG-I had no effect on HTLV-1-induced apoptosis (**Figures S7A, B**). Following HTLV-1 infection of monocytes, we detected IRF3-Bax complex formation at 3h pi, prior the induction of RIG-I expression, and complex formation was abrogated in monocytes when STING expression was silenced, thus identifying STING signaling as an additional

pathway involved in IRF3-Bax induction. That IRF3 activation can promote both apoptotic and antiviral signalling was also demonstrated in earlier studies in which constitutively active forms of IRF3 and IRF7 were transduced into primary macrophages (Goubau et al., 2009). Transcriptional profiling and biological characterization of transduced human macrophages demonstrated that IRF3 initiated an antiviral response, but also rapidly induced cell death through the up-regulation of a subset of pro-apoptotic genes (Goubau et al., 2009).

We observed similar apoptotic mechanisms associated with STING binding and IRF3-Bax induction of apoptosis when HIV-1 RTI were introduced into monocytes, arguing in favour of the generality of the SAMHD1-STING initiation of apoptotic signaling in human retroviral infection **(Figure 7).** This observation, however, is not applicable in the context of HIV-2 which expresses Vpx; the physical interaction of SAMHD1 with Vpx recruits the Cul4-DDB1-DCAF1 complex to drive its proteasomal degradation (Descours et al., 2012; Laguette et al., 2011; Lahouassa et al., 2012). Furthermore, the exonuclease TREX1 suppresses recognition of HIV-1 replicative intermediates by STING as a viral evasion mechanism (Yan et al., 2010).

No correlation was observed between HTLV-1-induced apoptosis and expression levels of SAMHD1 (data not shown), consistent with Descours *et al.* who demonstrated that HIV-1 restriction was not only associated with SAMHD1 expression levels (Descours et al., 2012). In fact, monocyte-derived DC are efficiently and productively infected by HTLV-1 (Abe et al., 2013; Jones et al., 2008), despite SAMHD1 expression (Laguette et al., 2011). Moreover, in macrophages and cycling $CD4^+$ T-cells, HTLV-1 infection was not blocked by SAMHD1 and led to Tax production (Gramberg et al., 2013), indicating that SAMHD1 function differs in other cell types. It is likely that regulation of SAMHD1 restriction depends on many retroviral and host parameters including post-translational modifications, cellular co-factors, or splice variations (Descours et al., 2012; Welbourn et al., 2012). In this regard, it was recently demonstrated that SAMHD1 activity is modulated by phosphorylation status. Cyclin A2/CDK1 phosphorylated SAMHD1 at Thr592 SAMHD1 in cycling cells, and phosphorylation at Thr592 correlated with loss of restriction (Cribier et al., 2013; White et al., 2013). Furthermore, type 1 IFN treatment reduced Thr592 phosphorylation, indicating a link between SAMHD1 phosphorylation, antiviral activity and innate immune signaling(Cribier et al., 2013). White *et al.* further demonstrated that T592 phosphomimetic SAMHD1 mutants did not restrict HIV-1 replication, despite dNTPase

activity, oligomer formation, and nuclear localization(White et al., 2013). These experiments identify phosphorylation of SAMHD1 at Thr592 by cyclin A2/CDK1 as an important regulatory mechanism and highlight the need for further studies to elucidate the mechanisms that regulate SAMHD1 activity and function.

The demonstration that cytoplasmic RTI contributes to the elimination of HTLV-1-infected monocytes may have important consequences for human retroviral infection and pathogenesis. For example, the clearance of infected monocytes may prevent transmission to $CD4^+$ T cells, but would also deplete the precursor pool of myeloid dendritic cells that play a crucial role in controlling HTLV-1 infection (Rahman et al., 2011). Individuals suffering from ATL or HAM/TSP have been shown to possess a lower absolute number of DC than healthy individuals (Azakami et al., 2009; Hishizawa et al., 2004) and HTLV-1-infected monocytes also exhibit defective differentiation into DC (Makino et al., 2000; Nascimento et al., 2011). These factors may influence the development of HAM/TSP or ATL in HTLV-1 infected individuals, thus emphasizing the need to better understand the early host restriction of HTLV-1 infectivity by SAMHD1.

EXPERIMENTAL PROCEDURES

Products

RPMI-1640 media, FBS and antibiotics were provided by Wisent Technologies (CA, USA). All monoclonal antibodies (mAbs) and products used for flow cytometry were purchased from Biolegend (CA, USA), except for anti-cleaved caspase 3-PE mAbs, Annexin-V buffer 10X and Annexin-V-V450 obtained from Becton Dickinson (NJ, USA). Anti-p19 mAbs (clone TP-7) was purchased from ZeptoMetrix Corporation (NY, USA), whereas anti-gp46 mAbs (clone 67/5.5.13.1) was purchased from Abcam (MA, USA). Anti-Tax-FITC mAbs (clone LT4) was generously provided by Dr. Yuetsu Tanaka (Kitasato University, Kanagawa, Japan). All antibodies included in western blotting analyses came from Cell Signaling Biotechnology Inc. (MA, USA), except for anti-Bax mAbs (Santa Cruz Biotechnology Inc.; TX, USA). Pan-caspase

inhibitor Z-VAD-FMK (ZVAD) was purchased from R and D Systems^{R} (MN, USA). All cell lines were obtained from the ATCC^{TM} (VA, USA).

Purification of monocytes

Leukaphereses from healthy donors were obtained from the Royal Victoria Hospital, Montreal (QC, Canada), with informed consent of the patients and in agreement with the Royal Victoria Hospital, the Jewish General Hospital, and McGill University Research Ethics Committee. PBMCs were isolated using Ficoll-Hypaque gradient (GE Healthcare BioScience Inc., Ontario, Canada). Monocytes were purified from PBMCs using the untouched monocyte isolation kit (EasySep® Human Monocyte Enrichment Kit without CD16 Depletion; StemCell Technologies, Vancouver, BC, Canada), allowing for more than 94% purification without any cell stimulation and apoptosis.

HTLV-1 purification and *in vitro* infection

HTLV-1 viruses were purified from MT-2 supernatants. Cells were seeded overnight in complete RPMI (2.10^6 cells/mL). Supernatants were collected and ultra-centrifuged for 2h at 30,000 g at 4ºC. The viral pellet was re-suspended in complete RPMI, and HTLV-1 particles were quantified by gag p19 ELISA assays (ZeptoMetrix). 200,000 purified monocytes were incubated with 2µg HTLV-1 for 3h at 37ºC in 0.5 mL RPMI with or without 100µM Z-VAD-FMK. To specifically block HTLV-1 infection, neutralizing anti-gp46 mAbs (at 10µg/mL; Abcam) were incubated 30 minutes at 4ºC with HTLV-1 prior *in vitro* infection. At 3h pi, monocytes were washed twice in complete RPMI and then co-cultured with 8.10^5 autologous monocyte-depleted PBMCs (1 ml complete RPMI; [HTLV-1] = 2µg/mL).

HTLV-1 DNA quantitation and nuclear extraction

2-4.10^6 monocytes, isolated using magnetic beads and infected or not with HTLV-1, were collected at several time points (3-120h pi), and washed with PBS. Cells were pelleted and stored at -80ºC, prior to various DNA extraction protocols. *Whole cell DNA:* DNA was extracted from stored pellets using the DNeasy® Blood & Tissue kit (Cat. Number: 69504; Qiagen Inc.) using the manufacturer's instructions. *Nuclear DNA:* nuclear material was extracted from stored

pellets; cells were lysed in 10mM Tris (pH 7.9), 10mM KCl, 0.1mM EDTA, and 0.1mM EGTA, for 15 minutes. NP40 was then added and the lysates were centrifuged 3 minutes at 13,000 rpm to obtain a nuclear pellet that was used for subsequent DNA extraction (Qiagen). *ALU PCR:* integrated HTLV-1 DNA was amplified from purified nuclear DNA as previously described (Liszewski et al., 2009), with the following modifications. Cloned Pfu DNA polymerase (Cat. Number: B0093; Bio Basic) was used, and the primer sequences for Alu (forward) and gag (reverse) are listed in **Table S1**. HTLV-1 DNA load was determined on total or nuclear cell extract, or ALU PCR products, using the real-time SYBR green PCR method. The assay was analyzed using the AB 7500 real-time PCR system (Applied Biosystems, CA, USA) with PerfeCTa® SYBR® Green FastMix® (Cat. Number: 95074; Quanta Biosciences, MD, USA) using ~200ng of extracted DNA, or 0.1µl of amplified integrated DNA. The endogenous erv-3 gene was used to normalize the amount of DNA per reaction. The real-time PCR conditions were: 95°C for 10 minutes, and 45 cycles of 95°C for 15 seconds followed by 60°C for 1 minute.

Measurement of viral genomic RNA

Following HTLV-1 infection, $4x10^6$ isolated monocytes were incubated 6 minutes with 0.25% trypsin at 37ºC to remove non-internalized HTLV-1 particles and washed twice in PBS. Cells were thereafter pelleted and stored at -80ºC in RLT buffer in the presence of β-mercaptoethanol for further use. Total RNA was extracted using RNeasy kit (Qiagen Inc.) according to the manufacturer's instructions. The RNA integrity and purity was assessed with the Thermo Scientific NanoDrop 1000 (Thermo Scientific, DE, USA). Complementary DNA (cDNA) was then produced by reverse transcription using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Qiagen Inc.). The subsequent real-time PCR parameters were described in the previous section, except for the fact that β-actin was used as the housekeeping gene.

Flow cytometry

At several time pi, collected cells were incubated for 10 minutes at 4ºC in PBS + 10% FBS to avoid any unspecific binding. *Measurement of viral infection:* Cells were then stained for 10 minutes at 4ºC with anti-CD14-APC H7, anti-CD3-PE Cy7 in PBS. Cells were washed twice and

fixed at room temperature (RT) in BD FACS Lysing Buffer (Becton Dickinson) and incubated with anti-Tax-FITC and anti-p19-Alexa 647 mAbs for 20 minutes at RT in 0.25% saponin. Anti-p19 IgG_1 antibody was conjugated to Alexa647 dye using the Zenon^R mouse IgG_1 labeling kit (number: Z25008; Life Technologies Inc., ON, USA) according to the manufacturer's protocol. After three final washes in PBS, we collected approximately 20,000 gated events on the BD Fortessa flow cytometer (Becton Dickinson) and analyzed the data using the DIVA software. *Apoptosis assay:* To assess apoptosis, cells were washed and labeled for 10 minutes at 4ºC with anti-CD14-APC H7, anti-CD3-PE Cy7 and Annexin-V-V450 in calcium buffer 1X (Becton Dickinson). ICS of $_{CL}$caspase-3 or Bax was the same as mentioned below for HTLV-1 antigens, except for the fact that all solutions were diluted in calcium buffer. *Mitochondria depolarisation:* This was evaluated through measurement of the loss of retention of the cationic fluorescent dye $DiOC_6(3)$ (used at 10nM), as previously described(Kalbacova et al., 2003). $DiOC_6(3)$-related fluorescence was finally analyzed on $CD3^-CD14^+$monocytes by flow cytometry.

Western blotting

Protein lysates (2-10 μg) from monocytes were subjected to Western blot analysis as previously described (Oliere et al., 2010).

Small interfering RNA assays

10^7 purified monocytes were electroporated in the presence of control or specific siRNA using Nucleofactor II technology according the manufacturer's protocol (Amaxa human monocyte nucleofactor kit). SAMHD1-, STING-, RIG-I- or Bax-specific siRNA as well as siRNA-A negative control were obtained from Santa Cruz Biotechnology (Cat. numb. sc-76442, sc-92042, sc-61480, sc-29212, and sc-37007 respectively; CA, USA). 300nM siRNA was transfected per condition in RPMI + 30% FBS overnight without antibiotics. Transfected monocytes were washed at 1500 rpm for 5 minutes to remove necrotic cells and cultured with autologous PBMC. For SAMHD1 and STING siRNA treatments, monocytes were washed and counted for further *in vitro* HTLV-1 infection 3 days post-transfection. RIG-I and Bax siRNA treated monocytes were similarly processed 3h post-transfection.

Biotinylated retroviral DNA_{90} assay

Retroviral DNA was produced by Integrated DNA technologies (IA, USA). HTLV-1 $ssDNA_{90}$ is the reverse complement of the 5'utr region (315-404 of complete HTLV-1 genome; NCBI) that is 90 bases long and conjugated with biotin on the 5' end. The sequence is: 5'-CTG TGT ACT AAA TTT CTC TCC TGG AGA GTG CTA TAG AAT GGG CTG TCG CTG GCT CCG AGC CAG CAG AGT TGC CGG TAC TTG GCC GTG GGC-3'. HTLV-1 biotinylated ssDNA-sense strand was annealed to the reverse complement to create $dsDNA_{90}$. As a negative control, we used an scrambled RTI (generated by randomizing the U5 sequence; 5'-ATT CAG CTC ACG GCG TCG AGT GCT GCT CGA TGG CTC CTT AGT CCT GCT AAG TCG AGG TGG CTA ATC CGG TAG TCG GTC GGA TGG AAT TCG-3'). HIV-1 $ssDNA_{90}$ is also the biotinylated reverse complement of the 5'utr region (97-186 of complete HIV-1 genome; NCBI). The sequence is: 5'-CGC CAC TGC TAG AGA TTT TCC ACA CTG ACT AAA AGG GTC TGA GGG ATC TCT AGT TAC CAG AGT CAC ACA ACA GAC GGG CAC ACA CTA CTT-3'. 10^7 monocytes were transfected with 10µg $vDNA_{90}$ for 24h in RPMI + 30% FBS in the absence of antibiotics. Monocytes were washed and then co-cultured in complete RPMI with $CD14^{neg}$PBMCs.

STING pull-down and Bax co-immunoprecipitation

Monocytes were infected with HTLV1 or transfected with biotinylated RTI for 6h (pull-down) or 3h (co-IP). Monocytes were lysed using CHAPS buffer with protease inhibitors as previously described (Samuel et al., 2010). 3µg protein was collected and referred to as the "input" fraction. *STING Pull-down:* ~200µg protein was incubated with 20µl streptavidin bead resin (Thermo Scientific) overnight at 4ºC while rotating. Bead-protein complexes were washed 6 times in CHAPS buffer and then incubated 10 minutes at 100ºC with 2X loading dye. Lysates were centrifuged 3 minutes at 13,000 rpm to remove the beads and subjected for western blotting analyses. *Anti-Bax co-IP:* Protein L-agarose beads (Santa Cruz Inc.; sc-2336) were washed with 0.2 mol/L triethanolamine pH 9.0. 30µg of anti-Bax mAbs was cross-linked to 300µL of L-agarose beads in the presence of dimethylpentylamine-Hcl for 1h at RT. After washes in triethanolamine, 2µg of cross-linked anti-Bax beads was incubated overnight with ~200µg protein in 1% CHAPS lysis buffer at 4ºC overnight while rotating. Immunoprecipitates were collected, washed 5 times in CHAPS buffer and then incubated 10 minutes at 100ºC with 1X loading dye. Samples were centrifuged and subjected to western blotting analyses.

Statistical analysis

Statistical analyses were performed using the non-parametric Mann-Whitney *U* test, assuming independent samples. However, differences among the treatment groups performed with n = 3 samples were analyzed by the parametric unpaired Student *t* test. *P* values of less than 0.05 were considered statistically significant. ***, $P < 0.001$; **, $P < 0.01$ and *, $P < 0.05$.

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AUTHOR CONTRIBUTIONS

AS performed most of the experiments and helped write the paper; SMB and RL aided in the STING pull-down and anti-Bax co-immunoprecipitation experiments; DO edited text and Figures; JH and JvG conceived the study, designed experiments, supervised the experiments, and wrote the paper.

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Chapter 4: HTLV-1 Tax-Mediated Inhibition of FOXO3a Activity is Critical for the Persistence of Terminally Differentiated CD4$^+$ T Cells

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1. Abstract

The mechanisms involved in the persistence of activated CD4$^+$ T lymphocytes following primary human T leukemia/lymphoma virus type 1 (HTLV-1) infection remain unclear. Here, we demonstrate that the HTLV-1 Tax oncoprotein modulates phosphorylation and transcriptional activity of the FOXO3a transcription factor, *via* upstream activation of the AKT pathway. *De novo* HTLV-1 infection of CD4$^+$ T cells or direct lentiviral-mediated introduction of Tax led to AKT activation and AKT-dependent inactivation of FOXO3a, *via* phosphorylation of residues Ser253 and Thr32. Inhibition of FOXO3a signalling led to the long-term survival of a population of highly activated, terminally differentiated CD4$^+$Tax$^+$CD27^{neg}CCR7^{neg} T cells that maintained the capacity to disseminate infectious HTLV-1. CD4$^+$ T cell persistence was reversed by chemical inhibition of AKT activity, lentiviral-mediated expression of a dominant-negative form of FOXO3a or by specific small interfering RNA (siRNA)-mediated silencing of FOXO3a. Overall this study provides new mechanistic insight into the strategies used by HTLV-1 to increase long-term maintenance of Tax$^+$CD4$^+$ T lymphocytes during the early stages of HTLV-1 pathogenesis.

Author summary

HTLV-1 infection contributes to the development of Adult T cell Leukemia (ATL) or the neurological disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). HTLV-1 principally targets CD4$^+$ T lymphocytes and causes profound changes in activation,

immune function and cell death. The molecular mechanisms involved in the persistence of infected $CD4^+$ T cells following primary HTLV-1 infection remain unclear. We demonstrate here that the Tax oncoprotein inactivates the FOXO3a transcription factor to facilitate the long-term survival of a population of highly activated and terminally differentiated T cells that maintain the capacity to spread infectious viral particles. Mechanistically, expression of Tax oncoprotein in primary human $CD4^+$ T cells resulted in the phosphorylation-dependent inactivation of FOXO3a, via the AKT kinase. Tax-mediated $CD4^+$ T cell persistence was also reversed by chemical inhibition of the AKT pathway, and reproduced by the expression of a dominant negative version of FOXO3a itself or by silencing its transcriptionally active form using specific siRNA. Overall this study provides new mechanistic insights used by Tax to potentiate the long-term maintenance of $CD4^+$ T lymphocytes following HTLV-1 infection and suggests that modulation of FOXO3a activity, using a range of inhibitors targeting the PI3K-AKT-FOXO3a pathway, may offer a valuable addition to current therapeutic approaches.

2. Introduction

Infection with the human T cell leukemia virus type I (HTLV-1) affects more than 20 million people worldwide [1] and HTLV-1-associated diseases are a major cause of mortality and morbidity in endemic areas where infection rates range from 2 to 30%. Chronic infection with HTLV-1 can result in a number of severe pathologies, including the aggressive adult T cell leukemia (ATL) and the progressive neurological disorder termed myelopathy/tropical spastic paraparesis (HAM/TSP) [1]. The majority of HTLV-1-infected individuals remain asymptomatic carriers (AC) of the virus but a proportion of AC (1-5%) will develop ATL or HAM/TSP. $CD4^+$ T cells are the main targets for viral infection [1, 2], although HTLV-1 can also infect cells of the myeloid lineage including dendritic cells and monocytes [3, 4].

HTLV-1-associated diseases are characterized by profound deregulation of $CD4^+$ T cells in terms of activation, immune function and apoptosis [5, 6], all of which are facilitated by the pleiotropic functions of the viral oncoprotein Tax [7-10]. In addition to controlling viral gene expression and replication, Tax contributes to malignant transformation of $CD4^+$ T cells by modulating host signalling pathways including NF-κB, PI3K-AKT, and JAK-STAT [7-10].

The chronic nature of retrovirus infection has been linked to the activity of the Forkhead box (FOXO) transcription factor family, and particularly to FOXO3a, which can alter the activation, survival and proliferative capacity of $CD4^+$ T-cells [10-15]. FOXO3a is constitutively expressed in most cell types including T lymphocytes, where it regulates apoptosis, tumorigenesis and inflammation [16-18], processes that are also deregulated in HTLV-1-associated diseases [5, 19, 20]. Specifically, FOXO3a stimulates expression of pro-apoptotic and anti-proliferative target genes such as *BIM*, *FASL* and *p130* [21]. The FOXO family is subject to numerous post-translational modifications [17] and FOXO phosphorylation can serve either an inhibitory or an activating role in FOXO functions; phosphorylation by JNK activates FOXO3a function[22] while phosphorylation of specific residues (Ser 253 and Thr32) by the serine/threonine kinase AKT inactivates FOXO3a [23].

Previous studies demonstrated that FOXO3a activity contributes to the progressive depletion of central memory $CD4^+$ T cells in HIV-1-infected patients [15]. Modulation of FOXO3a activity

also occurs during *de novo* HIV-1 infection, where HIV Tat protein induces FOXO3a activity leading to HIV-specific apoptosis [24, 25].

In the present study, we demonstrate that expression of HTLV-1 Tax in primary human $CD4^+$ T cells, either through productive HTLV-1 infection or through lentiviral-mediated transduction results in the phosphorylation-dependent inactivation of FOXO3a *via* the upstream kinase AKT. FOXO3a inhibition resulted in long-term survival of terminally differentiated, $Tax^+CD27^{neg}CCR7^{neg}$ $CD4^+$ T cells that were capable of disseminating infectious HTLV-1. These results provide insight into the mechanisms used by HTLV-1 to increase the long-term maintenance of Tax^+CD4^+ T lymphocytes during the early stages of HTLV-1 pathogenesis.

3. Results

i. Productive HTLV-1 infection is associated with phosphorylation of FOXO3a and persistence of infectious $CD4^+$ $CD27^+$ $CCR7^+$ T cells

Primary CD3/CD28 activated $CD4^+$ T cells were infected by HTLV-1 in a dose dependent manner (**Figure 1A**) using an *in vitro* trans-infection system in which $CD4^+$ T cells were co-cultured with HTLV-1 shedding MT-2 cells [26]. Following multiple rounds of T cell receptor (TCR) triggering, HTLV-1 infected T cells [Tax^+ cells; blue] persisted for 21-28 days without a significant reduction in cell number, ($P < 0.05$) (**Figure 1B**); in contrast, T cells that were not infected [Tax^{neg} cells; black] displayed a significant reduction in cell number by 14 days post-infection (pi) ($P < 0.01$). The half-life of gated $Tax^{neg}CD4^+$ T cells was 18.1 days pi, whereas a half-life calculation could not be determined for Tax^+ T cells before 28 days.

Using a combination of CD3, CD45RA, CCR7 and CD27 surface markers, we evaluated the generation and maintenance of terminally differentiated ($CD3^+CD45RA^{+/-}CCR7^{neg}CD27^{neg}$) T cells during a 28-day cycle of HTLV-1 infection (**Figure 1C**) [27, 28]. Repeated TCR triggering reduced the proportion of viable terminally differentiated $CD27^{neg}CCR7^{neg}$ effector cells among gated $CD3^+Tax^{neg}$ T cells, whereas the Tax^+ T lymphocytes increased in cell number and maintained activation status (55.7 ± 4.6% and 36.2 ± 1.3% of $CD3^+CCR7^{neg}CD27^{neg}$cells, respectively for Tax^+ and Tax^{neg} T cell population at 28 days; $P < 0.01$) (**Figure 1D** and **Figure S1A**). In addition, terminally differentiated Tax^+CD4^+ T cells produced infectious HTLV-1, even after four weeks in culture, based on their capacity to transmit virus to freshly isolated autologous $CD4^+$ T lymphocytes (**Figure 1E**). Overall these results demonstrate that HTLV-1 infection promotes the *in vitro* maintenance of terminally differentiated, virus-producing CD4+ T cells ($CD3^+CCR7^{neg}CD27^{neg}$).

We hypothesized that the enhanced cellular survival observed in HTLV-1 infected $CD4^+$ T cells may be associated with the deregulation of FOXO3a signalling, given its important role in regulating cell proliferation and apoptosis in other retroviral infections [14, 15, 24, 29]. We first investigated at 2 days pi the activation status of AKT, one of the major upstream kinases responsible for FOXO3a phosphorylation [23] (**Figure 2A**). Based on phosphorylation at the Ser473 residue, we found that upstream kinase AKT was dramatically activated in Tax-

expressing cells [9] ($P = 0.0317$ and $P < 0.001$, respectively for PhosFlow and western blotting approaches) (**Figures 2B-D**). HTLV-1 infection led to an increase in FOXO3a phosphorylation at residue S253 ($P < 0.01$) and Thr32 ($P < 0.001$) at 2 days pi **(Figures 2C,D**), residues that inactive FOXO3a function [23]. Consistent with this observation, productively infected cells displayed reduced expression of FOXO3a downstream target genes p130 and Bim [15, 23]. The phosphorylation status of IKKα, another upstream kinase of FOXO3a, was however unchanged (**Figures 2C,D**). Overall these results demonstrate that productive HTLV-1 infection provides a survival and proliferative persistence advantage to infected $CD4^+$ T cells, and is associated with AKT-mediated inactivation of FOXO3a transcriptional activity.

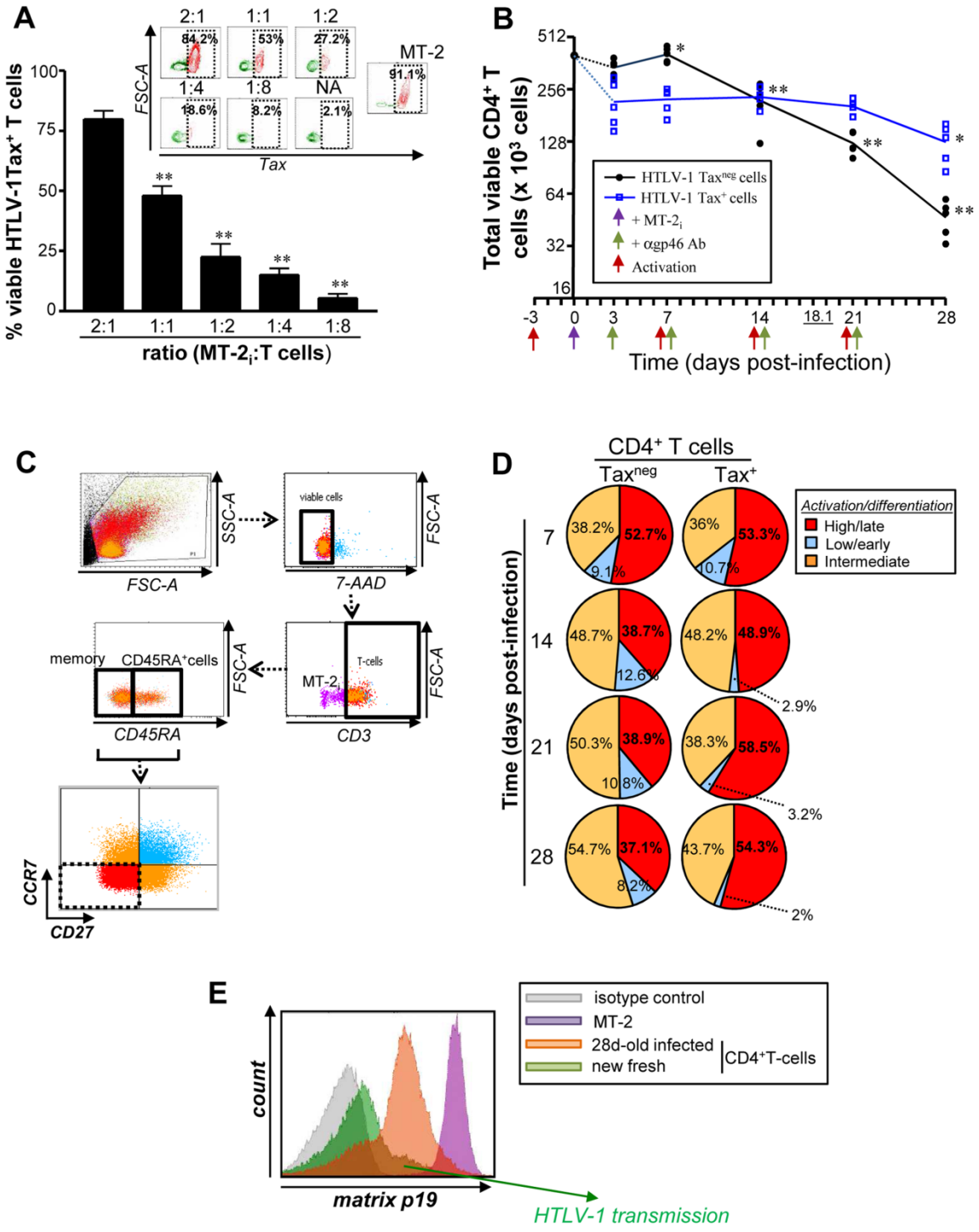

A
2:1
1:1
1:2
1:4
1:8
NA
MT-2
84.2%
53%
27.2%
8.6%
8.2%
2.1%
91.1%
FSC-A
Tax
% viable HTLV-1Tax+ T cells
ratio (MT-2i:T cells)
B
Total viable CD4+ T cells (x 10³ cells)
HTLV-1 Taxneg cells
HTLV-1 Tax+ cells
+ MT-2i
+ αgp46 Ab
Activation
Time (days post-infection)
C
SSC-A
FSC-A
viable cells
7-AAD
memory
CD45RA+cells
CD45RA
T-cells
MT-2i
CD3
CCR7
CD27
D
CD4+ T cells
Taxneg
Tax+
Activation/differentiation
High/late
Low/early
Intermediate
Time (days post-infection)
38.2%
52.7%
9.1%
36%
53.3%
10.7%
48.7%
38.7%
12.6%
48.2%
48.9%
2.9%
50.3%
38.9%
10.8%
38.3%
58.5%
3.2%
54.7%
37.1%
8.2%
43.7%
54.3%
2%
E
isotype control
MT-2
28d-old infected
new fresh
CD4+T-cells
count
matrix p19
HTLV-1 transmission

Figure 1. Productive HTLV-1 infection is associated persistence of infectious $CD4^+CD27^{neg}CCR7^{neg}$ T cells.

Briefly, activated $CD4^+$ T cells were productively infected with MT-2_i for 2–28 days to assess their persistence and stepwise differentiation following multiple rounds of TCR stimulation. (A) Tax expression on activated Annexin-V^{neg} $CD4^+$ T cells in response to various concentrations of irradiated MT-2 after 48 h pi. We used a CD4/MT-2_i ratio of 1:1 for subsequent experiments. Representative contour plots of five independent experiments were shown above (n=5). (B) Absolute numbers of total viable Tax^{neg} (black) and Tax^+ (blue) $CD3^+$T cells were determined by trypan blue exclusion. Results are expressed in $\log_2$ scale. *P* values were determined based on the comparison with cells at 3 dpi. The underlined number represents the half-life of cultured Tax^{neg} T cells. (C) Briefly, cells were collected at 7–28 days pi using long-term co-culture assay and then stained with 7-AAD and anti-CD3-PE, anti-CD45RA-ECD, anti-CD27-APC H7, anti-CCR7-PE Cy7 and anti-Tax-Alexa 647 Abs. Gating strategies to distinguish between three distinct T cell populations, based on their stepwise activation/differentiation status from low/early, intermediate to high/late ($CCR7^+CD27^+$ [blue], single $CCR7^+$ or $CD27^+$ [orange], $CCR7^{neg}CD27^{neg}$cells [red], respectively). (D) Distribution of activated/differentiated 7-$AAD^{neg}CD3^+$ T cells subsets based on CCR7 and CD27 staining at 7-28 days pi. Pie charts are representative of raw data from five independent experiments. (E) Transmission of HTLV-1 to fresh uninfected PE-stained T cells with 28 day-old HTLV-1-infected $CD3^+$ T cells from the same donor (n=3). Briefly, at 28 day pi infected $CD4^+$ T cells were isolated using a CD3 positive selection kit (StemCell Technology, >97.6% purity). These cells were then co-cultured (ratio 1:1) for 5 days with activated autologous $CD4^+$ T cells that were stained 1 h prior to co-culture with anti-CD3-PE Ab. After 5 days, HTLV-1 transmission was measured on new CD3-PE-stained targets using p19 intracellular staining. MT-2 cells were used as positive control.

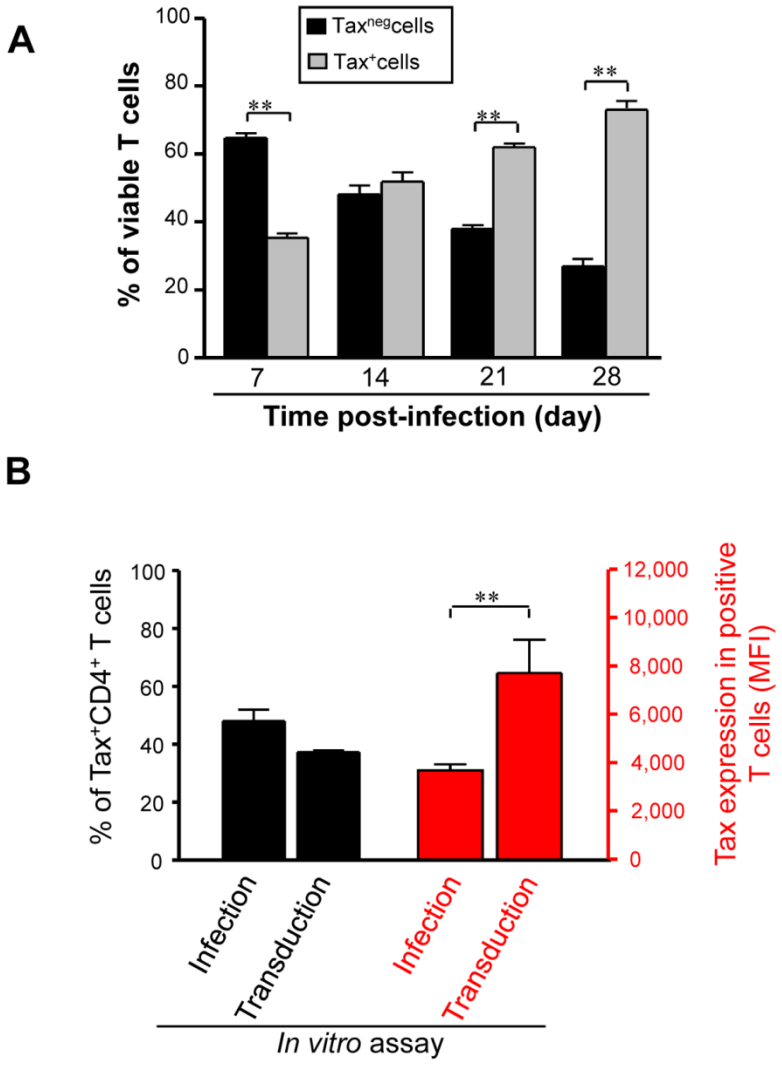


***Figure S1. In vitro* assay of primary T cells (trans-infection with MT-2$_i$ cells and transduction with LVP$_{Tax}$).**

(A) Activated CD4$^+$ T cells were productively infected with MT-2$_i$ for 7–28 days to assess the proportion of HTLV-1 infected T cells during the time course of the co-culture. Results shown represent the percentages of Annexin-V^{neg}Tax$^+$CD3$^+$ T cells at each time points (n=3) (B) Comparison of Tax expression levels between productively infected (n=5) and Tax-tranduced (n=3) CD4$^+$ T cells at 48 hours. The percent of Tax$^+$CD4$^+$ T cells (black) and the Tax expression levels on positive cells (red) are shown.

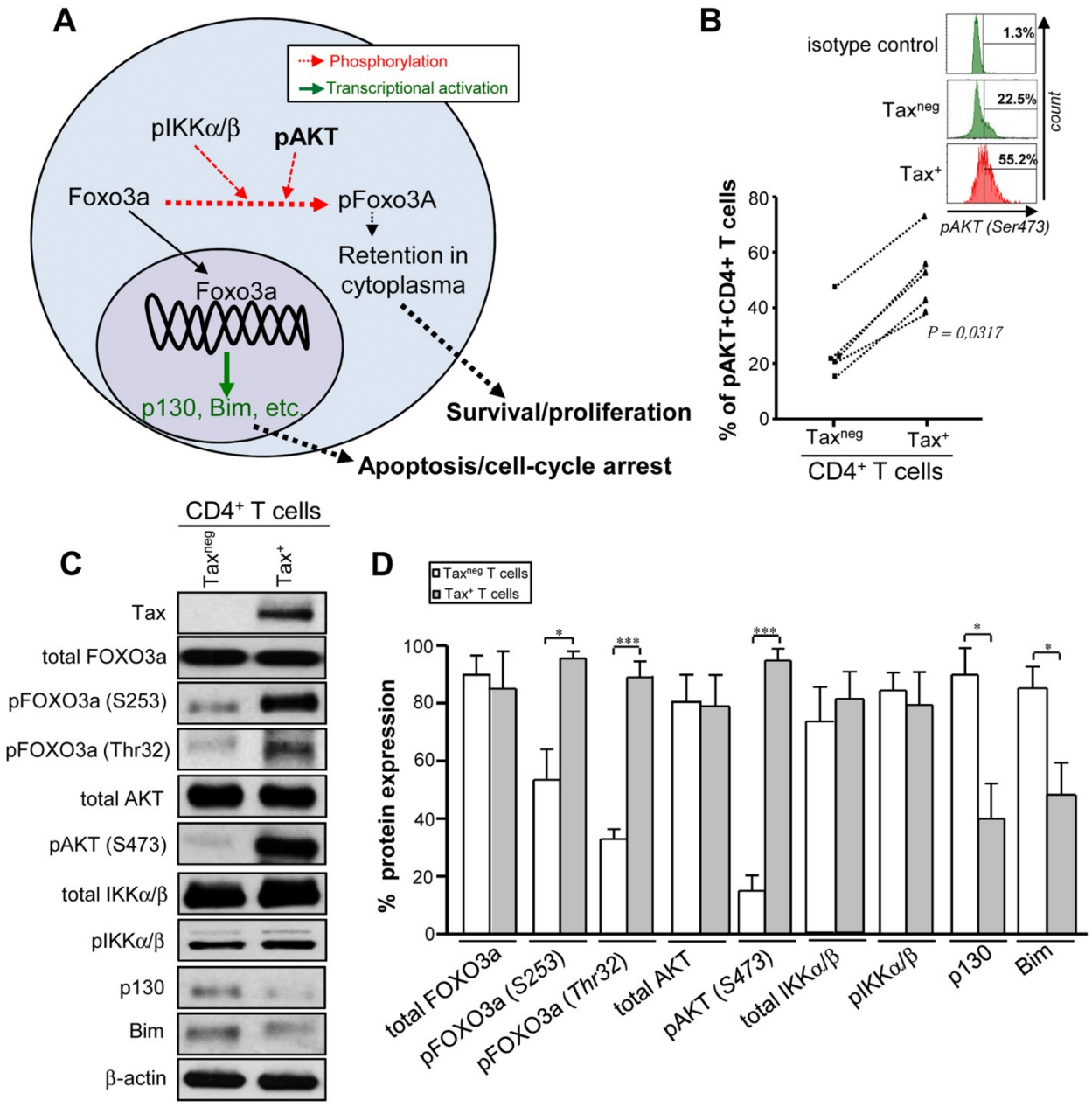


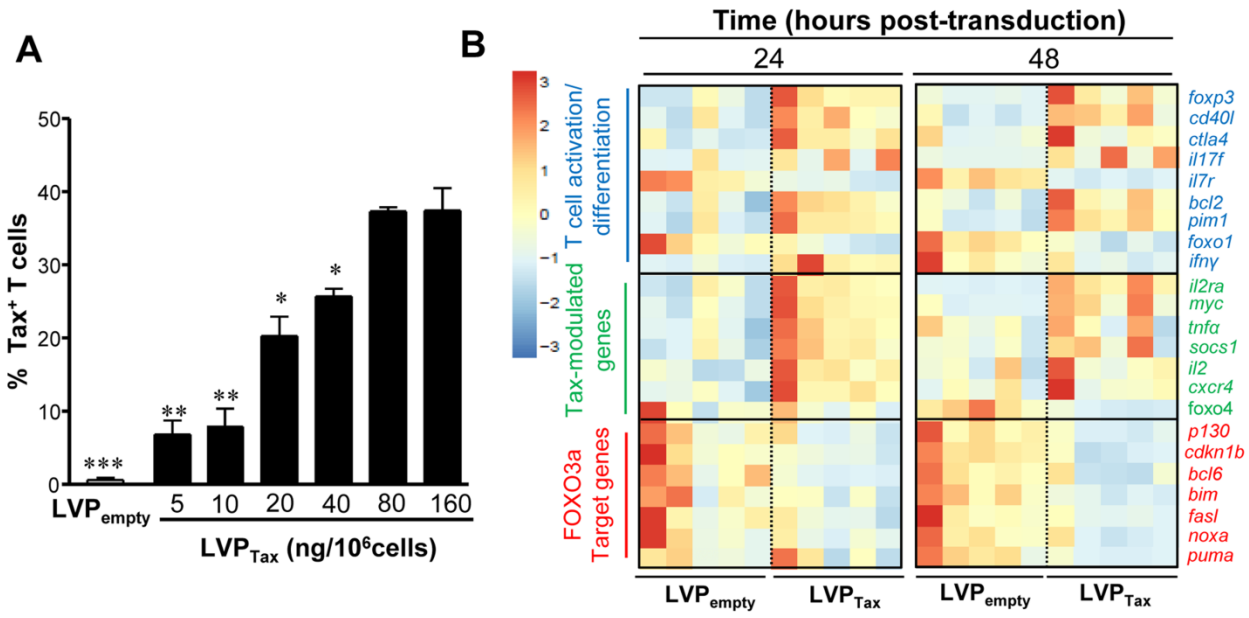
Figure 2. Tax^{+}CD4^{+}T cells display higher phospho-FOXO3a phenotype than the Tax^{neg}subset.

(A) Schematic showing the inhibition of FOXO3a signalling pathway by phosphorylation mediated by upstream pro-survival kinases. (B–D) FOXO3a signaling profile on Tax^{+} and Tax^{neg} T cells subsets that are derived from the same culture (in the presence of MT-2i; at ratio 1:1). (B) PhosFlow analysis of phospho-AKT (Ser473) levels on Tax^{+} and Tax^{neg}CD3^{+} T cells at 2 days pi. Representative histograms of pAKT status are also shown above (n=5). (C) At 2 days pi,

10μg of highly purified $CD3^{+}Tax^{neg}$ and $CD3^{+}Tax^{+}T$ cell subsets were subjected to immunoblotting to investigate the FOXO3a signalling pathway (n=3). (D) Densitometric quantification of the spots was performed in parallel using ImageJ software. Results shown represent the mean relative expression ± SD of 3 independent experiments.

ii. Tax expression inhibits FOXO3a activity *via* activation of AKT

Among the proteins encoded by HTLV-1, the Tax oncoprotein exerts its essential role in viral transcription, as well as in T cell transformation[30-32]. To determine whether Tax expression alone was sufficient to drive FOXO3a inactivation, HTLV-1 Tax was introduced into activated $CD4^+$ T cells using lentiviral particle (LVP)-mediated transduction. Tax was detected using intracellular staining by flow cytometry **(Figure 3A)** and a concentration of 80 ng LVP/10^6cells resulted in ~40% Tax^+ cells. LVP_{Tax}-transduced $CD4^+$ T cells displayed higher expression levels of HTLV-1 Tax when compared to infected cells (**Figure S1B**; fold increase ~2.07; P=0.0091). A BioMark transcriptional high throughput qPCR analysis of LVP-transduced T cells demonstrated that Tax expression modulated mRNA levels of several Tax-modulated genes[8, 31], including an increase in IL-2 and a decrease in type I IFN-associated genes. Tax expression led to higher mRNA expression levels of *CXCR4, SOCS1* and *myc* proto-oncogene, as previously shown [7, 33-35]. This analysis also demonstrated the activated/differentiated status of Tax-transduced $CD4^+$ T cells, based on the increased expression of *granzyme B*, *CD40L*, *CTLA-4*, *IFNγ* and *IL7R* mRNA (**Figures 3B**). Interestingly, Tax expression not only inhibited p130 and Bim expression (**Figures 3B,C**), but also down-regulated several other FOXO3a target genes; *BCL6*, *p27*, *BIM*, *FASL*, *NOXA* and *PUMA* were all downregulated at the mRNA levels at 24 and 48h post-transduction (**Figures 3B,C**). Tax transduction also induced AKT activation (P < 0.001) [9], phosphorylation of FOXO3a (P < 0.05) and inhibition of p130 (P < 0.05) at the protein level, all of which were significantly reversed by the addition of an AKT inhibitor, AKT inhibitor IV (AKT_i) (**Figures 4A,B**). Conversely, treatment of transduced $CD4^+$ T cells with 100 μg/mL IKK inhibitor II (Calbiochem) did not significantly alter the expression levels of phosphorylated FOXO3a (**Figure S2A,B**). Tax expression in $CD4^+$ T cells did not modulate FOXO3a stability, as we found no significant change in its expression in the presence or absence of Tax even after 6 days of transduction (**Figures S3A,B**). Nonetheless Tax transduction resulted in increased nuclear localization of inactive pFOXO3a forms (Ser253 and Thr32 residues) (**Figures S3C,D**) [13]. Overall this data demonstrate that Tax expression is sufficient to transcriptionally inactivate FOXO3a signaling, *via* upstream activation of AKT.



C

Symbol	Name	Function	Tax transduction			
			24h		48h	
			fold change	*P-value*	*fold change*	*P-value*
p130	retinoblastoma protein	cell-cycle arrest	**0.73±0.21**	**0.0470 (*)**	**0.34±0.08**	**0.0050 (**)**
p27, cdkn1b	cyclin-dependent kinase inhibitor 1B		0.56±0.17	0.0526 (*ns*)	**0.37±0.10**	**0.0003 (***)**
bcl6	B cell lymphoma 6	repressor of transcription	**0.53±0.18**	**0.0185 (*)**	**0.50±0.25**	**0.0091 (**)**
bim	Bcl-2-like protein 11	Apoptosis	**0.61±0.18**	**0.0406 (*)**	**0.24±0.04**	**0.0038 (**)**
FasI	Fas Ligand		0.70±0.21	0.0547 (*ns*)	**0.32±0.06**	**0.0412 (*)**
noxa	PMA-induced protein 1		**0.54±0.20**	**0.0155 (*)**	**0.25±0.11**	**0.0066 (**)**
puma	p53 upregulated modulator of apoptosis		0.86±0.23	0.1518 (*ns*)	**0.63±0.29**	**0.0320 (*)**

Figure 3. Tax expression inhibits FOXO3a activity *via* activation of AKT.

Briefly, activated T cells were transduced by lentiviral particles (LVP) expressing or not Tax. (A) Tax expression at 48 h on transduced T cells in response to LVP_{Tax} concentrations (5–160 ng/10^6 cells; n=3). (B) At 24 and 48 h post-transduction, total RNA was extracted, and subjected to Biomark analyses. Heatmaps of genes significantly modulated following Tax expression. (C) Table showing fold changes and *P*-values for several modulated FOXO3a targets genes on Tax-transduced T cells (n=5; paired *t* test).

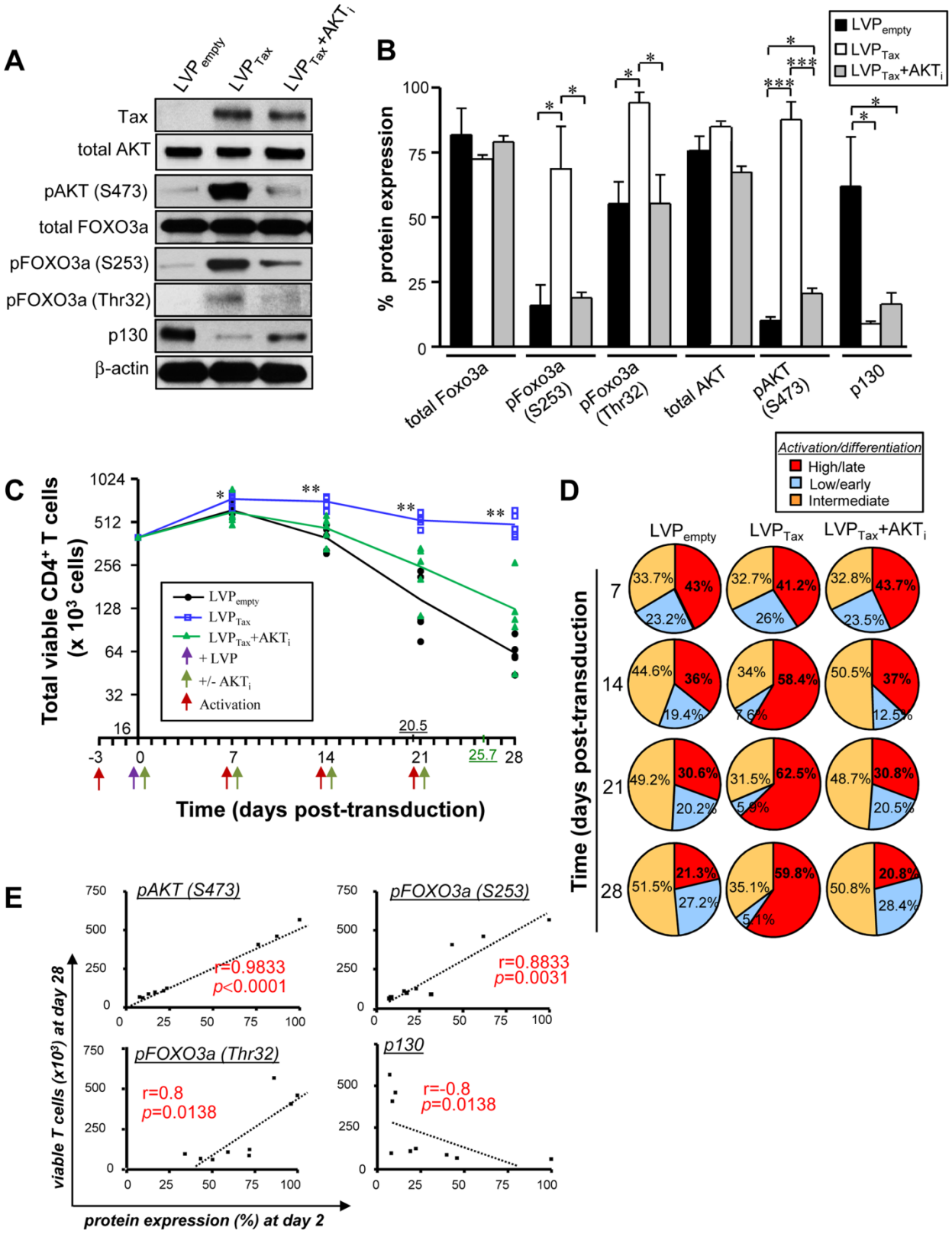

A
LVP empty
LVPTax
LVPTax+AKTi
Tax
total AKT
pAKT (S473)
total FOXO3a
pFOXO3a (S253)
pFOXO3a (Thr32)
p130
β-actin
B
LVPempty
LVPTax
LVPTax+AKTi
% protein expression
total Foxo3a
pFoxo3a (S253)
pFoxo3a (Thr32)
total AKT
pAKT (S473)
p130
C
Total viable CD4+ T cells (x 10³ cells)
LVPempty
LVPTax
LVPTax+AKTi
+ LVP
+/- AKTi
Activation
Time (days post-transduction)
D
Activation/differentiation
High/late
Low/early
Intermediate
LVPempty
LVPTax
LVPTax+AKTi
Time (days post-transduction)
E
pAKT (S473)
r=0.9833
p<0.0001
pFOXO3a (S253)
r=0.8833
p=0.0031
pFOXO3a (Thr32)
r=0.8
p=0.0138
p130
r=-0.8
p=0.0138
viable T cells (x10³) at day 28
protein expression (%) at day 2

Figure 4. Tax-mediated FOXO3a inactivation is responsible for $CD4^+$ T cell persistence.

Briefly, activated T cells were transduced by lentiviral particles (LVP) expressing or not Tax for 2–28 days. (A) FOXO3a signaling on transduced T cells expressing or not Tax at 48 h determined by immunoblotting (n=3). (B) Densitometric quantification of specific bands was performed using ImageJ software. Results shown represent the mean relative expression ± SD of 3 independent experiments. (C–E) Persistence and stepwise differentiation of activated $CD4^+$ T cells transduced with LVP expressing Tax in the presence or absence of AKT_i (n=5). (C) Absolute numbers of total viable $CD3^+$ T cells were determined by trypan blue exclusion. Results are expressed in $\log_2$ scale. *P* values were determined based on the comparison with LVP_{empty}-transduced cells. The underlined numbers represent the half-life of cultured LVP_{empty} (black) and LVP_{Tax}+AKT_i (green) conditions. (D) Differentiation status of transduced T cells subsets at 7–28 dpt. Pie charts are representative of raw data from five independent experiments. (E) Correlation between the absolute numbers of viable transduced T cells at 28 days and the levels of expression of FOXO3a-related proteins at 48 h are also shown (n=9; Spearman test).

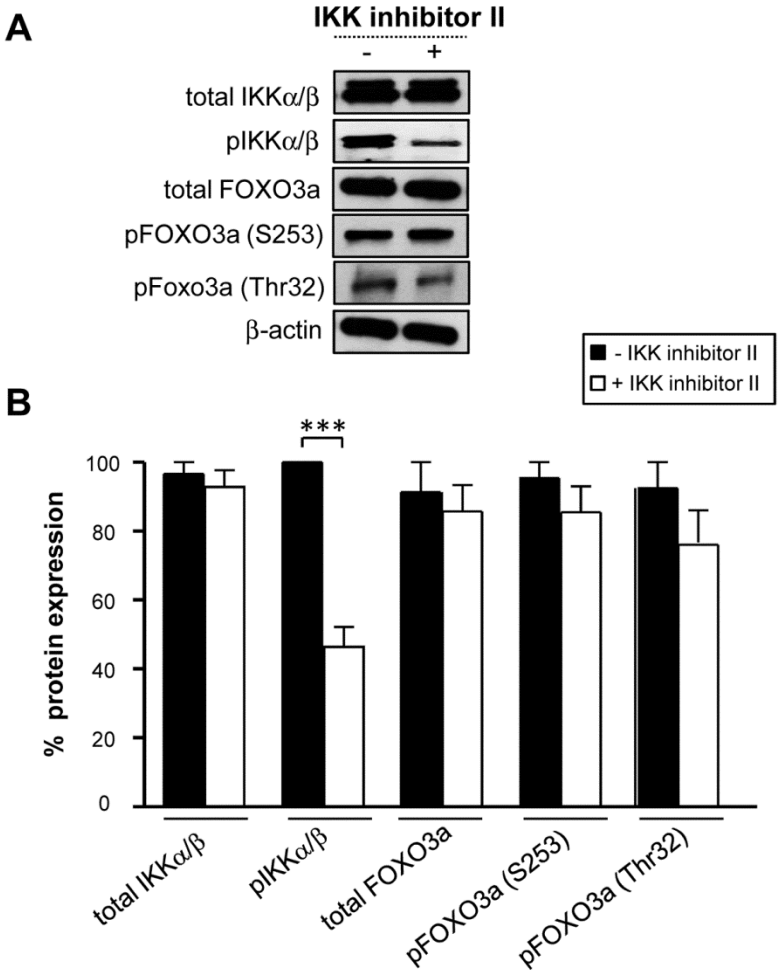


Figure S2. Inhibition of IKK does not impact the phosphorylation of FOXO3a.

(A, B) Briefly, activated $CD4^+$ T cells were transduced for 48 h with LVP_{Tax} in the presence or absence of IKK inhibitor II. (B) Representative blots at 48 h post-transduction are shown for pFOXO3a and pIKK expression profiles. (C) Densitometric quantification of three independent experiments was performed using ImageJ software (mean ± SD).

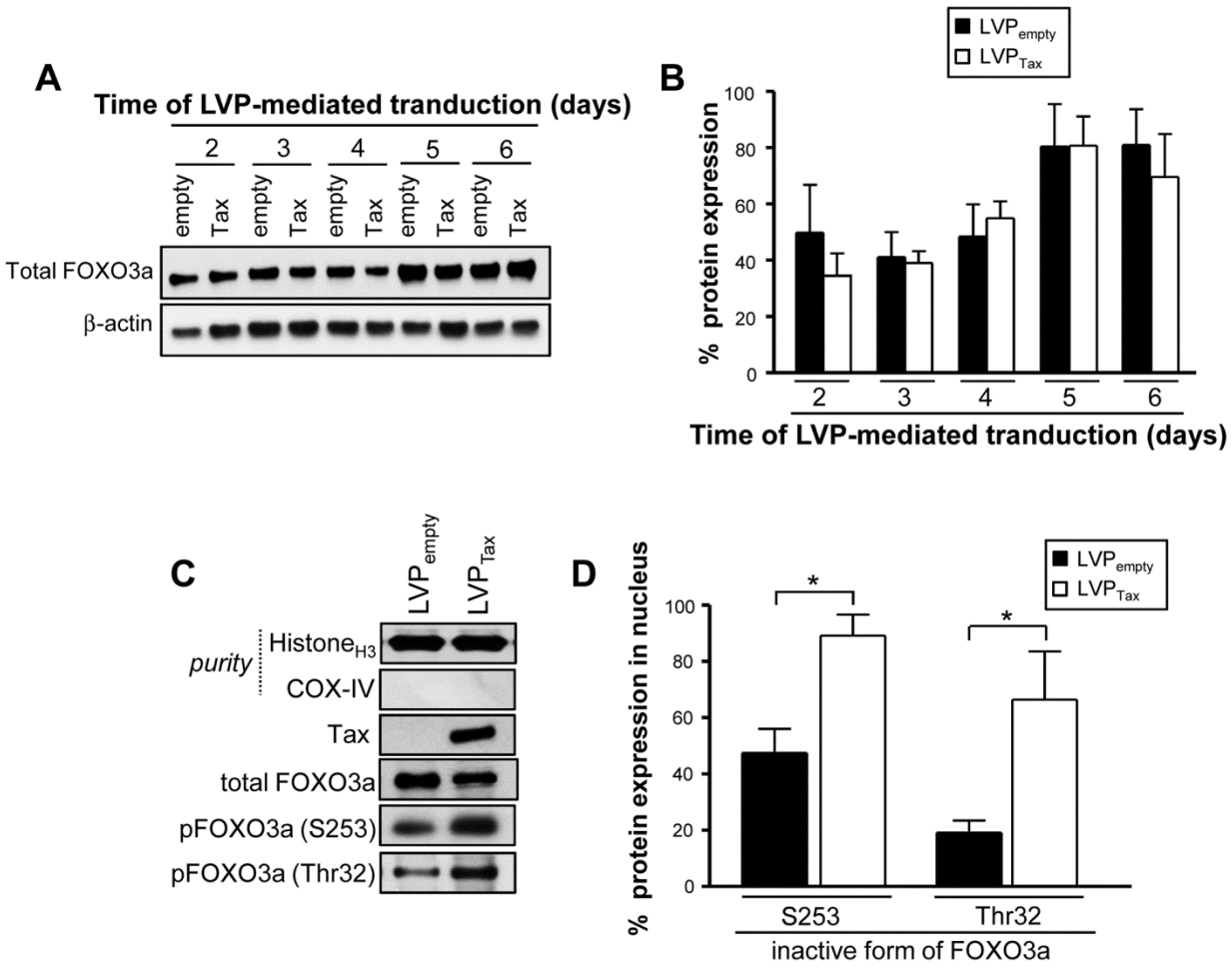


Figure S3. Tax expression does not alter the expression of total FOXO3a, but results in increased nuclear localization of inactive phosphorylated forms.

(A, B) Purified $CD4^+$ T cells were transduced with LVP_{empty} or LVP_{Tax} for 2 to 6 days and collected at each time points. (A) Western blot analysis of total FOXO3a expression was performed on transduced cells until 6 days. (B) Densitometric quantification of specific bands was performed using ImageJ software (n=3). (C, D) After two days of transduction, $CD4^+$ T cells were collected and subjected to nuclear extraction as previously performed [4]. (C) Western blots performed on purified nuclear fractions in the presence or absence of Tax expression. Purity of nuclear fractions was determined using antibodies against nuclear ($Histone_{H3}$) and cytosolic (COX-IV) proteins. (D) Densitometric quantification of three independent experiments for the expression levels of pFOXO3a forms in the nuclear fractions (mean ± SD).

iii. Tax-mediated FOXO3a inactivation is responsible for $CD4^+$ T cell persistence

We further investigated whether Tax promoted the persistence of activated $CD4^+$ T cells through AKT induction and subsequent FOXO3a inactivation. Tax transduction alone [blue] was sufficient to maintain T cell survival for 28 days, and maintenance of the differentiated T cell population was mediated through AKT signaling, since the addition of the AKT_i reduced T cell viability to basal levels (**Figure 4C**) (half-lives of transduced T cells with LVP_{empty} [black] and LVP_{Tax}+AKT_i [green] were 20.5 and 25.7 days, respectively). Tax expression was also associated with the terminally differentiated phenotype, similar to that of HTLV-1 productively infected-T cells (**Figure 4D**). A majority of the Tax-transduced $CD4^+$ T cells belonged to the $CD3^+CCR7^{neg}CD27^{neg}$subset at 28 days after Tax transduction (55.2±6.3% and 23.9±6.3% for LVP_{Tax} and LVP_{empty} infected T cells, respectively; $P<0.01$). In addition, a strong correlation was established between the number of viable primary $CD4^+$ T cells at 28 days post-transduction (**Figure 4C**) and the inhibition of FOXO3a signaling observed as early as 2 days pi (**Figures 4A,B**), and measured by phosphorylation of AKT ($P<0.0001$), pFOXO3a-S253 ($P=0.0031$), pFOXO3a-Thr32 ($P=0.0138$), and expression of p130 ($P=0.0138$) (**Figure 4E**). Altogether, these results demonstrate that long-term survival of activated $CD4^+$ T lymphocytes is mediated by a Tax-dependent, AKT phosphorylation and inactivation FOXO3a transcriptional activity.

iv. Specific inhibition of FOXO3a activity mimics Tax expression

Based on the above results, we rationalized that the T cell persistence observed during HTLV-1 infection or Tax transduction could be reproduced by introduction of a dominant negative form of FOXO3a, termed FOXO3a Nt[15], that encompasses the N-terminal DNA binding domain of FOXO3a (aa1–304) but lacks the C-terminal transactivation domain. FOXO3a Nt acts as a competitive DNA binding inhibitor of transcriptionally active FOXO3a[15, 36] and interferes with FOXO3a activation of pro-apoptotic and anti-proliferative target genes. As shown in Figure 5A, lentiviral-mediated transduction of FOXO3a Nt prevented primary T cells from undergoing apoptosis (**Figure 5A**) and thus mimicked Tax function. Expression of FOXO3a Nt also inhibited endogenous FOXO3a activity, as determined by reduced expression of p130 and Bim (**Figure 5B**). FOXO3a Nt expression resulted in persistence of a highly activated, terminally differentiated $CD4^+$ T cell population, similar to that observed with Tax expression (**Figures 5C,D**). In addition, we found an increased percentage of terminally differentiated $CD4^+$ T cells in the presence of specific FOXO3a siRNA at 14 days of culture (**Figures 5E,F** and **Figures S4**). Finally, we sought to determine if Tax physically interacted with an inactivated FOXO3a; by co-immunoprecipitation from Tax-transduced primary T cells, we did not observe an interaction between Tax and FOXO3a, although the interaction between Tax and PI3K was detected (**Figures S5**) [9] It is possible that Tax-PI3K association stimulates AKT activity and thus indirectly contributes to phosphorylation of FOXO3a by AKT. Collectively, these results demonstrate that Tax expression enhanced T cell longevity and activation, through the inhibition of FOXO3a transcriptional activity, mediated by AKT phosphorylation at S253 and Thr32 residues.

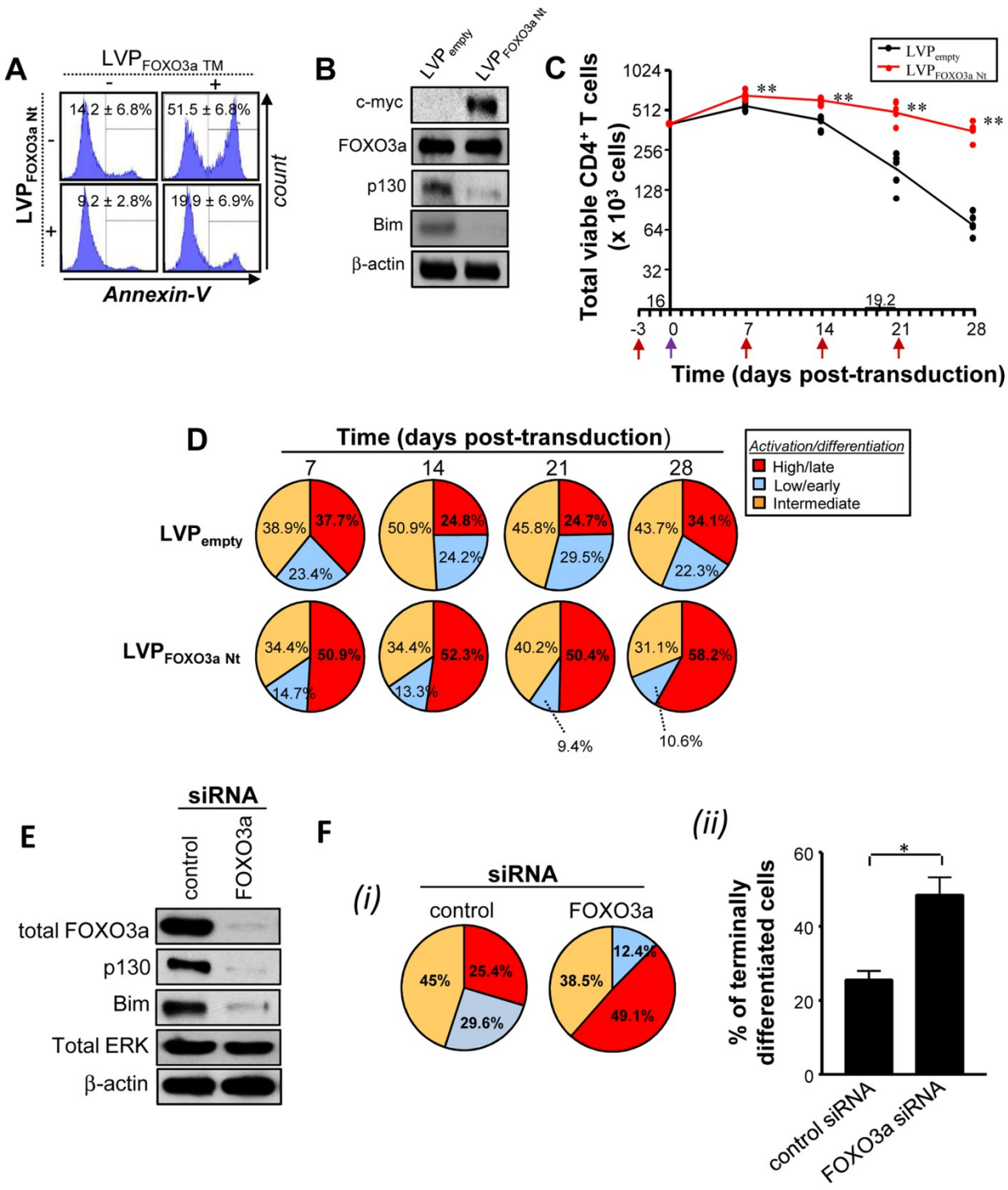


Figure 5. Specific inhibition of FOXO3a activity mimics Tax expression.

(A–D) Purified $CD4^+$ T cells were pre-activated for 3 days with anti-CD3 and anti-CD28 antibodies and then transduced or not with LVP a dominant-negative form of Foxo3a (Nt) for 2–

28 days. (A) Cells were transduced for 48 h with either LVP expressing FOXO3a Nt, or FOXO3a TM (dominant positive), or both. Histograms shown are representative of three independent experiments indicating Annexin-V staining. (B) Immunoblot analyses are performed at 2 days post-transduction to validate the expression of FOXO3a Nt form (c-myc tagged) and subsequent inhibition of endogenous p130 expression (n=3). (C) Absolute numbers of total viable $CD3^+$ T cells were determined by trypan blue exclusion at 7–28 days. *P* values were determined based on the comparison with LVP_{empty}-transduced cells. The underlined numbers represent the half-life of cultured LVP_{empty} condition (n=5). (D) Differentiation status of FOXO3a Nt-transduced T cell subsets at 7–28 days. Pie charts are representative of raw data from five independent experiments. (E, F) Purified $CD4^+$ T cells were transfected with control or FOXO3a-specific siRNA and then cultured for two weeks in the presence of TCR triggering. (E) The efficiency of FOXO3a silencing was monitored by immunoblotting after 72 hours of transfection. Western blot analysis of FOXO3a and its targets (p130 and Bim) was performed on transfected cells. The expression of total ERK was also assessed to appreciate the specificity of protein silencing. (F) Differentiation status of FOXO3a siRNA-transfected T cell subsets at 14 days. (*i*) Pie charts are representative of raw data from three independent experiments. (*ii*) % of terminally differentiated $CD45RA^{+/-}CCR7^{neg}CD27^{neg}$T cells in the presence or absence of FOXO3a silencing are also shown (mean ± SD).

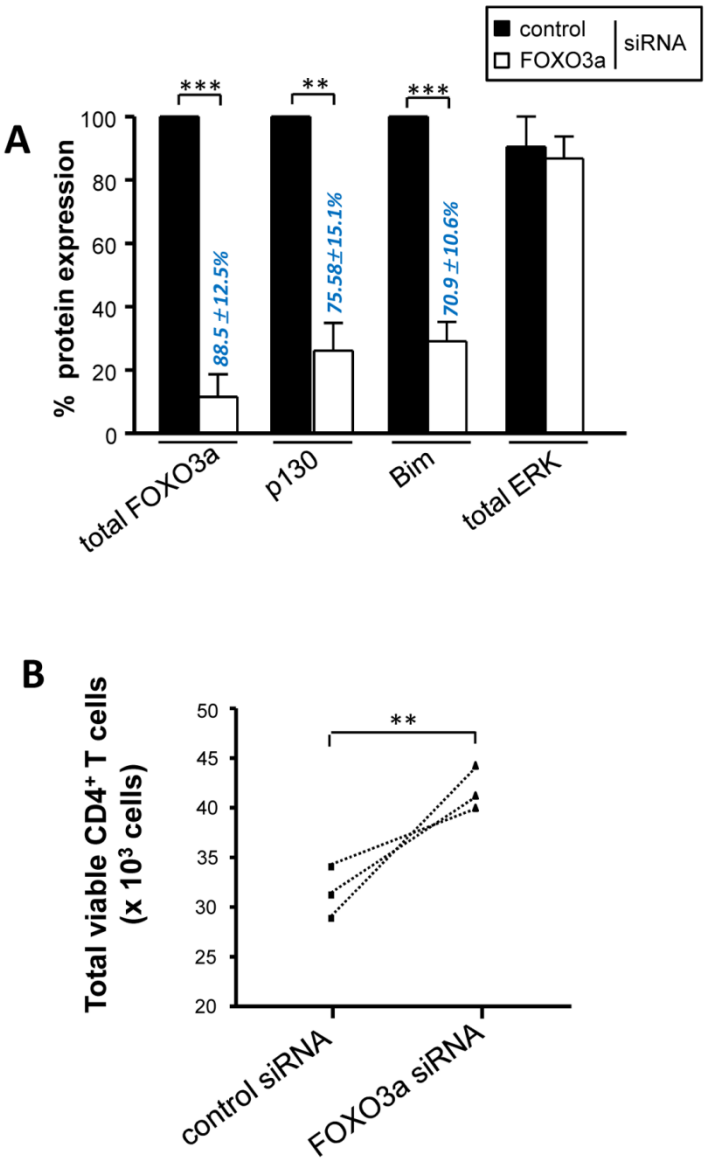


Figure S4. Silencing FOXO3a expression using small interfering RNA (siRNA) increased the number of CD4$^+$ T cells (n=3).

(A, B) Purified CD4$^+$ T cells were transfected with control or FOXO3a-specific siRNA and then cultured for two weeks in the presence of TCR triggering. (A) The efficiency of FOXO3a silencing was monitored by immunoblotting after 72 hours of transfection using Western Blotting. Results shown are the densitometric analysis of bands using ImageJ software. (B) At day 14 of culture, transfected CD4$^+$ T cells were also collected to assess numbers of total viable

$CD4^+$ T cells that were determined by trypan blue exclusion. *P* values were determined based on the comparison with LVP_{empty}-transduced cells.

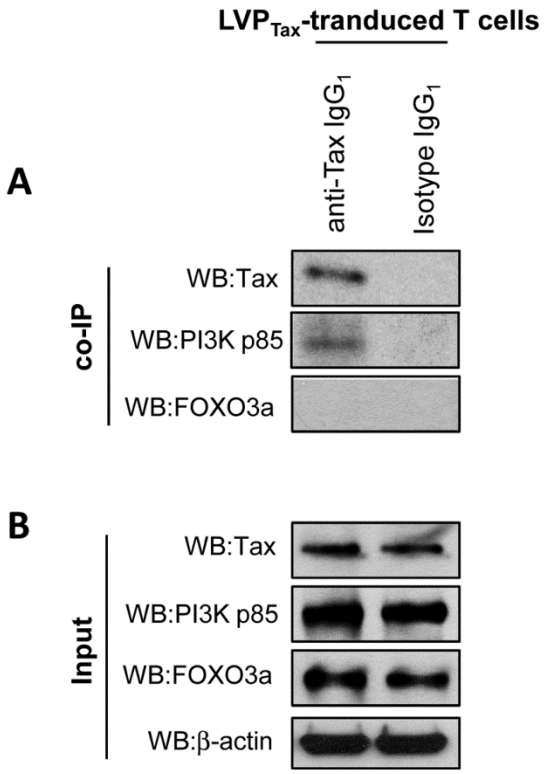


Figure S5. HTLV-1 Tax interacts with PI3K but not FOXO3a (n=4).

Primary $CD4^+$ T cells were transduced for 48 h with LVP_{Tax}, then collected and lysed using CHAPS buffer to assess (A) anti-Tax co-immunoprecipitation (co-IP). Representative blots from two separate experiments are shown, including (B) the "input" fractions. We also included lysates that were immunoprecipitated with an isotype IgG1 (instead of anti-Tax antibody) as a negative control to help differentiate non-specific background signal from specific antibody signal.

4. Discussion

HTLV-1 infection is associated with the expansion and leukemic transformation of $CD4^+$ T lymphocytes, driven in large part by the chronic disruption of host signaling networks by the HTLV-1 Tax oncoprotein[8, 31, 37, 38]. In the present study, we demonstrate that HTLV-1 infection enhances the *in vitro* cellular persistence of activated $CD4^+$ T cells, the expansion of terminally differentiated ($CD3^+CCR7^{neg}CD27^{neg}$) cells and the functional inactivation of the FOXO3a pathway (illustrated by the increased localization of inactive FOXO3a in the nucleus and the inhibition of several targets such as Bim and p130). Mechanistically, both *de novo* HTLV-1 infection and Tax transduction stimulated AKT activation and downstream phosphorylation of FOXO3a at residues S253 and Thr32 (**Figures 2** and **4A,B**). Mechanistically, we did not observe an interaction between Tax and FOXO3a (**Figure S5**), although the interactions between Tax and PI3K was detected as previously reported [9]. It is possible that Tax-PI3K association stimulates AKT activity and thus indirectly contributes to phosphorylation of FOXO3a by AKT. Also, we cannot exclude the possibility that post-translational modifications other than phosphorylation (such as acetylation, methylation, ubiquitination) may impact FOXO3a activity[36, 39]. In addition, since mRNA and protein levels of Tax are generally barely detectable in ATL cells displaying constitutively active AKT [40, 41], it is possible that other Tax-independent mechanisms of FOXO3a inactivation may be used by HTLV-1. Nevertheless, Tax-transduced T cells displayed a global inhibition of FOXO3a activity, illustrated by reduced expression of many pro-apoptotic and anti-proliferative target genes such as *BIM, FASL, NOXA, p27 and p130* at 24–48 h post-transduction (**Figures 3B,C**). Overall this study provides new mechanistic insights by which Tax potentiates the long-term maintenance of $CD4^+$ T lymphocytes following HTLV-1 infection.

FOXO3a activity is also targeted by another HTLV-1 accessory protein, HBZ, which was shown to inhibit FOXO3a by interfering with its localization and ability to bind DNA[13]. The mechanistically distinct, yet functionally redundant, inhibition of FOXO3a signaling may be explained by the distinct kinetics of expression of these two regulatory proteins. It has been reported that, in contrast to Tax, HBZ is transcribed at high levels in chronically infected patient samples [42], Conversely, even though Tax mRNA expression is relatively moderate, it is at its highest during the early stages of infection, specifically within the first week [43]. Additionally,

Tax controls FOXO4 activity through degradation by the proteasome during ATL development [11]. The inhibition of FOXO3a or FOXO4 activity by distinct HTLV-1 accessory mechanisms also highlights the importance of FOXO inactivation as a strategy to perpetuate HTLV-1 infected $CD4^+$ T lymphocytes and to contribute in the ATL development.

Using a BioMark^{TM} high throughput qPCR analysis (**Table S1**), we demonstrated that Tax not only mediated $CD4^+$ T cell persistence through the inactivation of the FOXO3a pathway, but also down regulated type I IFN responses **(Figure S1A)**, in part mediated by the negative regulator of the JAK-STAT-1 pathway SOCS1 [7, 34]. Taken together with our findings, these data indicate an involvement of Tax oncoprotein in targeting FOXO3a to concomitantly modulate the cell survival, as well as the type I IFN antiviral responses in $CD4^+$ T cells and thus facilitate HTLV-1 infection.

The identification of a pivotal role for FOXO3a in *de novo* HTLV-1 infection of $CD4^+$ T cells in terms of cellular differentiation and persistence survival may have important consequences for retroviral pathogenesis. For instance, alterations in the microenvironment mediated by HIV infection significantly increase FOXO3a activity, with a major impact on T and B cell immunity and survival[14, 15, 44, 45]. Kino *et al*. reported that the HIV accessory protein Vpr inhibited the ability of insulin to induce FOXO3a phosphorylation via AKT, thus interfering with its exclusion from the nucleus [46]. The expression of HIV-1 regulatory molecule Tat in specific T cells and macrophages also induced FOXO3a-mediated apoptosis[24, 47]. FOXO3a activity also impacts the pathogenesis and the outcome of Abelson murine leukemia virus[29].

Several gene networks/pathways that were deregulated in Tax expressing $CD4^+$ T cells were similarly disrupted in transcriptome analyses of PBMC from HTLV-1 infected individuals[34, 48, 49] (**Figure S5B**). For instance, transcriptional analysis of differentially regulated pathways demonstrated that cytokines IL15, IL17R, IL7R were down regulated, and chemokine CXCR4 was up regulated in ATL patients; in contrast TNFRSF17 was down regulated while granzyme B and IL-2 were up regulated, in HAM/TSP and AC individuals (**Figure S5B**). It is tempting to speculate that disruption of signalling mechanisms identified early after *de novo* HTLV-1 infection are also important in the development and maintenance of HTLV-1 associated pathologies and could be targeted for clinical treatment.

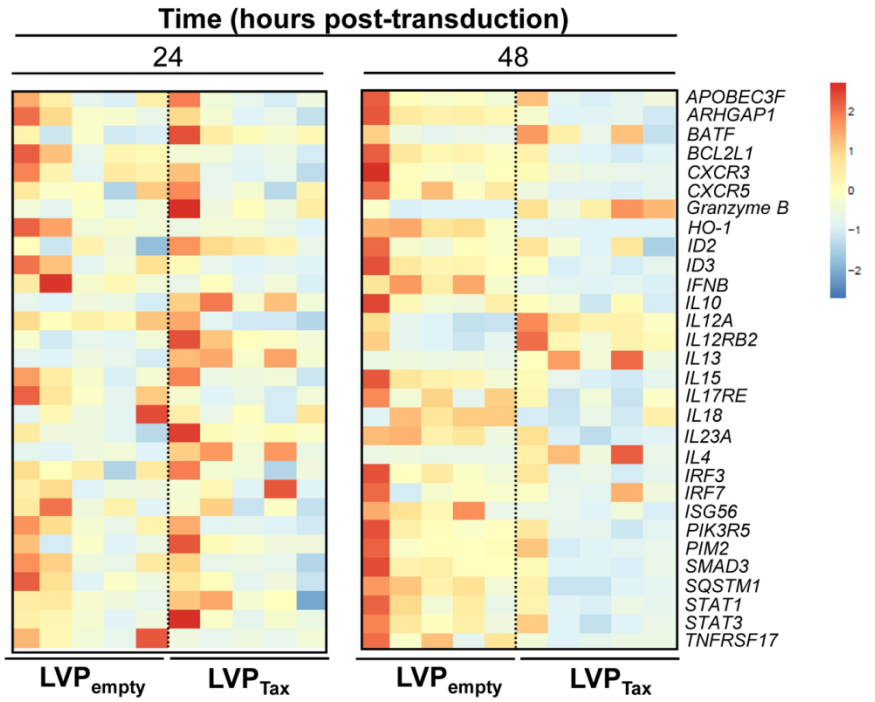
Due to poor prognosis of patients diagnosed with ATL, coupled with limited therapeutic options, novel immunological approaches including recombinant IL-7, IFN-α, and neutralizing anti-CD25 or anti-CXCR4 antibodies are currently being used to treat ATL and HAM/TSP patients [50-53]. The present study indicates that the PI3K-AKT-FOXO3a pathway may also represent a potential therapeutic target in ATL patients. Since AKT inhibitors are already in clinical development[54], they may offer a valuable addition to current therapeutic approaches.

SUPPLEMENTAL TABLE 1				
Gene		Sequence (5' to 3')	Probe	Accession
ACTB	F	attggcaatgagcggttc	11	NM_001101.3
	R	tgaaggtagtttcgtggatgc		
AKT1	F	ggctgaagagatggaggtgt	3	NM_005163.2
	R	ggatcaccttgccgaaagt		
AKT3	F	ttgctttcagggctcttgat	22	NM_005465.4
	R	cataatttcttttgcatcatctgg		
APOBEC3F	F	agcctggagcagaaagtgaa	50	NM_145298.5
	R	tcgctccactgtgtttctga		
APOBEC3G	F	gagcgcatgcacaatgac	18	NM_021822.3
	R	gccttcaaggaaaccgtgt		
ARHGAP1	F	ccagcatccacttgaaacct	10	NM_004308.2
	R	tccatcacacgctgtagctc		
B2M	F	ttctggcctggaggctatc	42	NM_004048.2
	R	tcaggaaatttgactttccattc		
BATF	F	acacagaaggccgacacc	85	NM_006399.3
	R	cttgatctccttgcgtagagc		
BCL2	F	agtacctgaaccggcacct	75	NM_000633.2
	R	gccgtacagttccacaaagg		
BCL2L1	F	gctgagttaccggcatcc	55	NM_138578.1
	R	ttctgaagggagagaaagagattc		
BCL6	F	cgaatccacacaggagagaaa	9	NM_001706.4
	R	acgcggtattgcaccttg		
BIM	F	catcgcggtattcggttc	70	NM_138621.4
	R	gctttgccatttggtctttttt		
CCR6	F	tggtgagctggagtcatcag	41	NM_031409.3
	R	cagcggtagcaggaaagtaga		
CCR7	F	ggggaaaccaatgaaaagc	77	NM_001838.3
	R	acctcatcttgacacaggcata		
CD4	F	gttgcatcaggaagtgaacct	50	NM_000616.4
	R	cccacacctcacaggtcaa		
CD40L	F	tcatgaaaacgatacagagatgc	2	NM_000074.2
	R	cttcgtcctctttgtttaacatt		
CDKN1B	F	tttgacttgcatgaagagaagc	60	NM_004064.3
	R	agctgtctctgaaagggacatt		
CTLA4	F	tcacagctgtttctttgagca	21	NM_005214.3
	R	aggctgaaattgcttttcaca		
CXCL13	F	ctgaatggatacaaagaatgatgg	47	NM_006419.2
	R	tcagcatcagggaatctttctc		
CXCR3	F	ccatggtccttgaggtgag	79	NM_001504.1
	R	tccatagtcataggaagagctgaa		
CXCR4	F	ggtggtctatgttggcgtct	18	NM_001008540.1
	R	actgacgttggcaaagatga		
CXCR5	F	gccatgaactacccgctaac	65	NM_001716.3
	R	tctgtccagttcccagaaca		
DDB1	F	tgtcgatatgtgcgtggtg	80	NM_001923.3
	R	cttccttgaaagccccaga		
FASL	F	tggggatgtttcagctcttc	17	NM_000639.1
	R	tgtgcatctggctggtagac		
FOXO1	F	aagggtgacagcaacagctc	11	NM_002015.3
	R	ttccttcattctgcacacga		
FOXO4	F	acgagtggatggtccgtact	18	NM_005938.3
	R	gtggcggatcgagttcttc		
FOXP3	F	gagaagctgagtgccatgc	20	NM_001114377.1
	R	agcccttgtcggatgatg		
GADD45	F	gtgtacgaagcggccaag	40	NM_001924.2
	R	agccacatctctgtcgtcgt		
GAPDH	F	agccacatcgctcagacac	60	NM_002046.3
	R	gcccaatacgaccaaatcc		
GATA3	F	cgagcaacgcaatctgac	70	NM_002051.2
	R	agtccctcctcgggtcac		
GRANZYME A	F	gattttcaggttgattgatgtgg	5	NM_006144.3
	R	ggagttacttcatttcctccaataa		
GRANZYME B	F	gggggacccagagattaaaa	37	NM_004131.4
	R	ccattgtttcgtccataggag		
GRANZYME K	F	caaagaatgaggcctccaaa	47	NM_002104.2
	R	gagttttgcggctgtttga		
HMOX-1	F	ggcagagggtgatagaagagg	15	NM_002133.2
	R	agctcctgcaactcctcaaa		
ICOS	F	ggatgcatacttatttgttggctta	47	NM_012092.3
	R	tgtattcaccgttaggtcgt		
ID2	F	atatcagcatcctgtccttgc	5	NM_002166.4
	R	aaagaaatcatgaacaccgctta		
ID3	F	catctccaacgacaaaaggag	59	NM_002167.4
	R	cttccggcaggagaggtt		
IFNb	F	ctttgctattttcagacaaagattca	20	NM_002176.2
	R	gccaggaggttctcaacaat		
IFNg	F	ggcattttgaagaattggaaag	21	NM_000619.2
	R	tttggatgctctggtcatctt		
IKAROS	F	caatgtgctcatggttcacaa	47	NM_006060.4
	R	gttgcccttctgggtgaat		
IL10	F	gatgccttcagcagagtgaa	67	NM_000572.2
	R	gcaacccaggtaacccttaaa		
IL12A	F	cactcccaaaacctgctgag	50	NM_000882.3
	R	caatctcttcagaagtgcaagg		
IL12RB2	F	ggagcagagatcttcgttgg	50	NM_001559.2
	R	cccctgttctcccttctgta		
IL13	F	cagtgccatcgagaagacc	89	NM_002188.2
	R	ggacatgcaagctggaaaac		
IL15	F	caaacaacagtttgtcttctaatgg	65	NM_037840.2
	R	gacaatatgtacaaaactctgcaaaaa		
IL17A	F	tgggaagacctcattggtgt	8	NM_002190.2
	R	ggatttcgtgggattgtgat		
IL17F	F	ggcatcatcaatgaaaacca	39	NM_052872.3
	R	tggggtcccaagtgacag		
IL17RE	F	cacactgtagagctgccttatgaa	29	NM_153480.1
	R	tcctcttgcaggtaggatgc		
IL18	F	caacaaactatttgtcgcagga	66	NM_00124321
	R	caaagtaatctgattccaggttttc		
IL1RL2	F	tggagtgtccacagcatacat	24	NM_003854.2
	R	gcgataagccctcctatcaa		
IL2	F	aagttttacatgcccaagaagg	65	NM_000586.3
	R	aagtgaaagttttgctttgagcta		
IL21	F	aggaaaccaccttccacaaa	7	NM_021803.2
	R	gaatcacatgaagggcatgtt		
IL23A	F	agcttcatgcctccctactg	30	NM_016584.2
	R	ctgctgagtctcccagtggt		
IL23R	F	ctgggctaacagttgcttcc	82	NM_144701.2
	R	caaagacgatcattcccaataaa		
IL2RA	F	acggaagacaaggtggac	54	NM_000417.2
	R	tgcctgaggcttctcttca		
IL2RB	F	acccctcgaagttcttttcc	25	NM_000878.2
	R	agggcgaagagagccact		
IL4	F	gaaacggctcgacaggaac	57	NM_000589.2
	R	ctctggttggcttccttcac		
IL4R	F	cgtctgcctgttgtgctatg	9	NM_000418.2
	R	ggaatctgatcccaccattc		
IL7	F	aatggtcagcatcgatcaatta	56	NM_000880.3
	R	aattcattattcaggcaattgctac		
IL7R	F	ggagaaaagagtctaacctgcaa	12	NM_002185.2
	R	gatgtattaaatgtcaccacaaagtca		
IRF3	F	cttggaagcacggcctac	18	NM_001571.4
	R	cgggaacatatgcaccagt		
IRF7	F	agctgtgctggcgagaag	72	NM_001572.3
	R	ttggagtccagcatgtgtg		
ISG56	F	gcctaatttacagcaaccatga	50	NM_001548.3
	R	tcatcaatggataactcccatgt		
MAF	F	agcggcttccgagaaaac	55	NM_005360.4
	R	gcgagtgggctcagttatg		
MYC	F	caccagcagcgactctga	34	NM_002467.4
	R	gatccagactctgaccttttgc		
P130	F	ccatgaagtactcacaggcaaa	25	NM_005611.3
	R	ggggagcctgttcttacaaat		
P21	F	ccgaggcactcagaggag	70	NM_000389.3
	R	agctgctcgctgtccact		
PDCD1	F	cggccaggatggttctta	7	NM_005018.2
	R	gtgaaggtggcgttgtcc		
PIK3CA	F	cacgagatcctctctctgaaatc	15	NM_006218.2
	R	ggtagaatttcggggatagttaca		
PIK3R5	F	tgagcagatcctgcagaagac	2	NM_00114263
	R	gggtgagcaggtcgtaggt		
PIM1	F	atcaggggccaggttttc	13	NM_002648.3
	R	gggccaagcaccatctaat		
PIM2	F	gtggccatcaaagtgattcc	26	NM_006875.3
	R	ttcgagtgggcatgtgact		
PUMA	F	gacctcaacgcacagtacga	68	NM_00112724
	R	gagattgtacaggaccctcca		
PUMAbeta	F	tccctgccagatttgtg	30	NM_00112724
	R	cgactccaggtgctgctc		
SMAD3	F	gtctgcaagatcccaccag	79	NM_005902.3
	R	agccctggttgaccgact		
SOCS1	F	cccctggttgttgtagcag	50	NM_003745.1
	R	gtaggaggtgcgagttcagg		
SOD2	F	aatcaggatccactgcaagg	3	NM_000636.2
	R	taagcgtgctcccacacat		
SQSTM1	F	agctgccttgtacccacatc	14	NM_003900.4
	R	cagagaagcccatggacag		
STAT1	F	ttggcacctaacgtgctgt	68	NM_007315.3
	R	agttcgtaccactgagacatcct		
STAT3	F	ctgcctagatcggctagaaaac	65	NM_139276.2
	R	ccctttgtaggaaacttttgc		
Tbet	F	gtccaacaatgtgacccaga	34	NM_013351.1
	R	aaagatatgcgtgttggaagc		
TGFb1	F	gagcccaagggctaccat	29	NM_000660.4
	R	gggttatgctggttgtacagg		
TNFa	F	tccagacttccttgagacacg	36	NM_000594.2
	R	cccggtctcccaaataaatac		
TNFRSF10B	F	gtgtgtcagtgcgaagaagg	2	NM_003842.4
	R	gaccatccctctgggaca		
TNFRSF13B	F	tgcctgaaacactacccaataa	58	NM_006573.3
	R	agcagtttcaatgcaccaaa		
TNFRSF17	F	aggacgagtttaaaaacacagga	85	NM_001192.2
	R	atttcatcaccagtcctgctc		
TRAIL	F	cctcagagagtagcagctcaca	6	NM_003810.3
	R	cagagcctttcattcttgga		

Table S1: List of primers used for the Biomark analyses.

This list includes sequences and appropriate gene nomenclature.

A



B

	ACUTE (/LVP_{empty})		CHRONIC (/healthy)			Clinical treatments
	24h Tax transduction	48h Tax transduction	AC	HAM/TSP	ATL	
IL15		↓			↓	
IL17R	↓	↓			↓	
IL7R	↓	↓			↓	recombinant IL-7
CXCR4	↑				↑	anti-CXCR4 Abs
SOCS-1	↑		↑	↑		recombinant type I IFN ± AZT
granzyme B		↑	↑	↑		
TNFRSF17		↓	↓	↓		
IL-2	↑	↑		↑		anti-CD25 Abs ± AZT
IL-2R	↑	↑				

Figure S6. Transcriptome analysis of HTLV-1 Tax expressing $CD4^+$ T cells.

(A) Briefly, activated T cells transduced or not with LVP_{Tax} were collected at 24 and 48 h post-transduction and subjected to Biomark analysis (n=5). Heatmap analysis of genes (not shown in Fig. 3B) that were significantly modulated following Tax expression. (B) Table listing several genes that were dysregulated during both acute Tax transduction (blue) and in chronically

infected individuals (red). List includes genes which are currently involved as therapeutics in ATL and HAM/TSP patients.

Materials and methods

Products

RPMI-1640 media, FBS and antibiotics were provided by Wisent Technologies (CA, USA). Unconjugated anti-Tax mAbs (clone LT4) was generously provided by Dr. Yuetsu Tanaka (Kitasato University, Kanagawa, Japan). MT-2 cell lines were obtained from the ATCC^{TM} (VA, USA). All antibodies used for flow cytometry were purchased from BD Biosciences, except for the antibody to CD45RA-ECD, which was from Beckman Coulter. All primary antibodies used in Western Blots (anti-phospho forms of FOXO3a, anti-Bim, and anti-phospho-IKK Abs) were purchased from Cell Signaling Technology Inc., whereas anti-p130 and anti-actin were purchased from Sigma Aldrich; anti-FOXO3a from Abcam. 7-Aminoactinomycin D (7-AAD) came from Invitrogen.

Purification and activation of CD4$^+$ T cells

Leukaphereses from healthy donors were obtained from the Royal Victoria Hospital, Montreal (QC, Canada), with informed consent of the patients and in agreement with the Royal Victoria Hospital, the Jewish General Hospital, and McGill University Research Ethics Committee. PBMCs were isolated using Ficoll-Hypaque gradient and CD4$^+$ T cells were then purified using the untouched CD4 isolation kit (EasySep® Human CD4$^+$ T cell Enrichment Kit; StemCell Technologies, Vancouver, BC, Canada), allowing for more than 94% purification without any cell stimulation and apoptosis. CD4$^+$ T cells were then activated 72 hours in RPMI complete in the presence of 1 μg/mL anti-CD28 (BD Biosciences) in 6 well plates pre-coated 24 hours earlier with 0.5 μg/mL anti-CD3 (clone: OKT-3, BioLegend; 2.10^6 cells/well).

HTLV-1 trans-infection

Cell-cell transmission of HTLV-1 was performed essentially as previously described [26]. 20.10^6 HTLV-1 produced cell line MT-2 were first irradiated at 15,000 rads and then mixed at various ratios (2:1 to 1:8) of irradiated MT-2 to activated CD4$^+$ T cells. At several time points post-infection (pi), collected cells were treated with Cell Dissolution Solution non-enzymatic

according to the Sigma manufacturer's protocol and finally filtered (70μm), prior further analyses.

Flow cytometry

Surface staining: At day 2 pi, cells were first stained in calcium buffer with anti-CD3-PE Cy7 antibody (Ab) and Annexin-V-V450 in the presence or absence of anti-CD25-APC Cy7 or anti-HLA DR-Alexa700 Abs for 10 minutes at 4ºC. *HTLV-1 Tax quantitation*: Cells were washed twice and fixed at room temperature (RT) in BD FACS Lysing Buffer (Becton Dickinson) and incubated with anti-Tax-FITC and anti-p19-Alexa647 mAbs for 20 minutes at RT in 0.25% saponin. Anti-p19 IgG_1 antibody was conjugated to Alexa647 dye using the Zenon^R mouse IgG_1 labeling kit (number: Z25008; Life Technologies Inc., ON, USA) according to the manufacturer's protocol. *pAKT measurement*: AKT phosphorylation was measured with BD Bioscience PhosFlow using anti-pAKT (S473 residue) specific Ab as previously described [23].

Western blotting

Protein lysates (2-10 μg) from highly purified $CD4^+$ T cell subsets were subjected to Western blot analysis as previously described [4]. Densitometric quantifications of protein of interest (normalized to β-actin whose expression level was used as loading control) were calculated using ImageJ software.

Generation of recombinant lentiviruses

The lentiviral vector pWPI (empty vector), packaging plasmid psPAX2 and envelope plasmid pMD2G were generously provided by VGTI-Florida, whereas pCLXSN-Tax vector was purchased from Addgene (ref: 44038; MA, USA). The FOXO3a N-terminal (Nt) fragment was cloned into pWPI, and as previously described [15], lentiviral particles were produced in 293T cells that harboured either pWPI or pCLXSN (empty controls), pWPI-FOXO3a Nt, or pCLXSN-Tax expression vector. Titers (ng/mL) of lentiviral constructs were assessed using HIV p24 ELISA (Zeptometrix Corporation, USA).

Long-term persistence assays

$CD4^+$ T cells ($4X10^4$) were co-cultured in the presence of irradiated MT-2 cells (ratio = 1:1) in complete RPMI. At 3 days pi, cells were washed to remove the maximum of dead MT-2_i and cultured in the presence of 1µg/mL anti-gp46 Abs to avoid any *de novo* infection. On days 7, 14 and 21 pi, cells were re-stimulated by adding fresh anti-CD3 and anti-CD28 Ab in the presence of anti-gp46 Abs to avoid any *de novo* infection. The efficiency of neutralizing anti-gp46 Ab was confirmed by the absence of p19 staining on primary cells that were treated since the onset of co-culture (*data not shown*). At days 3, 7, 14, 21 and 28 pi, viable cultured cells were counted by trypan blue exclusion and stained with 7-AAD, anti-CD3-PE, anti-CD45RA-ECD, anti-CD27-APC H7, anti-CCR7-PE Cy7 and anti-Tax-Alexa 647 Ab for flow cytometry analyses.

Fluidigm BioMark™ assays

Total RNA was isolated from cells using RNeasy Kit (Qiagen, Valencia, USA) as per manufacturer's instructions. RNA was reverse transcribed using the SuperScript® VILO cDNA synthesis kit according to manufacturer's instructions (Invitrogen, Carlsbad, USA). PCR primers were designed using Roche's Universal Probe Library Assay Design Center (www.universalprobelibrary.com) and ordered from the Integrated DNA Technology company (IDT, USA) (Supplementary Table 1). cDNA along with the entire pool of primers were pre-amplified for 14 cycles using TaqMan® PreAmp Master Mix as per manufacturer's protocol (Applied Biosystems, Foster City, USA). cDNA were exonuclease treated to get rid of excess primers using Exonuclease I (*E. coli*) (New England Biolabs, Ipswich, USA). cDNA samples were prepared with 2X FastStart TaqMan® Probe Master (Roche, Penzberg, Germany), GE sample loading buffer (Fluidigm, San Francisco, USA) and Taq Polymerase (Invitrogen, NY, USA). Assays were prepared with 2X assay loading reagent (Fluidigm, NY, USA), primers (IDT) and probes (Roche, Penzberg, Germany). Samples and assays were loaded in their appropriate inlets on a 48.48 BioMark chip. Chip was run on the Biomark™ HD System (Fluidigm, San Francisco USA) and enabled quantitative measurement of up to 48 different mRNAs in 48 samples under identical reaction conditions. Raw Ct values were calculated after 40 cycles by the real time PCR analysis software (Fluidigm, San Francisco, USA) and software designated failed reactions were discarded from analysis. All data are presented as a relative quantification with efficiency correction based on the relative expression of target gene versus the mean of (*gapdh+actin+β2 microglobulin*) as the invariant control. The N-fold differential

expression of mRNA gene samples was expressed as $2^{\Delta\Delta Ct}$. The heatmap was produced with the R package pheatmap (http://CRAN.R-project.org/package=pheatmap) and gene expression is shown as gene-wise standardized expression (Z score).

Statistical analysis

Statistical analyses were performed as previously described [4]. ***, $P < 0.001$; **, $P < 0.01$ and *, $P < 0.05$.

Author contributions

DO and AS performed experiments and helped write the paper; SBH and ZH designed recombinant lentiviral constructs; CC and CS completed the Biomark™ analyses and generated heatmaps and ingenuity network; RL made corrections to the text and figures; JH and JvG conceived the study, designed experiments, supervised the experiments, and wrote the paper.

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Chapter 5: Discussion

The objectives of this thesis as described in the Rationale and Objectives section were met and published in two distinct primary research articles. HTLV-1 infection led to depletion and persistence in monocytes and $CD4^+$ T-cells respectively. This data coincides with *in vivo* observations regarding the outcomes of cellular fates for these cells, and elucidates the early molecular events following *de novo* HTLV-1 infection. Considerable development has recently been made on the topic of SAMHD1 and retroviral infection of myeloid cells, and is the focus of the following discussion. The role of senescence and FOXO3a inactivation by Tax during HTLV-1 infection will also be discussed in a later subsection.

1. The Detection of Reverse Transcribed Intermediaries by STING

An important outstanding question raised in chapter 3 remains regarding which sensor was responsible for the detection of the RTI (reverse transcribed intermediaries) that induced apoptosis. While many DNA receptors have been identified such as TLR7, cGAS and IFI16, cGAS seems to have taken a prominent role in the detection of cytosolic DNA[1], and STING remains absolutely vital for downstream activation following DNA stimulation[2]. Many groups have identified cGAS as the sensor for HIV-1 DNA[3-6], and our own unpublished results suggest a similar conclusion in terms of HTLV-1. Interestingly, HIV-1 strong stop DNA, the first single stranded DNA product made during reverse transcription, was analyzed to discover what sequence motifs were important for IFN production[7]. Herzner *et al.* discovered that stem loop structures and guanosines were vital for the induction of IFN. The strong stop DNA of HTLV-1 contains several SL (stem loop) structures, and the synthetic RTI used in chapter 3 and contained a guanosine trimer overhang that is vital for IFN activation (unpublished results). Most relevant, was the data that this cGAS mediated response to HIV-1 infection in MDM (monocyte derived macrophages) was strongly correlated with the production of negative strand DNA, the same type of ligand likely detected during HTLV-1 infection in primary monocytes.

The apoptosis observed in our system was similar to the one described by several other groups studying abortive HIV-1 infection in primary human T-cells. One group demonstrated that once

$CD4^+$ T-cells isolated from PBMC were infected with HIV-1, a significant portion of them died via apoptosis, and little productive infection was recorded. The cells could be rescued from apoptosis by viral entry inhibitors, but not other drugs affecting later steps of the viral lifecycle. Cell death could also be reduced via mitogenic stimulation[8], likely causing SAMHD1 inactivation and the cellular pool of dNTP to rise[9, 10].

A series of papers from the laboratory of Dr. Greene using primary tonsil $CD4^+$ T-cells also demonstrated that abortive HIV-1 infection resulted in an inflammatory form of cell death called pyroptosis. Here too cellular activation increased survival, and the addition of various anti-retroviral agents strongly suggested that long incomplete cytoplasmic HIV-1 DNA was the cause of apoptosis[11]. Intriguingly, in their experiments they found that cell-free virus, at comparable concentrations to our own experiments, did not induce apoptosis, and cell-to-cell infection was required[11]. These results were expanded in a subsequent report, which demonstrated that a small number of cells were required to be productively infected and physically interact with uninfected cells in order to induce cell death. Increasing infectivity via spinoculation did not result in cell death when a single-round virus was used, but did when a multiple-round virus was used[12].

This data suggests some difference in the apoptotic stimuli between monocytes and $CD4^+$ T-cells. While RTI alone are sufficient to induce apoptosis in monocytes, additional cell-to-cell signals are required for T-cell pyroptosis. However, the source of HTLV-1 virus in chapter 3 was concentrated supernatants of MT2 cells that produce viral biofilms. These supernatants were not filtered, suggesting that infection could have been mediated by viral biofilms instead of cell-free virions, which have significantly different infectious properties[13]. Consequently, unknown extracellular signals may be present in HTLV-1 biofilms that may play an important role in HTLV-1 induced apoptosis in monocytes. It is also possible that only cell-to-cell infection causes enough RTI to accumulate in the target cell to activate cell death pathways in $CD4^+$ T-cells.

Additional follow-up reports identified IFI16 as the receptor responsible for the induction of pyroptosis, cGAS was undetectable in tonsil $CD4^+$ T-cells and intriguingly, cell death was independent of STING despite its expression[14]. Pyroptosis was mediated by caspase-1 activation in non-productively infected cells, as caspase-3 and type-1 IFN inhibitors did not

improve cell viability[15]. Several studies have shown that caspase activation, including caspase-1[16, 17], can dampen the cGAS-STING signaling pathway[18, 19], suggesting the caspase-1 inhibitors may, in addition to preventing $CD4^+$ T-cell depletion, enhance immune detection of RTI in these abortively infected cells.

Many reports have begun to elucidate the DNA signaling pathway induced by various DNA receptors in different cell types. One group reported that IFI16 and cGAS cooperated in human MDM to activate STING and IFN signaling in response to HIV-1 DNA[20]. A subsequent report confirmed the role of cGAS in IFN signaling following retroviral infection in activated $CD4^+$ T-cells and MDDC, but did not investigate IFI16[3]. Ku70 may also play a role in DNA detection through STING in MDM during HTLV-1 infection[21]. In human fibroblasts, IFI16 activation induced antiviral cytokine production including IFN, but was somewhat distinct from cGAS-STING activation that could induce apoptosis[22]. Indeed several groups have now demonstrated that the c-GAS-STING-TBK1-IRF3-Bax pathway can be activated by numerous stimuli to induce apoptosis[23-25]. Overall this indicates a complex antiviral response that varies depending on the cell type and the profile of DNA receptors and downstream adaptor molecules[26].

Peripheral blood $CD4^+$ T-cells were largely resistant to pyroptosis following infection, which was associated with decreased viral DNA production and IFI16 expression. Tonsil $CD4^+$ T-cells displayed higher levels of activation that while insufficient to allow productive infection, was capable of producing enough viral DNA to induce pyroptosis[27]. Although SAMHD1 has been identified as key factor responsible for abortive infection in blood $CD4^+$ T-cells, its activity has never been associated with pyroptosis[28, 29]. As the activity of SAMHD1 is closely tied to the activation status of the cell[9, 10], it seems likely that SAMHD1 is an important player in the depletion of $CD4^+$ T-cells observed during HIV-1 infection as the primary inducer of RTI accumulation.

Another mechanism of retroviral-mediated cell death was also identified, this time in activated $CD4^+$ T-cells, that also relies on a DNA signaling pathway[30]. Here DNA damage induced by retroviral integration was detected by DNA-PK, and induced p53-mediated cell death. 2-LTR

circle formation and viral protein production did not induce apoptosis, while integrase inhibitors could prevent cell death.

2. The Interactions between Innate Immune Signaling and SAMHD1

Several studies have characterized SAMHD1 as a negative regulator of the immune response. Supporting this are numerous pieces of data, such as non-functional SAMHD1 mutations leading to inflammatory conditions such as Aicardi-Goutèires syndrome[31]. As discussed in chapter 2, HIV-2 is significantly less pathogenic than HIV-1, and packages Vpx that can degrade SAMHD1 in order to productively infect myeloid cells. It has been proposed that productive infection of myeloid cells by HIV-2 would increase *in vivo* antigen presentation, and explain why an HIV-1 ancestor lost the *vpx* gene[32, 33].

However, one report showed that DC co-culture with other cells types such as T-cells stimulated the DC and reduced their restriction to HIV-1 infection via downregulation of SAMHD1[34]. These cells were then able to induce IFN production in response to HIV-1, and suggest that in *in vivo* conditions retroviral infection of myeloid cells is possible. It also indicates that the loss of the *vpx* gene may not be related to immune evasion in myeloid cells.

Many groups have demonstrated that the removal of SAMHD1 in myeloid cells such as MDDC and MDM, is required during HIV-1 infection to render the cells responsive to infection in a cGAS dependent manner that induces IFN production[5, 35, 36]. Intriguingly, the study by *Puigdomènech et al.* demonstrated that immune activation occurred due to a post-integration step, suggesting that the initial wave of RTI was undetectable. This is similar to what was reported in tonsil T-cells that required cell-to-cell infection[12]. In activated $CD4^+$ T-cells and MDDC, cGAS was responsible for IFN production, but this was mediated by *de novo* viral protein synthesis after integration. A single-cycle virus could also not induce immune signaling, again suggesting the RTI production was undetectable in this model as well[3].

Indeed the sensitivity of primary human monocytes to RTI described in chapter 3 is likely the result of the repertoire of DNA receptors and adaptor molecules uniquely expressed in them. For instance, one report demonstrated that $CD4^+$ T-cells express significantly higher levels of STING than MDM and responded differently to the same stimuli; apoptosis versus IFN production

respectively[26]. However, overexpression of STING in MDM resulted in similar induction of apoptosis. A comparison between various monocyte derived cells was performed by *Paijo et al.* and showed that MDDC and MDM express relatively low levels of STING[37]. Our own unpublished results suggest that monocytes express high levels of STING like $CD4^+$ T-cells, suggesting that MDDC and MDM could be unresponsive to RTI due to relative low expression of STING and other unknown factors responsible for RTI detection.

This was exemplified in a recent report investigating the different consequences of HIV-1 infection in MDDC and mature-MDDC, which were produced with different cytokine cocktails[38]. Consistently, MDDC only responded to HIV-1 infection when treated with Vpx to degrade SAMHD1. Mature-MDDC however, demonstrated the opposite result and only initiated an immune response to HIV-1 when SAMHD1 was present. Viral entry was comparable in both cell types, but MDDC expressed less cGAS. Altogether mature-MDDC behaved much more similarly to monocytes than MDDC did during retroviral infection.

These observations may also be due to intrinsic differences between HTLV-1 and HIV-1. HIV-1 capsid interaction with cyclophilins may sequester viral nucleic acids in such a way that greatly attenuate viral DNA detection[4, 6]. It is currently thought that HTLV-1 possesses different mechanisms related to cyclophilin binding and uncoating.

3. The Involvement of SAMHD1 in Cancer and NRTI Drugs

Since the manuscript presented in chapter 2 was published in late 2013, there have been additional publications on SAMHD1 that have expanded our knowledge regarding its function. One of the most intriguing developments was the link between SAMHD1 and cancer[39]. SAMHD1 is often down regulated[40-42], or mutated in cancer[41, 43-46]. The mechanism seems to be related to changes in dNTP pool that impact DNA replication fidelity[46, 47]. SAMHD1 expression is linked to inhibition of cellular proliferation and apoptosis induction[40, 47, 48]. Furthermore, SAMHD1 activity is tightly tied to the cell cycles. SAMHD1 Interacts with cell cycle proteins, including CDK1 which phosphorylates and inactivates it[9, 47, 49]. Indeed,

Depletion of SAMHD1 can arrest the cell cycle as this leads to a deregulated dNTP pool, an important cell cycle checkpoint[50, 51].

SAMHD1 activity may also protect malignant cells from certain chemotherapies by degrading anti-proliferative nucleoside analogs[52, 53]. Similarly, SAMHD1 silencing causes HIV-1 to be significantly less sensitive to NRTI (Nucleoside reverse transcriptase inhibitors), including AZT[54-56]. These common antiretroviral drugs are incorporated into reverse transcription DNA products, but terminate DNA elongation and thus restrict viral replication. Once inside the cell, they are phosphorylated and made active by cellular kinases. SAMHD1 increases the efficacy of NRTI by lowering the concentration of the competing dNTP. However, the exact mechanism is more complicated, as while SAMHD1 tends to have little effect on anti-retroviral NRTI and does not cleave them, it does influence the efficiency of their activation/phosphorylation by cellular enzymes[57].

The precise beneficial mechanism of AZT treatment during ATL is not known. Although a recent study demonstrated that it along with ABC (abacavir), another NRTI used during HIV-1 infection, induced significant cell death of ATL cells. This appears to be mediated by the incorporation of ABC into genomic DNA, where it induced severe DNA damage due to the weakened DNA repair machinery[58]. A subsequent study indicated that the HTLV-1 accessory protein HBZ suppresses the key machinery used to prevent ABC mediated cytotoxicity in healthy cells[59]. Since SAMHD1 seems to potentiate the anti-retroviral activity of ABC[54, 56], it may serve an important role in mediating the efficacy of NRTI treatments in ATL patients.

To my knowledge, no direct evidence exists of the capacity of HTLV-1 to modulate SAMHD1 function or expression, but several lines of indirect evidence supports this possibility. Firstly, HTLV-1 deregulates the cell cycle, which could have indirect alterations on SAMHD1. Secondly, the SAMHD1 promoter can also be inhibited by miR-30a, miR-181a, and miR-155[60]. miR-181a, and miR-155 seem to be deregulated during HTLV-1 infection[61, 62]. Overall further study is required to establish if HTLV-1 directly modulates SAMHD1.

4. RNAse Functions of SAMHD1

While the general consensus of the scientific community appears to be that SAMHD1 prevents retroviral replication by depleting the intracellular dNTP pool required for reverse transcription, there exists some data that would suggest additional mechanisms of restriction. Phosphorylation of SAMHD1 controls its anti-HIV-1 properties, but not its ability to hydrolyze dNTP[49, 63, 64]. Furthermore, SAMHD1 seems to possess exonuclease activity that could mediate additional antiretroviral functions[65-67]. Dr. Ahn's group demonstrated that SAMHD1 restricted HIV-1 replication via ribonuclease activity that degraded the gRNA of incoming viral particles[68, 69].

However this data remains controversial, as many independent studies have shown no RNAse activity is associated with SAMHD1-mediated retroviral restriction[70-72]. It was proposed that residual contamination was the reason for the observed RNAse function of SAMHD1[73]. Furthermore, as a nuclear protein[74], it remains unclear how SAMHD1 would interact with gRNA in the cytoplasm. One of the key findings supporting the RNAse paradigm was repeated, whereby a dNTPase deficient mutant of SAMHD1 that could not bind to activating GTP ($SAMHD1_{D137N}$) could still restrict HIV-1 replication, presumably through non-dNTPase functions[75]. However an independent group demonstrated that $SAMHD1_{D137N}$ could still hydrolyze dNTP, indicating that the previously observed retroviral restriction was in fact mediated by dNTPase function, and not through RNAse activity.

It is known that SAMHD1 D137 mutants have impaired dNTPase activity[76-78]. This site is part of the Allo-site 1 that binds to the activating GTP/dGTP. The interaction is mediated by the formation of 6 hydrogen bonds between the SAMHD1 amino acids at D137 (2 bonds), Q142 (1 bond), R145 (2 bonds), and V117 (1 bond) and GTP, while dGTP forms 5 bonds at the same residues except V117[79]. Another purine base like adenine would simply not be able to form most of these interactions and does not activate the dNTP triphosphate hydrolase activity of SAMHD1[80]. When mutated, most groups have demonstrated SAMHD1 inactivity via D137A mutations, whereby the aspartic acid was replaced with an alanine amino acid[76-78]. This likely

abolishes a significant portion of the overall binding capacity between GTP/dGTP and SAMHD1, as alanine cannot form any hydrogen bonds[80].

Curiously, Dr. Ahn's group that demonstrated the RNAse function of SAMHD1 developed and used a D137N mutant instead of the D137A. Replacing aspartic acid with asparagine, and in their hands it controversially retained no dNTPase function. However, the side chain of asparagine is the same length as aspartic acid, and it is similarly capable of forming two hydrogen bonds via its terminal oxygen and nitrogen, indicating that it may still bind to GTP. This analysis would suggest that despite a mutation in Allo-site 1, $SAMHD1_{D137N}$ could still hydrolyze dNTP.

Dr. Ahn responded to these criticisms by stating that different conditions are likely the reason they observe RNAse activity[81]. They have subsequently shown that SAMHD1 is a phosphorolytic ribonuclease instead of the presumed hydrolytic one[82].

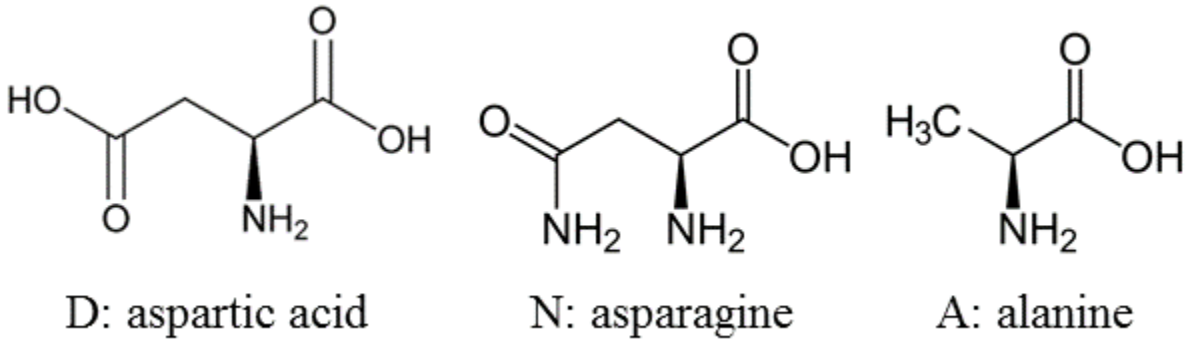

Figure 1: Structures of various amino acids mutated at side 137 in SAMHD1.

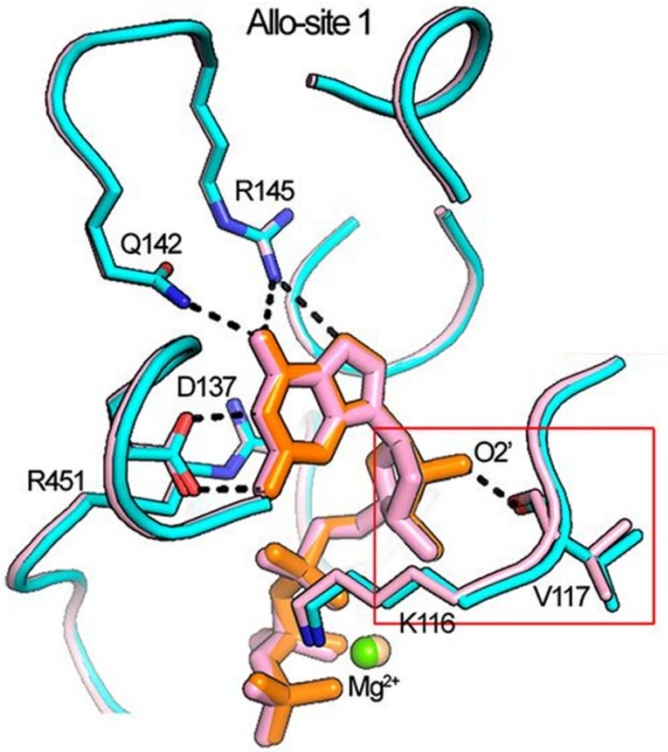

Figure 2: Schematic representation of the Allo-site 1 of SAMHD1. Presented here is aspartic acid at residue 137 among other binding sites. Mutation at this residue to an asparagine could likely maintain both hydrogen bonds, indicated here as dashed lines. In blue; SAMHD1 protein, in orange; activating GTP. Highlighted in the orange box is the additional hydrogen bond formed between SAMHD1 and GTP, which will not occur during dGTP activation. Adapted from[78].

5. Senescence Mediated Infection

It is known that HTLV-1 infection can lead to senescence in most cells via Tax-mediated NF-κB activation[83-85]. One of the main observations we recorded in chapter 4 was that the infected primary $CD4^+$ T-cells persisted in the long term over the non-infected samples, but did not seem to proliferate, suggesting that most of these cells were senescent. The cells were capable of viral transmission however, and suggest that Tax-induced senescence is not quite a dead end for HTLV-1. Senescent cells have previously been reported to attract macrophages[86] and $CD4^+$ T-cells[87], which could lead to their infection in *in vivo* settings[88]. The downregulation of FOXO3a helps to protect these infected senescent cells from cell death, promoting viral transmission for as long as possible. It is also likely that FOXO3a deregulation protects the infected cells from the powerful anti-Tax CTL response during the intermittent cycles of Tax expression. The subsequent burst of HBZ that downregulates Tax would then no longer necessitate the anti-FOXO3a mechanism for survival.

Surprisingly, we did not detect an upregulation in activated IKK during HTLV-1 infection between Tax positive and Tax negative cells. We did however record an overall increase in IKK phosphorylation in the infected samples compared to controls, although this data was not included in the publication as it was statistically weak. We determined that in our model, the PI3K/AKT pathway was primarily responsible for FOXO3a deactivation, while other studies have also involved NF-κB[89-91].

6. Concluding Remarks

The goal of this project was to discover the consequences of early HTLV-1 infection in primary human monocytes and $CD4^+$ T-cells. Infection of inactivated monocytes was found to be non-productive and resulted in their depletion, a finding that has recently been confirmed in patient samples[92]. Cell-to-cell infection of activated $CD4^+$ T-cells lead to sustained cellular survival in those that were productively infected, mediated by Tax activation of the PI3K/AKT pathway that inactivated the pro-apoptotic transcription factor FOXO3a. This may represent an important deregulated cellular pathway exploited during HTLV-1 infection. These HTLV-1 infected Tax

expressing cells likely form an apoptosis-resistant viral reservoir that spreads HTLV-1 infection in the host by possibly taking advantage of cellular senescence.

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