Multiple levels of Protein Kinase R inhibition during Human Immunodeficiency Virus replication by double-stranded RNA binding proteins and their relationship to the innate cell response to viral infection

Presented by

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Sometimes our fate resembles a fruit tree in winter. Who would think that those branches would turn green again and blossom, but we hope it, we know it.

Johann Wolfgang von Goethe

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CONTRIBUTION TO KNOWLEDGE

During the course of this PhD program, I worked on the host innate immunity against the immunodeficiency virus type 1, HIV-1. We are the first to report that the protein kinase RNA-activated, PKR, which is central to host antiviral innate response, is activated following HIV-1 infection. Overall, our data show an active innate response against HIV-1 replication, which is regulated at multiple levels. My work on ADAR1 interaction with PKR and its implication during HIV-1 replication was the subject of one published primary paper. It will constitute the bulk of another primary publication that will be submitted in the next few weeks. I am currently writing a review on PKR regulation during HIV replication and its relationship to innate immunity. It will also be submitted shortly. Furthermore, I have contributed to several other publications and one review .

Contributed papers:

1. **Clerzius G**, Daher A, Gélinas J-F, Boulassel MR, Routy J-P, Mouland AJ, Patel R, Gatignol A. 2010. The PKR activator, PACT is a PKR inhibitor during HIV-1 replication. Tentative title. In preparation.

2. **Clerzius G**, Gélinas J, Gatignol A. 2010. Multiple levels of PKR inhibition during HIV replication. Review. Tentative title. In preparation.

3. **Clerzius G**, Gelinas JF, Daher A, Bonnet M, Meurs EF, Gatignol A. 2009. ADAR1 interacts with PKR during human immunodeficiency virus infection of lymphocytes and contributes to viral replication. *J Virol* 83: 10119-28

4. Laraki G, **Clerzius G**, Daher A, Melendez-Pena C, Daniels S, Gatignol A. 2008. Interactions between the double-stranded RNA-binding proteins

TRBP and PACT define the Medipal domain that mediates protein-protein interactions. *RNA Biol* 5: 92-103

5. Bannwarth S, Laine S, Daher A, Grandvaux N, **Clerzius G**, Leblanc AC, Hiscott J, Gatignol A. 2006. Cell-specific regulation of TRBP1 promoter by NF-Y transcription factor in lymphocytes and astrocytes. *J Mol Biol* 355: 898-910

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7. Ong CL, Thorpe JC, Gorry PR, Bannwarth S, Jaworowski A, Howard JL, Chung S, Campbell S, Christensen HS, **Clerzius G**, Mouland AJ, Gatignol A, Purcell DF. 2005. Low TRBP levels support an innate human immunodeficiency virus type 1 resistance in astrocytes by enhancing the PKR antiviral response. *J Virol* 79: 12763-72

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ABSTRACT

Following infection by most viruses, an antiviral state is induced in the host cells characterized by the expression of interferon (IFN) and several IFN-stimulated genes (ISGs). IFN treatment is effective to inhibit HIV replication in infected cells, but shows no significant improvement of HIV-infected patients. Currently, the discrepancy between the *in vitro* and the *in vivo* findings remains largely unresolved. The IFN-induced RNA-dependent protein kinase PKR is activated via trans-phosphorylation and plays a central role in the IFN-induced antiviral pathway. Our results show that PKR is transiently activated following HIV-1 infection of Jurkat and peripheral blood mononuclear cells. The kinase is then inactivated at the viral peak, when HIV replication is highly active. By immunoprecipitation, we found that PKR forms a ribonucleoprotein complex with cellular double-stranded RNA binding proteins (dsRBPs), the TAR RNA Binding Protein (TRBP), the adenosine deaminase acting on RNA (ADAR)1 and the PKR Activator (PACT) during HIV replication. Over-expression of PKR is sufficient to inhibit HIV production in HEK 293T cells. This inhibition is reversed by expression of the ADAR1, another ISG. By using mutants of ADAR1, we show that this activity is linked to the ability of the protein to bind PKR. In astrocytes that do not replicate HIV efficiently due to an enhanced PKR response, ADAR1 partially restores viral expression. Surprisingly, PACT binds to and inhibits PKR activity. All three dsRBPs, TRBP, ADAR1 and PACT prevent PKR activation and the phosphorylation of its downstream target, eIF2α. Together, our results highlight the key function of PKR in innate immunity and its multiple-level of regulation during HIV-1 replication.

RÉSUMÉ

L'infection d'une cellule par un virus induit un état antiviral, caractérisé par l'expression de l'interféron (IFN) et de plusieurs gènes induits par l'IFN. Le traitement par l'IFN est efficace pour inhiber la réplication du virus de l'immunodéficience humaine (VIH) dans des cellules infectées en culture, mais ne montre aucun effet bénéfique chez les patients infectés par ce même virus. Cette disparité qui existe entre les résultats de recherche *in vivo* et *in vitro* n'est toujours pas résolue. PKR, une protéine kinase induite par les IFNs, est activée par phosphorylation et joue un rôle central dans le mécanisme antiviral de l'IFN. Nos résultats démontrent que PKR est activée de manière transitoire suite à l'infection de cellules lymphocytaires Jurkat ou de lymphocytes/monocytes primaires du sang périphérique par le VIH. Par contre, la kinase n'est plus activée durant et après le pic d'infection, lorsque la réplication du virus est intense. Par immunoprécipitation, nous avons démontré que PKR forme un complexe ribonucleoprotéique avec plusieurs protéines cellulaires qui lient l'ARN double-brin, soit la protéine liant l'ARN TAR, TRBP, l'adénosine déaminase ADAR, ainsi que la protéine activatrice de PKR, PACT, pendant la réplication virale. La surexpression de PKR est suffisante pour inhiber la production du VIH dans les cellules HEK 293T. Cette inhibition est supprimée par l'expression d'ADAR1, une des protéines induite par les IFNs. Par différentes mutations dans la séquence protéique d'ADAR1, nous avons démontré que cette activité d'ADAR est liée à sa capacité de lier PKR. Dans les astrocytes, qui ne répliquent pas le VIH efficacement en raison d'une activation accrue de PKR, ADAR1 rétablit partiellement l'expression virale. Étonnamment, PACT se lie à PKR et inhibe son activité dans les cellules infectées par le VIH. Testées en parallèle, TRBP, ADAR1 et PACT empêchent l'activation de PKR ainsi que la phosphorylation de sa principale cible, eIF2α, dans les cellules infectées par le VIH. Pris ensemble, nos résultats mettent en valeur la fonction clé de PKR dans l'immunité innée ainsi que sa régulation qui survient à différents niveaux durant la réplication du VIH.

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ABBREVIATIONS

ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ADAR	Adenosine deaminase acting on RNA
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ALV	Avian leukemia virus
CD	Cluster of differentiation
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DR	Death receptor
ds	Double-stranded
EBV	Epstein-Barr virus
eIF2α	Eukaryotic initiation factor 2 alpha
FDA	Food and Drug Administration
GALT	Gut-associated lymphoid tissue
GCN2	General control non-de-repressible 2
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HEK	Human embryonic kidney
HHV	Human herpes virus
HIV	Human immunodeficiency virus
HPV	Human papillomavirus

HRI	Heme-regulated inhibitor
KSHV	Kaposi sarcoma-associated herpesvirus
HSV	Herpes simplex virus
IFN	Interferon
IFNAR	Interferon alpha receptor
IKK	IκB kinase
IRF	Interferon regulatory factor
ISG	Interferon stimulated gene
kb	Kilobase
kDa	Kilo Dalton
LC	Langerhand cell
LTR	Long terminal repeat
Mx	Myxovirus-Resistance
Mϕ	Macrophage
Nef	Negative effector
NK	Natural killer
OAS	Oligoadenylate synthetase
PACT	PKR activator
PBMC	Peripheral Blood Mononuclear Cell
PDC	Plasmacytoid dendritic cell
PERK	PKR-like endoplasmic reticulum inhibitor
PIC	Preintegration complex
PKR	Protein kinase R
PRR	Pattern recognition receptor

RNA	Ribonucleic acid
RNAi	RNA interference
RISC	RNA interference silencing complex
RT	Reverse transcription/Reverse transcriptase
si	Small interfering
SIV	Simian immunodeficiency virus
SV40	Simian vacuolating virus 40
STAT	Signal transducer and activator of transcription
TANK	TRAF3 associated NF-κB activator
TAR	*Trans*-activation-responsive
Tat	*Trans*-activator of transcription
TIR	Toll/Interleukin-1 receptor-like
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-related Apoptosis-Inducing ligand
TRBP	TAR RNA binding protein
TRIF	TIR domain-containing adaptor inducing IFN-beta
TRIM	Tripartite motif
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U

CHAPTER 1

LITERATURE REVIEW

1.1 RETROVIRUSES

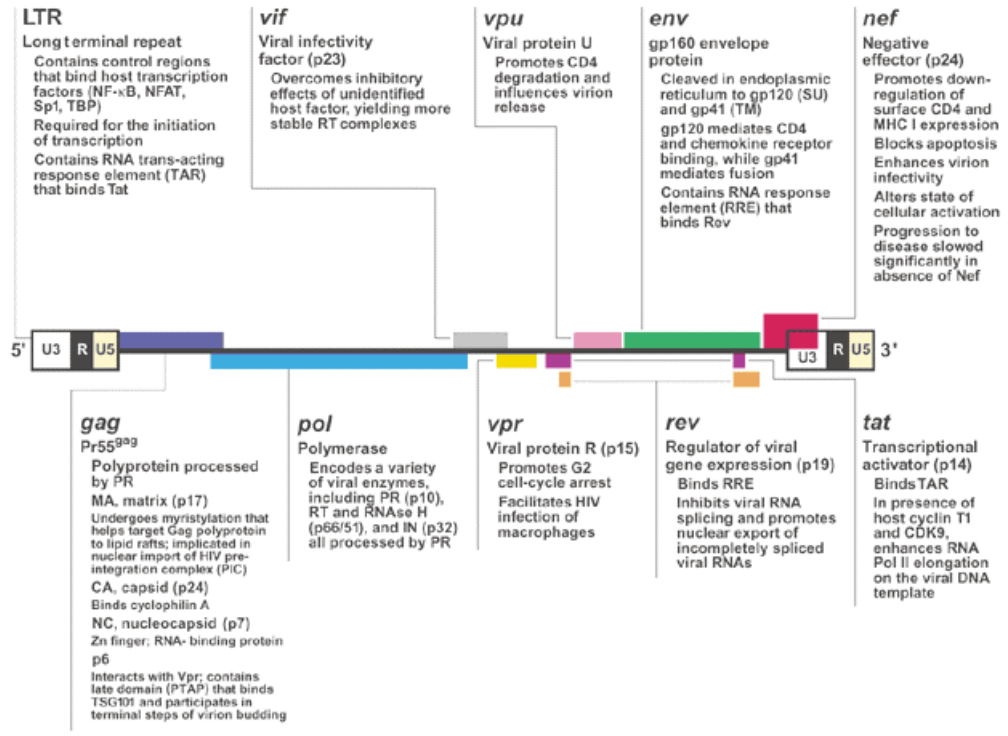
1.1.1 RETROVIRUS FAMILY

HIV and the closely related simian immunodeficiency virus (SIV) belong to the lentivirus genus of retroviruses. Retroviruses are a large group of structurally and phylogenetically related enveloped RNA viruses found naturally infecting a wide range of vertebrate species. Two subfamilies have been recognized: (1) the Orthoretrovirinae, comprising six genera, and (2) the Spumavirinae subfamily, only comprising the spumavirus genus (Table 1.1). Retroviruses were named due to their ability to reverse transcribe their single-stranded RNA genome into dsDNA that is incorporated into the infected cell chromosome. Retroviruses contain two copies of their RNA genomes and their genome organization and composition fall into two categories—simple and complex (1). The simple genome contains the three major coding domains specific to all retroviruses and coding for *gag*, which directs the synthesis of internal virion proteins that form the matrix, the capsid, and the nucleocapsid; the *pol* reading frame, encoding the information for the reverse transcriptase and integrase enzymes; and *env*, coding for the surface and transmembrane subunits of the envelope protein (Fig. 1.1) (2). All retroviruses also contain *pro*, a smaller domain coding for the protease enzyme. In complex retroviruses, *pro* is part of the *pol* gene, whereas in simple retrovirus it constitutes a gene on its own. In addition, retroviruses with a complex genome organization—comprising the Human T-cell leukemia viral group from the Deltaretrovirus subfamily, the Lentivirus, and Spumaviruses genus—code for several proteins which are not incorporated into the virion that are involved in regulatory and pathogenesis processes.

Genus	**Type species**
Subfamily *Orthoretrovirinae*	
Alpharetrovirus	Avian leukosis virus
Betaretrovirus	Mouse mammary tumor virus
Gammaretrovirus	Murine leukemia virus
Deltaretrovirus	Bovine leukemia virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Lentivirus	Feline immunodeficiency virus
Subfamily *Spumavirinae*	
Spumavirus	Human foamy virus

Table 1.1: Classification of the *Retroviridae* family.

A



B

Figure 1.1: Retroviral genome organization. (A) Overview of the organization of the HIV-1 complex provirus and a summary of the functions of its nine genes and 15 encoded proteins. Figure modified from (2) (B) Avian leukemia virus (ALV) simple retroviral genome.

1.1.2 MECHANISM OF PATHOGENICITY

Retroviral infection results in a broad range of pathogenicity separated into three different mechanisms: (i) malignancies by transfer of active oncogenes or (ii) activation of oncogenes by insertional mutagenesis, and (iii) direct killing of cells. Of the seven recognized retrovirus subfamilies, only the spumavirus and Lentivirus subfamilies are not classified as oncogenic retroviruses. Many strains of Rous sarcoma virus (RSV) harbor the *src* oncogene in their genomes, derived from the cellular proto-oncogene that has been captured from the host cell during co-evolution, and induce sarcoma in chicken. Other oncogenic retroviruses induce transcriptional activation of specific cellular genes following insertion. ALV, which induces bursal lymphoma in chickens, often activates the cellular proto-oncogene c-*myc*. Lentiviruses are cytopathic, causing diseases by killing target cells, resulting in loss of function. As for Spumaviruses, their discovery is based on the observation of the characteristic foamy-appearance that they induce on infected cells under the microscope. So far, they have not been linked to any disease or symptom.

1.2 HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1)

1.2.1 HISTORY

1.2.1.1 First Historical Manifestation

From 1979 to 1981, several cases of an acquired immunodeficiency syndrome afflicting young homosexual men and intravenous drug abusers in San Francisco and New York areas were reported (3, 4). These cases set the base for what we now know was the beginning of the HIV pandemic. The term “acquired immunodeficiency syndrome” (AIDS) was introduced in September 1982 and adopted by the Center for Disease Control (CDC) the same year. It describes the

markedly reduced circulating $CD4^+$ T cell count resulting in a combination of opportunistic infections and tumors. Since its first isolation in 1983 by Barré-Sinoussi and Montagnier (5), billions of dollars have been invested worldwide in HIV research and treatment. The virus has so far taken the lives of more than 25 million of individuals, and 33 million people currently live with the virus. Sub-Saharan Africa remains the most affected region, with a seroprevalence of more than 40% in some regions (Fig. 1.2) (6, 7).

1.2.1.2 Origin

The earliest known case of HIV infection in the human population is from a frozen plasma sample from a man living in Léopoldville from the Belgian Congo, now Kinshasa in the Democratic Republic of Congo, in 1959 (8). Phylogenetic analysis suggests that the virus might have been introduced in the human population around 1930 (9), as early as 1890, or perhaps a few hundreds years earlier. The virus is believed to have remained localized and confined inside a small group of individuals in Central Africa before its explosion following socio-economic changes around 1950 in Africa, and other unknown factors.

Two types of HIV are known to infect men, HIV types 1 and 2. Both have been established to arise in humans via zoonotic transmission of SIV from primates in Africa from between 3 and 7 independent cross-species transmission events respectively. For HIV-1, these events have given rise to the four phylogenetic lineages: the M (major), O (outlier), and the two newest groups N (for non-M, non-O, and P (for pending the identification of further human cases) (10, 11). The M group is responsible for the current pandemic. Naturally occurring SIV infection is found in more than 20 species of primates, all from Sub-Saharan Africa (9). Compelling evidence has definitively identified SIVcpz, harbored by the chimpanzee (*Pan troglodytes troglodytes*) in the wild, as the natural ancestor for HIV-1's three groups (Fig. 1.3) (9, 12, 13). However, the

possibility of a gorilla intermediate for transfer from chimpanzees to humans has not been ruled out for the O group (12, 14). Strains of SIV closely related to HIV-2 have only been found naturally in the sooty mangabeys (*Cercocebus atys*) from Western Africa (15). Phylogenetic analyses of MHC class I suggest that the current ape populations are contemporary offsprings of AIDS-resistant animals. An AIDS-like pandemic caused by SIVcpz or a related retrovirus is believed to have taken place 2-3 million years ago resulting in severe selective sweep of an ancient, genetically more MHC-I-diverse population of apes (16). These results suggest that SIV was pathogenic in its natural hosts in the past, just as HIV is now in humans. The fact that infected apes in the wild show no sign of disease, even with viral loads exceeding those found in human in final stages of AIDS, suggest that co-evolution with its host has made the virus non-pathogenic.

Hunting and consumption of primates and use of orphan animals as pets are believed to be contributing factors to the cross-species transmission of SIVs to human. Iatrogenic transmission to human from contaminated preparations of oral polio vaccine developed by a team led by Dr. Hilary Koprowski, and administrated to more than one million individuals in the Belgian Congo in the late 1950s, have long been suspected to be the source of the pandemic (17). However, phylogenetic data, in addition to several major inconsistencies do not support this hypothesis (18).

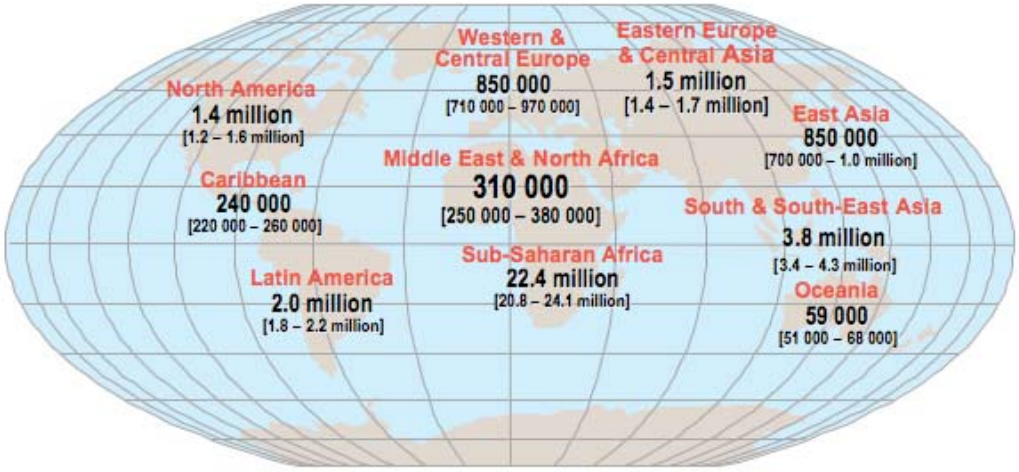


Figure. 1.2: Worldwide HIV-1 epidemic. Estimated number of individuals living with HIV-1, based on 2008 statistics from the United Nations Program on HIV/AIDS (UNAIDS). A total of 33.4 million (31.1 – 35.8) adults and children are infected worldwide.

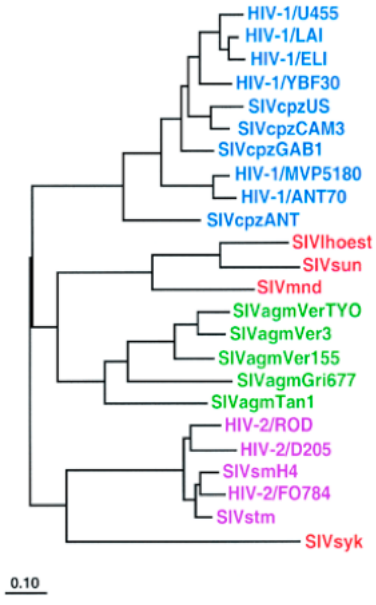


Figure 1.3. Primate lentiviruses evolutionary relationship based on maximum-likelihood phylogenetic analysis of full-length Polymerase protein sequences. The five major lineages are color-coded. The scale bar indicates 0.1 amino acid replacement per site after correction for multiple hits. Figure from (9) .

1.2.2 HIV REPLICATION

Dependence of retroviral replication on cell proliferation shows great variation among the different classes. While Gammaretroviruses such as the murine leukemia virus (MLV) infection are strictly dependent on cell proliferation, the Alpharetrovirus subfamily shows intermediate phenotype. HIV and other lentivirus replication is independent of the cell cycle and these viruses infect most non-dividing cells nearly as well as dividing cells. However, naïve quiescent $CD4^+$ T cells or G0 monocytes isolated from peripheral blood are refractory to HIV infection. The HIV replication cycle involves a series of twelve sequential steps: (1) attachment, (2) fusion and entry, (3) uncoating, (4) reverse transcription, (5) nuclear transport and translocation of the pre-integration complex (PIC), (6) integration, (7) transcription, (8) mRNA export into the cytoplasm, (9) translation, (10) assembly, (11) budding and (12) maturation (Fig. 1.4) (19).

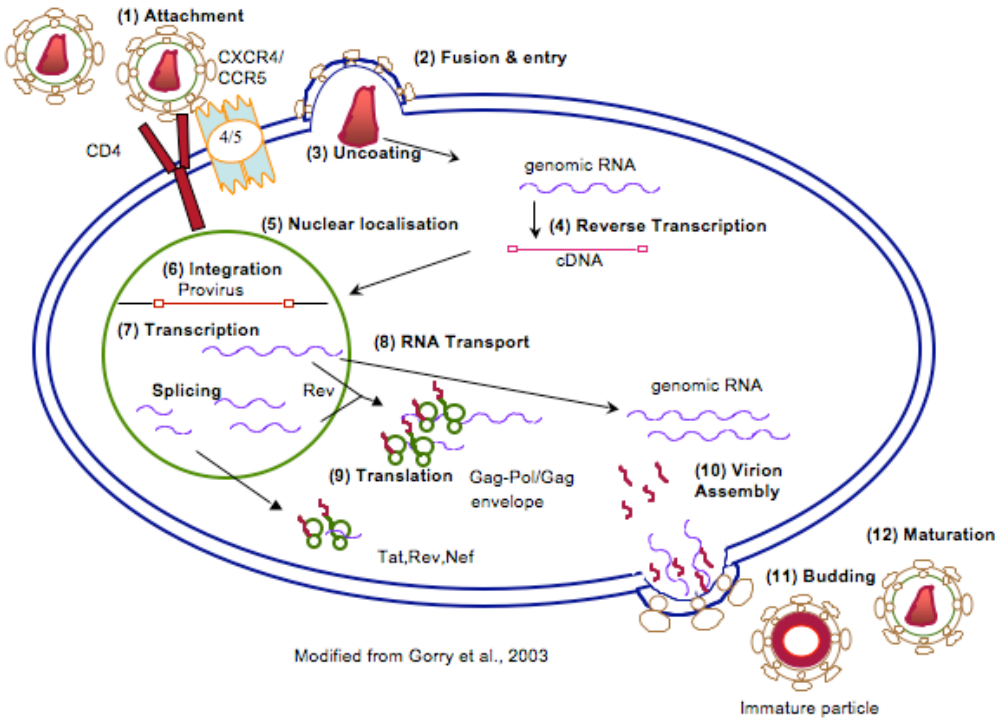


Figure 1.4: Overview of HIV-1 replication cycle. See text for details. Modified from (19).

1.2.2.1 Viral attachment and entry

The first step of infection, viral entry, is a complex series of sequential processes involving attachment, co-receptor binding, and fusion. Attachment of virions to the cell is mediated by the specific binding of HIV gp120 subunit to the CD4 receptor at the cell surface. This induces a conformational change exposing a high-affinity binding site located within the third variable loop (V3), essential for C-C chemokine receptor type 5 (CCR5) and CXC-chemokine receptor 4 (CXCR4) co-receptor binding (20). Further conformational rearrangements of gp120 and gp41 occur following co-receptor binding, exposing the fusion-peptide domain within gp41. This brings the viral and cellular membranes into close proximity, ultimately creating a fusion pore through which the viral core passes into the cell (21). Recent research in therapy has targeted the HIV entry, with Enfuvirtide being the first approved drug for therapy by the Food and Drug Administration (FDA) in 2003, followed by Maraviroc in 2007. Several other molecules are in phase I or II clinical trials.

1.2.2.2 Uncoating, reverse transcription and nuclear translocation

Immediately after the release of the viral core into the cytoplasm, the elusive and poorly defined uncoating step occurs. The partial and progressive disassembly of the core matrix leads to the generation of subviral particles called reverse-transcription complexes (RTCs) and pre-integration complexes (PICs). Reverse transcription of the viral genome into dsDNA is mediated by the viral reverse transcriptase (RT) enzyme and is believed to be coupled with the onset of uncoating of the core (22). RT inhibitors constitute the first class of antiretroviral drugs developed for HIV therapy. The enzyme tendency to commit errors during reverse transcription is the major cause of the virus' ability to mutate and diversify, resulting in a high immune evasion capacity and generation of drug resistance. Attempts to precisely define the composition of the PIC depend largely

on the technique used, but its main components have been identified. Besides the viral cDNA, the PIC is constituted of three different viral proteins, the integrase (IN), the matrix (MA), and the viral protein R (Vpr), all of which contain unique but redundant nuclear import signals (23). In addition, the DNA flap produced during reverse transcription is central to the nuclear import step of the virus life cycle (24). The PIC is more than twice the size of the central channel within the nuclear pore complex, which represents a challenge for its entry into the nucleus. The redundancy of nuclear import signals in the viral components very likely contributes to its successful entry into the nucleus (25).

1.2.2.3 Integration

Once inside the nucleoplasm, the viral double stranded DNA genome is integrated into the host chromosome. This step is mediated by the viral IN, which catalyses two chemical reactions – 3' processing and DNA-strand transfer – at different times during infection (26). Several cellular proteins have been shown to be part of the integration process. Most notably the lens epithelium-derived growth factor (LEDGF)/p75, which binds to HIV-1 IN, plays a role in targeting the virus to active genes for integration (27). Because it is absolutely essential for viral replication and has no functional equivalent in human cells, IN is an ideal target for drug design. Raltegravir, which targets the strand transfer step of the integration process, is the first approved IN inhibitor (28), and several drugs have since been approved or are undergoing clinical trials.

1.2.2.4 Transcription and viral mRNAs export

Integration gives the viral genome the status of a cellular gene. Therefore, transcription and subsequent steps of viral replication cycle mainly occur using the cellular machinery. Transcription of the HIV genome takes place from a single promoter located in the 5' long terminal repeat (LTR) and requires the viral

trans-activation protein Tat (29). HIV transcription is characterized by an early, Tat-independent, and a late, Tat-dependent, phase, where transcription level is increased several fold. Three types of HIV transcripts are found in the cell after transcription of the provirus; (i) the genomic full-length mRNA, which is used for encapsidation and also directs the synthesis of the Gag and Gag-Pol polyproteins, (ii) different forms of singly-spliced mRNAs coding for Vif, Vpr, Vpu, and Env, and (iii) subsets of multiply-spliced mRNAs coding for Tat, Nef, and Rev. In the early step, in the absence of Rev, the full-length RNA is produced but cannot be exported. It is spliced to form the doubly-spliced 1.8-2.0 kilobase (kb) RNAs that are passively exported to the cytoplasm where they are translated to produce Tat, Rev, and Nef (30). Tat and Rev then go to the nucleus where Tat increases transcription and Rev allows the export of the unspliced (9 kb) and singly-spliced (4 kb) RNA to the cytoplasm. HIV splicing is regulated by the Rev protein, which shuttles between the cytoplasm and the nucleus. Nuclear localized Rev binds to full-length and singly-spliced transcripts harboring the Rev response element (RRE). The Rev-RNA complexes are transported to the cytoplasm using the CRM-1 pathway before the cellular splicing machinery gains access, the mRNAs are then translated to form the structural proteins (31, 32).

1.2.2.5 Translation

HIV mRNAs are translated on free polyribosomes in the cytoplasm by the cellular translational machinery. This process is modulated by the HIV Transactivation Response (TAR) RNA structure present at the 5'-end of all the mRNAs. *In vitro* and *in vivo* studies have shown that the presence of TAR RNA on mRNA transcripts downmodulates their translation. This process is mediated both by a block by the TAR structure and through activation of the protein kinase R (PKR) direct binding to the TAR structure, resulting in subsequent translation inhibition through the phosphorylation of the alpha subunit of the eukaryotic initiation factor 2 (eIF2α). The TAR RNA Binding Protein (TRBP) increases the

translation of TAR RNA-containing mRNA transcripts through direct binding to the TAR RNA and PKR (33). The full-length 9 kb transcript serves as the mRNA for synthesis of Gag and Gag-Pro-Pol polyproteins, to form the structural and enzymatic proteins. Singly spliced mRNAs direct the synthesis of the Vpr, Env and Vif proteins, while the different multiply spliced mRNAs produced during the late phase of transcription direct the synthesis of the remaining viral proteins.

1.2.2.6 Assembly and final stages

HIV-1 assembly step is mainly mediated by the Gag polyprotein. In cells and *in vitro* , expression of Gag polyprotein alone is sufficient for the formation of virus-like particles (VLP) that are morphologically indistinguishable from HIV-1 immature virion particles. Gag polyprotein monomers and low gradient polymers are diffused and transported to phosphoinositide-rich regions at the cytoplasmic face of the cell plasma membrane where assembly proceeds (34, 35). Gag's intrinsic property to multimerization into spherical particles drives the assembly of the nascent viral particle, which is sufficient to initiate the budding process. Two sequences, PTAP and YPLTSL, present in the p6 domain of the Gag polyprotein, named late-budding (L) domains, are absolutely required for release of the immature HIV particle from the plasma membrane. The PTAP and YPLTSL sequences bind to the cellular proteins Tsg101 and ALIX respectively, which are involved in the endosomal sorting complex required for transport (ESCRT) pathway. This pathway normally mediates budding of vesicles into the late endosomal lumen (36, 37). In the final stages of HIV replication cycle, release from the plasma membrane results in a dramatic morphological maturation process. Multiple cleavages of the Gag precursor by the viral protease allow the release of the matrix, capsid, and nucleocapsid structural proteins and their subsequent reassembly to form the core of the mature virion, yielding an infectious viral particle. Several inhibitors of the late stages of HIV replication

have been developed and are currently available. These inhibitors target the viral protease and act essentially at the maturation step, by targeting the viral protease.

1.3 COURSE OF DISEASE AND THERAPY

In the absence of antiretroviral treatment, the course of HIV-1 infection in most individuals is illustrated in Figure 1.5 (38). Three distinctive phases are usually recognized; (i) primary infection phase; (ii) asymptomatic or chronic phase, and (iii) symptomatic phase or AIDS, in which the virus induces a progressive depletion of $CD4^+$ T cells that invariably, over the course of years, leads to the fatal destruction of the immune system. Both the innate and adaptive immunity, with evidence of an effective viral-specific $CD8^+$ T lymphocytes, are highly activated during the course of HIV disease (39).

1.3.1 Acute Primary Infection

The initial stage of HIV-1 infection starts immediately following viral entry into the body and is often accompanied by an acute retroviral syndrome lasting only a few weeks. It is often heralded by a clinical illness characterized by rash, fever, and lymphadenopathy. This phase is associated with seroconversion following elevated amount of plasma HIV-1 RNA, a sharp decrease in peripheral blood $CD4^+$ T cell counts, establishment of a latently infected $CD4^+$ T cell reservoir, and development of an HIV-1-specific immune response (40).

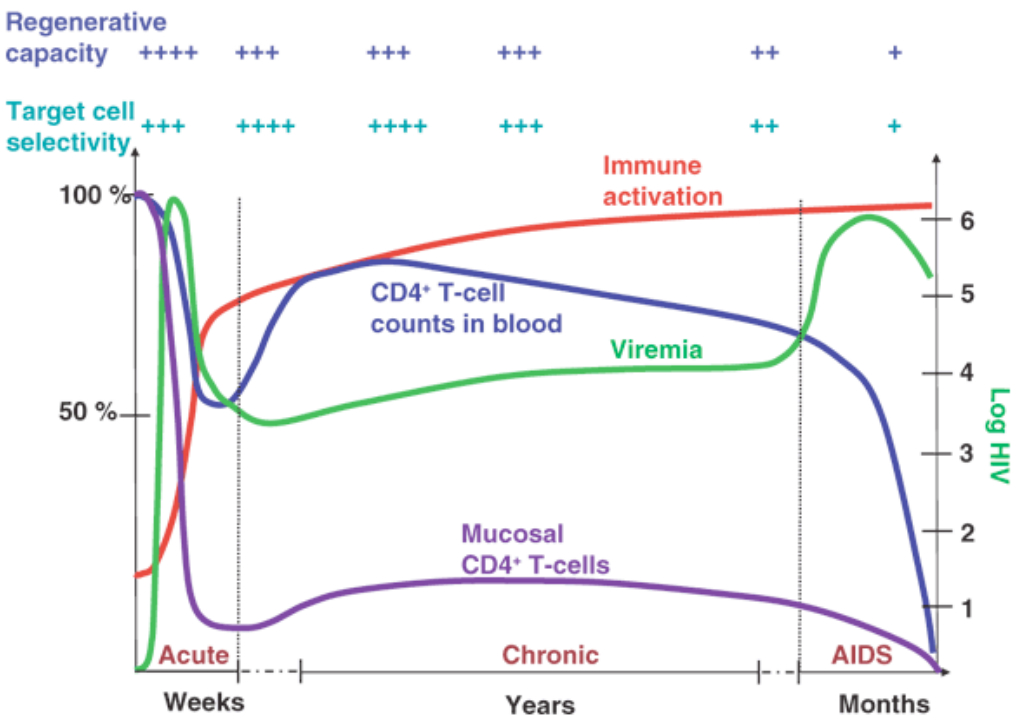


Figure 1.5. HIV disease progression during primary infection, chronic and late stages showing changes in mucosal and blood CD4+ T cell counts and in viremia level. The relative state of immune activation and in the 'regenerative capacity' and 'target-cell selectivity' are also shown. See text for details. Figure from (38).

1.3.2 Establishment of Infection

Establishment of HIV infection is dependent on target cell expression of CD4 receptor and the chemokine CCR5 and CXCR4 co-receptors, with CCR5 almost always being the natural target co-receptor for naturally transmitted virus. Relatively little is known regarding the natural route of viral dissemination following sexual transmission of HIV infection in individuals, what tissue compartments outside of the blood and lymph nodes are primarily targeted before the virus causes a systemic infection. Most experimental data of mucosal HIV

infection are from primate models. Primary sexual infection of HIV occurs with the CCR5 viral strain. Cells in the lamina propria of the cervicovaginal mucosal are the first viral targets and HIV replication could be detected two to three days following vaginal inoculation, dendritic cells (DCs), macrophages (Mϕs), natural killer (NK) cells, and $CD4^+$ T cells are the first cells to be infected (41, 42).

Different mechanisms allow the virus to cross the epithelial barrier. In the absence of mucosal disruption, the virus requires active transport, by means of transcytosis, to cross the tight epithelial barrier (43) and the specialized epithelial mast (M) cells. Breach of the epithelium barrier, as well as direct infection of intraepithelial lymphocytes, and transport by DCs, give the virus access to other tissues.

1.3.2.1 Infection of dendritic cells (DCs)

DCs are professional antigen presenting cells (APCs) and are the sentinels of the immune system. They monitor pathogen infection and trigger innate and adaptive immune responses. With the natural killer cells, the DCs form the main cellular component of the innate system and participate in the first line of defense against pathogens and viruses. Capture of foreign antigens in the periphery by immature DCs triggers their maturation. Once matured, DCs migrate to the lymphoid tissues where they present the processed antigens on MHC to naïve T cells, resulting in the activation of the adaptive immune response against the pathogen. In humans, two major dendritic subsets exist: the conventional $CD11c^+$ myeloid DCs (cDCs) and the plasmacytoid DCs (PDCs). cDCs are strategically localized in the skin, in the genital/gut mucosa, and in the blood and are important players during acute HIV infection. PDCs, which are found in blood, thymus, inflamed skin and mucosa, and lymph intervene later during HIV primary infection (44). HIV-DCs interactions involve contact of viral components with pattern recognition receptors (PRRs) at the surface of the DCs. This triggers the

activation of the innate response to HIV infection where immature DCs process and present viral antigens on MHC to naïve T cells, leading to their activation. Direct HIV infection and capture of HIV particles by DCs are two other outcomes of HIV-DCs interactions primordial in establishment of infection and subsequent disease progression.

The cDCs and PDCs all express relatively low levels of the HIV CD4 receptor and the co- receptor CCR5/CXCR4 (45, 46). Thus, they are all susceptible to HIV infection. However, infection of DCs *in vitro* is very low and no more than 1-3% of the DC population being productively infected (47, 48), with very low viral productivity (49). Nonetheless, the low levels of virus produced are sufficient for DCs to efficiently *cis*-transfect T cells with HIV. Proposed reasons behind the moderate infection are attributed to DCs intrinsic restriction strategies, low levels of HIV receptors and co-receptors, rapid and extensive degradation of internalized HIV particles in intracellular compartment (50). Endocytosis of HIV by PDCs is followed by their activation via interaction of Toll-like receptor 7 (TLR7) with viral RNA. Patients undergoing acute HIV infection show a 50% reduction in their PDCs counts and impaired interferon (IFN) production on *in vitro* stimulation with herpes simplex virus type 1, compared to healthy individuals (51, 52). In addition, endocytosis of HIV by cDCs fails to activate these cells, even though they express TLR7. cDC infection by HIV may route the virus to endosomal compartments that are TLR7-independent. DCs derived from HIV-infected patients' PBMCs show a significantly reduced efficiency in stimulating allogeneic T cell stimulation (53), and show a generally reduced expression of co-stimulatory molecules, which impairs their maturation process (54).

Two subsets of cDCs are found at the site of HIV-1 infection: the Langerhans DCs (LCs) and a subset characterized by the expression of DC-SIGN (DC-SIGN^{+}-DCs), a C-type lectin receptor. The Langerhans subset of DCs represents 2–3% of the cells found in the skin and the stratified squamous

epithelia of the vagina, ectocervix and foreskin, including glans penis and skin (55-59). LCs are the first subset that HIV encounters in intact genital epithelial tissues and, in contrast to other subsets of DCs, the LCs don't promote HIV-1 transmission. Instead, they form a protective barrier aimed to prevent HIV infection and account for the low transmission rate of HIV in general (57). Langerin, another C-type lectin, is only expressed on LCs where it serves as a receptor for HIV entry. Infection of LCs results in rapid internalization of the virus in Birbeck granules, which promotes HIV degradation, viral clearance, and inhibition of transmission (59).

1.3.2.2 Capture of HIV particles

Damage within the epithelial LC-rich layer as a result of trauma or ulcerative anogenital co-infections (60), expose the subepithelial DC-SIGN$^+$-DCs, macrophages, and CD4$^+$ T cells to HIV. In the subepithelia mucosa, HIV subverts DCs' key function as a tool to reach the CD4$^+$ T cells and facilitate their immune evasion. DC-SIGN$^+$-DCs are found in the dermis, submucosa, rectal epithelium, as well as in other tissues (58). As mentioned, DCs show low susceptibility to HIV-1 infection. The major route of HIV dissemination is through infection of CD4$^+$ T cells, which is believed to occur from transfer of intact infectious particles from the DCs to the T cells. Three discrete steps are involved in DC presentation of virus to CD4$^+$ T cells: (i) capture of viral particles, (ii) migration of DC-HIV particle complex, and (iii) transfer to T cells. The carbohydrate-recognition domain of DC-SIGN receptors at the surface DC-SIGN$^+$-DCs interacts with HIV gp120 (61), resulting in HIV internalization. Then, within the DCs, the virus migrates to secondary lymphoid organs where it is presented to CD4$^+$ T cells. The latter are then *trans*-infected with the virus through infectious synapses. Direct cell-cell interaction is required in DC-SIGN-mediated HIV transmission, and is also cell-type dependent (62). Recently, Lambert *et al.* reported that another C-type lectin surface receptor, the DC immunoreceptor

(DCIR), serves as an attachment factor for HIV-1 in DC and promotes viral propagation (63).

1.3.2.3 Macrophages (Mϕs):

The monocyte-macrophage lineage is widely recognized as the second major target of HIV-1 infection, in addition to $CD4^+$ T cells. Just like DCs, Mϕs play a central role in host immunity and participate in pathogen processing and antigen presentation. They express the CD4 receptor and the CCR5 co-receptor necessary for HIV entry, as well as C-type lectin receptors that encourage virus capture and entry. Breach of the epithelial first line of defense gives HIV access to Mϕs. These cells are usually highly resistant to the cytopathic effects of HIV. Therefore, they can productively be infected and harbor the virus for a longer period. In addition, with their ability to cross the blood-tissue and the blood-brain barriers, infected Mϕs are potent agents for delivery of HIV-1 to all tissues and organs, including the brain, where they contribute to systemic dissemination throughout the body (64, 65).

1.3.2.4 Impairment of Innate Immunity: DC and Natural Killer (NK) cells:

DCs promote HIV replication and serve as a vehicle to *trans*-infect $CD4^+$ T cells in the lymph nodes. However, DCs function as potent antigen-presenting cells and, therefore, are bound to activate antiviral responses during infection. Undoubtedly, these responses are ineffective in preventing HIV spread and infection. With the high productivity of $CD4^+$ T cells, virus dissemination is likely to be more rapid before an effective antiviral immunity can be established (66, 67). HIV has been shown to prevent DC maturation (68) and, unlike other pathogens, HIV does not activate DCs, a necessary step to enhance these cells' function, and to mount an effective antiviral immunity (69). Several studies have

shown that both immature and mature DCs are capable of HIV processing and antigen-presentation *in vivo* (70, 71). Comparison of virus-carrying mature and immature DCs *in vitro* revealed that mature DCs are able to stimulate $CD4^+$ and $CD8^+$ T cells responses while immature DCs can activate virus-specific $CD4^+$ T cells (70, 72). By only stimulating $CD4^+$ T cells, immature DCs antigen-presentation could potentially result in exacerbation of viral spread to the virus-specific T cells in the $CD8^+$ T cells cytotoxic activity. Furthermore, despite virus-carrying mature DCs ability to stimulate $CD8^+$ T cells, virus would still be spread to nearby $CD4^+$ T cells more rapidly than any virus-specific T cell response could be initiated (66). PDCs are found in lymphoid follicles, where they encounter HIV virions and their numbers in blood decrease in HIV-infected individuals (73, 74). *In vitro* studies revealed that PDCs produce type 1 IFN in response to HIV infection (66). Increasing number of *in vitro* studies showed the importance of a HIV viral protein in modulating immature DCs functions to favor viral spread. Specifically, Nef, one HIV accessory protein, was shown to induce release of several cytokines such as IL-6, -8, -12, TNFα, MIP-1α, -1β, and RANTES. These cytokines are usually secreted by mature DCs and promote HIV dissemination (75, 76). Similar results were also reported with macrophages (77).

NK cells exert their innate immune function against viral infection using their high cytotoxic potential and their capacity to release several cytokines and chemokines, including TNFα and IFN, CCL4/MIP-1β and CCL5/RANTES (78). They have been reported to kill HIV-infected cells directly or through antibody-dependent cellular cytotoxicity (ADCC) (79). NK cell-mediated ADCC against HIV is dependent on the presence of gp120 Env antibodies in the serum or at the mucosal level. ADCC and circulating levels of NK cells have been reported to increase during primary HIV infection, and their correlate with patients' disease progression (80, 81). However, high HIV viremia seems to correlate with a functional defect of NK cells, resulting in downregulation of the activation of the natural cytotoxicity receptors (NCR) and their capacity to induce production of CCR5-binding chemokines early during HIV infection (82).

1.3.2.5 Infection of $CD4^+$ T cells

$CD4^+$ T cells represent a population of cells that have central functional roles in immunoregulation. However, $CD4^+$ T cells cannot kill pathogens directly and have no phagocytic activity. They are involved in antibody class switching for B cells, activation and mobilization of cytotoxic T lymphocytes (CTLs). They also activate mononuclear phagocytes' phagocytic and intracellular killing activities (83). $CD4^+$ T cells are the major HIV cellular targets and their progressive depletion is the hallmark of HIV-1 infection. HIV-1's first encounter with $CD4^+$ T cells is believed to occur in the subepithelial mucosa. In this area, $CD4^+$ T cells can be directly infected by the virus, or infected in *trans* by internalized virions via DC-SIGN. Infection of lymphocytes is active and mainly accounts for the burst of viremia that occurs during primary infection and results in viral dissemination to the lymphoid organs.

1.3.3. Dissemination of HIV to tissues

1.3.3 .1 Infection of the gut-associated lymphoid tissue (GALT)

After establishment of infection in the mucosa, extensive $CD4^+$ T cells infection accounts for a rapid spread of the virus in both local and distant lymphoid tissue (41, 84) over the following two weeks. This results in the establishment of a pool of latently infected $CD4^+$ T cells (41, 84, 85). Cell-cell transmission accounts for the majority of *de novo* infections, making propagation mainly dependent on cell interaction and local target cell densities (40, 86, 87). $CCR5^+$ $CD4^+$ memory T cells accounts for all the T cells found on mucosal surfaces but are relatively infrequent in peripheral blood and lymph nodes, are the main targets (88-90). The GALT is the largest lymphoid organ and is estimated to contain, at steady state level, more than 60% of the body's total T cell load (Fig. 1.6) (91, 92). Consequently, acute HIV infection profoundly disturbs the GALT

dynamic and during infection this organ is crucial in sustaining massive viral replication levels, resulting in viral replenishment, establishment of a viral reservoir and irreversible damage to the $CD4^+$ T cell-mediated immunity. Within days of infection, an estimated 60% of mucosal memory $CD4^+$ T cells become infected at the peak of viremia (93). The massive increase in plasma viral load during primary HIV infection is characterized by a dramatic and selective loss of 75% of the total memory $CD4^+$ T cells (91, 94), within 4-6 weeks post-infection in acutely HIV-1 infected patients. The GALT is, by far, the most affected organ (95). The depletion of $CD4^+$ memory T cells has also been reported in other lymphoid tissues and in peripheral blood (93, 96). However, they are affected to a lesser extent with only 15% of blood and lymph node T cells that express the CCR5 (90).

Because of their ability to generate faster and more effective immune responses against all kinds of pathogens, the extent of memory T cell depletion determines disease outcome (97). Decrease in CD4 expression at the surface or internalization of CD4 receptor following HIV-1 infection cannot explain CD4 memory T cell depletion. Rather, a direct cytopathic effect of the virus on $CD4^+$ T cells accounts for the major mechanism leading to memory $CD4^+$ T cells depletion during acute infection (93). Moreover, viral infection causes a widespread activation and apoptosis of uninfected bystander T cells in both lymphoid tissues and peripheral blood (98). Activated T cells are short-lived in order to limit inflammatory damage inflicted by sustained activation of the immune system after infection clearance. Because HIV infection is rarely cleared, widespread activation of non-antigen-specific T cells occurs, resulting in constant draining of naïve and resting memory $CD4^+$ T cells pools, leading to failure of T cell homeostasis and depletion (99, 100). Being a major target of HIV infection, the GALT is also involved in the initiation of an antiviral immune response. The massive loss of $CD4^+$ memory T cells does not compromise the overall regenerative capacity of the immune system because naïve and most central memory T cells are spared (38). However, preferential infection and depletion of

HIV-specific $CD4^+$ T cells does compromise the body's ability to mount an effective immune response.

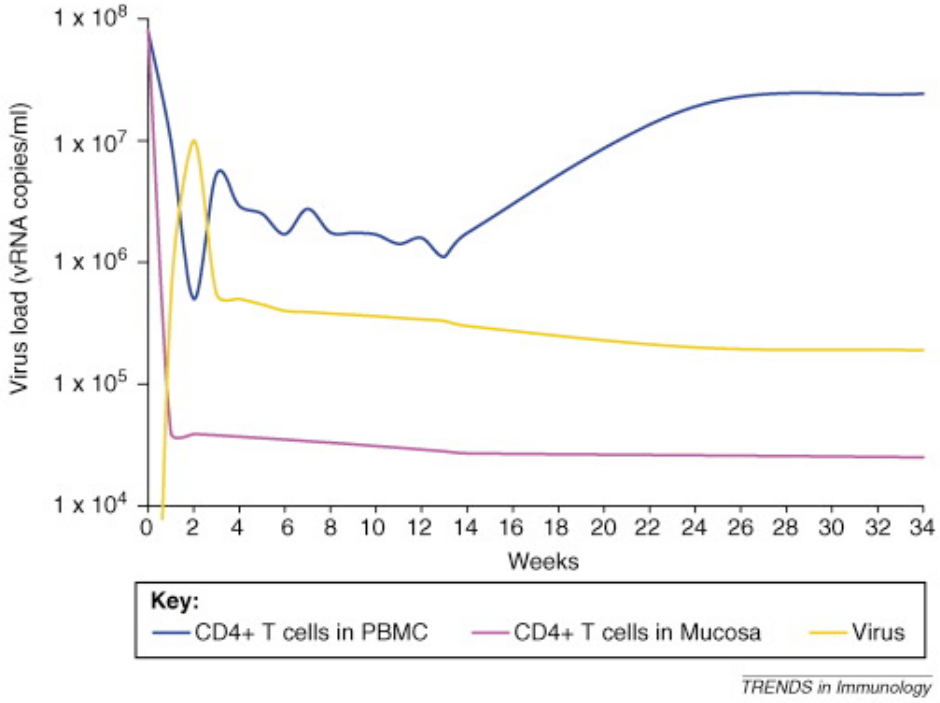


Figure 1.6. Rapid selection and depletion of GALT CD4+ T cells in the intestine of HIV-infected patients correlated with high viremia within 1–2 weeks. Early treatment with HAART results in CD4+ T-cell recovery in peripheral blood and lymph nodes (PBMCs), but not in mucosal tissues. Figure from (91).

Soon following infection, the virus can be recovered from most tissues, including the brain. Studies have demonstrated that virus isolated from brain tissue is predominantly macrophage tropic (101). The exact sources of early systemic infection are not clear, but might be a reflection of trafficking infected monocytes from the blood stream to the cerebrospinal fluid (CSF). Furthermore, macrophages' ability to cross the blood-tissue barrier is believed to be a potent agent for delivery of HIV-1 to all tissues and organs. Therefore, HIV-1-infected macrophages are of critical importance for early viral spread, persistence, and virus dissemination throughout the body of the host (102). Potential cellular targets for infection in the brain include perivascular and parenchymal macrophages/microglia, oligodendrocytes, neurons, endothelia, and astrocytes (103). However, HIV replication and production in the brain is extremely limited, and is believed to act as reservoir.

1.3.3.2 Establishment of Viral Reservoirs

Persistent and high levels of viral replication in GALT lead to replenishment and maintenance of viral reservoirs throughout the course of the disease. Therefore, it is the major active reservoir and viral source for constant *de novo* virus synthesis and viral transmission. However, the ability of the virus to remain latent in a susceptible subpopulation of cells is the major obstacle to HIV eradication. Latent reservoir leads to viral immune escape and persists for long periods of time, even in the presence of successful highly active antiretroviral therapy (HAART). Memory $CD4^+$ T cells long life span and ability to be reactivated given the right stimuli, make them crucial for the maintenance of latent HIV reservoirs. Two different latency mechanisms have been described: (i) pre-integration latency and (ii) post-integration latency (104). Pre-integration latency occurs in the presence of cellular blocks in reverse transcription and/or nuclear transport of the pre-integration complex, resulting in the transient state of pre-integration latency. Pre-integrated provirus is usually short-lived and labile in

nature, thus it is likely that its contribution to viral persistence is limited. In post-integration latency, expression of the integrated viral genome is repressed, resulting in no or very limited viral production. Limited transcription factor availability and integration of the viral genome into a repressive heterochromatin environment are factors that can lead to repressed gene expression (105). Memory $CD4^+$ T cells, cells of the monocyte-macrophage lineage, hematopoietic progenitor cells (HPCs), as well as some cells of the central nervous system (CNS) are the major pools of HIV-1 persistence that are established early during HIV primary infection.

1.3.3.1 Infection of resting T cells

Naïve and memory $CD4^+$ T cells are the two populations of resting $CD4^+$ T cells. Lack of or limited expression of the CCR5 co-receptor, as well as blocks during reverse transcription and nuclear import, greatly impair HIV-1 primary infection of naïve and memory quiescent T lymphocytes (106). Interestingly, HIV-1 infected patients reportedly harbor latently infected resting T cells early in disease progression (104), before the appearance of X4 strains. Given their long life span, this reservoir is believed to be the most physiologically relevant source of rebounding virus after cessation of highly active antiretroviral therapy HAART. The latently infected quiescent T cell pool is believed to arise from activated $CD4^+$ T cells that become infected while in the process of becoming memory T cells (107). Furthermore, some lines of evidence suggest that quiescent T cells might not be completely refractory to HIV infection, and interactions with autologous DCs or B cells, or with follicular dendritic cells may render them permissive to HIV-1 infection (108). Pre-integration latency accounts for most of the resting $CD4^+$ T cells reservoir (109).

1.3.3.4 Infection of Monocytes and Mϕs

Monocytes and Mϕs are infected early during infection. Monocytes circulate in the blood to migrate to various tissues for several days where they differentiate into macrophages. Contrary to $CD4^+$ T cells, infection of monocytes/macrophages is not cytopathic in nature and does not impact their viability. As a result, and as mentioned above, viral replication is more persistent, leading to sustained viral production. In addition, limited expression of viral proteins may enable the infected cells to escape immune responses. With less than 1% of cells infected, due to low expression of CD4 receptor and barriers to reverse transcription and nuclear import, the monocyte/macrophage reservoir is relatively small (110).

1.3.3.5 Infection of the CNS

Infection and activation of macrophages in the brain has long been associated with HIV-associated dementia (111). Of the four different subsets of macrophages found in the CNS, the perivascular macrophages and the microglia account for most of the production of HIV in the CNS. The CNS resident macrophages are the main phagocytic and antigen presenting cells of the CNS and intervene in immune responses. They all harbor the CD4 receptor and the CCR5 co-receptor, and are susceptible to HIV infection. Because of their longer life-span and their ability to proliferate *in situ,* the microglial cells are believed to contribute to the CNS macrophage-derived reservoir (112).

Astrocytes are the most abundant cell type of the CNS, representing about 80% of the brain cells. Their function is crucial in the CNS homeostasis, nourishment, neuronal function and repair, maintenance of the blood-brain-barrier, as well as in immune defense. Astrocytes express several chemokine receptors, including CCR5 and CXCR4, but lack expression of CD4 receptor. Therefore, HIV enters astrocytes in a CD4-independent manner where it

establishes a persistent infection with very limited virus production and predominant expression of nonstructural HIV components (19, 113, 114). However, viruses produced from *in vitro* HIV-infected astrocytes are fully infectious and can give rise to fully productive infection, suggesting the notion that this reservoir can be a source of persistence and dissemination of virus in the brain (115). Moreover, *in vitro* studies have reported recovery of HIV virus from latently infected astrocytes following stimulation with tumor necrosis factor (TNF)-α and IL-1β (116, 117).

1.3.4 The Chronic Phase of Infection

The chronic phase immediately follows the primary infection and generally starts around one to two months after initial infection. It is characterized by a 100- to 1000-fold decrease in plasma viremia, a partial rise in $CD4^+$ T cells, and a long asymptomatic phase of chronic infection (40). This chronic phase, lasting on average ten years, is marked by the slow decline of peripheral blood $CD4^+$ T cell counts, concomitant with the slow rise in viral load, where the immune system slowly shuts down before the onset of AIDS. Emerging data have begun to shed new light on how HIV causes immune deficiency, departing from the conventional view of a gradual decrease in immunocompetence (38, 95, 118). The studies showed that the massive systemic loss of $CCR5^+$ $CD4^+$ T cells by direct productive infection or cytotoxic T cell–mediated cytolysis, had a profound crippling effect on the immune system from which the individual never fully recovers. AIDS is now viewed as a ‘tale of two infections’, a highly destructive acute infection leading to massive depletion of mucosal $CD4^+$ memory T cells, setting the stage for the chronic phase in which a likely crippled immune system slowly shuts down (Fig. 1.7) (119).

Following the massive systemic depletion of $CCR5^+$ $CD4^+$ T cells during acute infection, a state of intense immune activation is induced. This results in a significant increase in the frequency of activated, short-lived, mucosal-homing, CCR5-expressing $CD4^+$ memory T cells, with a higher level of proliferation and a higher turnover (119). Increase in serum concentration of pro-inflammatory cytokines, T and B lymphocytes, and NK cell turnover have also been reported (120, 121). This constant immune activation profoundly impairs the immune system competence, perturbing the $CD4^+$ memory T cells dynamics. Illustrating a perfect example of how HIV co-opts the immune system for its own benefit, the constant immune activation leads to two major consequences. First, it brings a continuous supply of short-lived T cell targets and second, production and activation of longer-lived latently infected memory $CD4^+$ T cells continuously provide new viruses to maintain the infection. Therefore, HIV switches from the high-frequency infection of resting memory $CD4^+$ T cells during primary infection, to a low level infection of activated T cells. This accounts for the drop in viral viremia to a plateau, and partial T cells replenishment observed at the beginning of the chronic phase. However, the partial renewal in mucosal memory $CD4^+$ T cell count is transient, as constant immune activation is a double-edge sword as it also promotes surface chemokine and adhesion molecules expression on T cells, increasing infectability and T-cell trafficking to lymphoid tissues where most HIV infection occurs. Therefore, viral replication during the chronic phase is continuously sustained, promoting further $CD4^+$ T cells loss through HIV direct cytopathic effects and apoptosis of bystander cells.

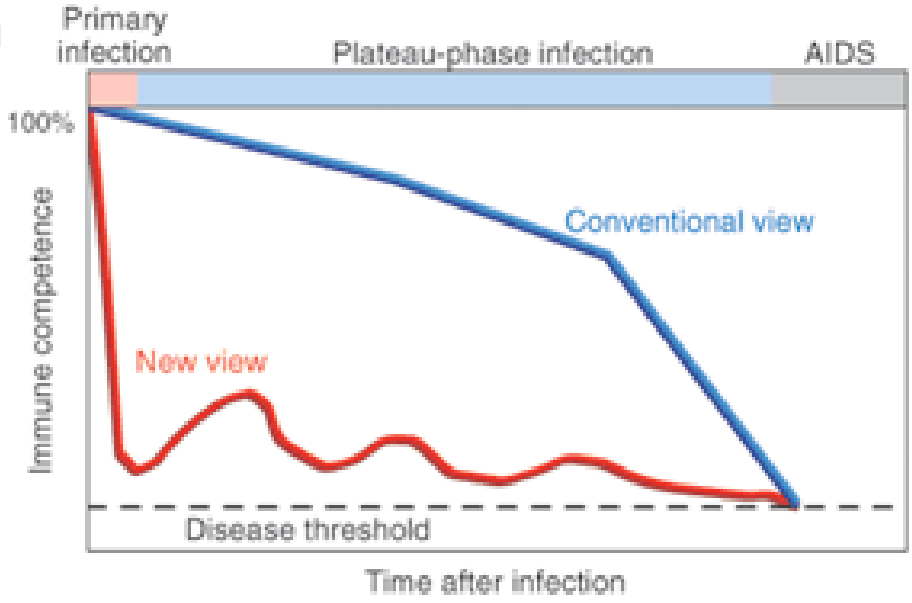


Figure 1.7. Shift in immune competence understanding during HIV infection. The conventional view of a slow decay in immune competence (blue line) contrasted with the new view (red line), in which the massive and irreversible destruction of immune competence takes place during acute infection. Figure from (119).

Throughout the chronic phase, the number of mucosal $CD4^+$ T cells remains small despite their continuous influx. Some explanations might be a consequence of viral infection and their short lives, which prevent a rapid accumulation in mucosal tissues to compensate for their initial loss. Moreover, the chronic phase begins with an already massively depleted memory $CD4^+$ T cell pool, and its reconstitution depends on input from thymus-derived naïve T cell pool. However, thymus function and thymocyte proliferation are rapidly suppressed early during HIV-1 infection (122), crippling the host's ability to replace the $CD4^+$ T cells. Therefore, because the source of naïve $CD4^+$ T cells is finite, persistent rounds of activation and death eventually deplete the pools below the threshold required to keep opportunistic pathogens at bay (38). Furthermore, the massive depletion of $CD4^+$ T cells during primary infection immensely compromises the integrity of the mucosal tissue and lymph nodes' architecture. In

addition, constant immune activation leads to chronic inflammation, a situation that is worsened by an increase in microbe translocation across the HIV-damaged gut mucosa, further amplifying immune hyperactivation and destruction of the mucosa and lymph node tissues (123, 124).

1.3.5 The Acquired Immunodeficiency Syndrome (AIDS) Phase

A peripheral blood $CD4^+$ T cell count less than 200 cells/μl is the diagnostic threshold for AIDS. As $CD4^+$ T cell counts decrease during chronic infection, viral load rises. Several events underlying the route leading to AIDS have been suggested, but their individual significance and roles is not yet known. They include (i) insufficient regeneration of central memory T cells, (ii) loss of naïve cells due to excessive differentiation of memory cells, and (iii) suppressed thymic function (38). During this stage, the virus undergoes accelerated evolution, resulting in the appearance of X4 viral strains. CXCR4-tropic viruses target and kill naïve and resting T cells, greatly broadening the virus' tropism. This accelerates the irreversible loss in regenerative capacity that also characterizes the AIDS phase, as the loss of a specific population of cells further compromises the architectural integrity of tissues. X4 viruses are believed to be more susceptible to antiviral immunity, which would explain their appearance during late stages when the immune system is more compromised (125). However, Delwart *et al.* reported that loss of target-cell susceptibility leads to a global nature of the infection, resulting in a major decrease in viral diversity and sharp increase in plasma viremia (126). These results contrast to the appearance of X4 strains that is usually seen in the latter stages of AIDS. At this stage, patients show an overall susceptibility to opportunistic tumors and infections, eventually leading to death.

1.3.6 Therapy

Integration into the host genome, the establishment of latently infected cellular reservoirs, and the reverse transcriptase's high error rate have long been recognized as the three major hurdles impeding the discovery of a cure for HIV infection. The development of combination highly active antiretroviral therapy (HAART) for HIV in the mid-1990s, in order to combat the virus at different stages of its life cycle, has remarkably altered the clinical course of HIV disease. Nucleoside, and non-nucleoside reverse transcriptase inhibitors, fusion/entry inhibitors, integrase inhibitors, and protease inhibitors are the common classes of drugs available to physicians. The CCR5 or CXCR4 fusion/entry inhibitors are the first class of drug directly targeting the host proteins and, therefore, may not be susceptible to viral escape mutations that are usually seen when viral proteins are targeted. What used to be a death sentence is now a fairly manageable disease. However, although HAART reduces HIV-1 viremia to undetectable levels, decreases HIV-related morbidity and prolongs survival, improves quality of life, restores and preserves immune function, and prevents vertical transmission, HAART is not a cure (127, 128). The virus persists in reservoirs in the face of HAART, and easily rebounds to high level within weeks after cessation of therapy. An estimated 60 years of sustained and effective anti-retroviral therapy would be required to completely eradicate the stable latent reservoirs (129, 130). Thus, the latently infected cells provide a mechanism for lifelong persistence of HIV-1.

Furthermore, viral escape mutants have been reported in patients undergoing HAART. In addition, in some patients, the increased $CD4^+$ T cell count that is generally observed with HAART does not occur, due to extensive damage to the thymus before the start of therapy. These patients are subject to the development of an AIDS-related illness. This fuels the ongoing debate on when to start HAART. The current recommendations set 350 $CD4^+$ T cells/μl as the minimum threshold for initiation of antiretroviral therapy. However, recent

studies showing the massive depletion of mucosal $CD4^+$ T cells and thymus defect during primary infection have prompted the debate on whether HAART should be initiated early during chronic phase or even during acute infection, when possible. Several reports have shown the benefits of early HAART intervention during primary infection in limiting immune activation and restoring immune function in peripheral blood and lymph nodes (123, 131). However, Mehandru *et al.* showed no improvement in GALT related immune reconstitution after prolonged treatment (132). Development of resistance and morbidity associated with prolonged use of HAART are often cited as reasons not to intervene during primary infection. Clearly, much data are missing regarding the beneficial outcome of early interventions and the first randomized controlled trial, SPARTAC, which stands for Short Pulse Anti Retroviral Therapy at HIV Seroconversion, to address those issues is currently underway and will report in one or two years (133).

1.4 INNATE IMMUNITY AND INTERFERON RESPONSE TO INFECTION

1.4.1 IFN System

Interferons (IFNs) and their antiviral activity were discovered more than 50 years ago (134, 135). They are a family of cytokines produced in response to the invasion of pathogens, and are key components of innate immunity. They are a part of the first line of defense mounted against viral infections, and also intervene in adaptive immune pathways. They non-specifically inhibit viral growth by inducing an antiviral state in surrounding cells. IFNs are found in most vertebrates, including humans, birds and fish (136, 137). Based on amino acid homology and receptor usage, mammalian IFN are divided into three types: IFN types I to III.

In humans, type I IFN usually refers to IFNα, which is mainly secreted by leukocytes, and IFNβ, which is produced by fibroblasts. They are also named antiviral IFNs, due to their known essential function in mounting a robust host response against viral infection. So far, 17 genes coding for type I IFN have been identified in human. They are all intronless and clustered on chromosome 9, comprising the 13 known subtypes of IFNα and the single IFNβ gene (138). Type I IFNs, which also include the lesser known IFNε, IFNω, and IFNκ in humans, mediate their action through the ubiquitously expressed IFNAR (IFNα receptor), composed of IFNAR1 and IFNAR2 subunits (139). Type I IFN importance is illustrated by the extreme susceptibility to viral infection of individuals who are deficient in components of IFNR pathways, and who die at an early age (140, 141).

IFNγ is the only known member of the type II IFN, and maps to chromosome 12 in humans. It is exclusively produced by immune cells, such as activated T cells and NK cells and also plays a role in the activation of these cells, as well as macrophages (142). It mediates broad immune responses to pathogens other than viruses through the heterodimeric IFNγ receptor (IFNGR) complex. The discovery of type III IFNs was first reported in two different talks at the ‘‘Cytokines and Interferon’’ meeting in Turin, Italy, in 2002. The results were published in the beginning of 2003 (143-145). In humans, type III IFNs comprise a group of three subsets, named IFN-λ_1, $_2$, and $_3$. They are also known as interleukin (IL)-29, IL-28A, and IL-28B respectively. Type III IFNs have been proposed to be ancestral to type I IFN (146), but they are structurally and genetically distinct. Nevertheless, type III IFNs exhibit similar features as type I IFNs on antiviral and antiproliferative activities, but bind to distinct membrane receptors (143).

1.4.2 Viral-Induced IFN Responses

Toll-like receptors (TLRs), and retinoic acid-inducible gene 1 (RIG-1)-like helicase receptor (RLR) are major cellular sensors used by most cells to recognize incoming viral infection. These pattern-recognition receptors (PRRs) are present at the cell surface, and on the lumen side of endosomal membranes. They recognize conserved molecular patterns of dsRNAs, which activate the cell to produce IFN types I and III. In response to viral infection and cytosolic dsRNA, nearly all nucleated cells are capable of type I IFN production. However, PDCs, which are found in blood and in lymphoid tissues, produce up to 1000-fold more IFNα than any other leukocytes following activation by vi ruses (73, 147). Even though they only constitute 0.5-0.8% of blood leukocytes, they are most likely the sole source of IFNα following viral infection. Viral infection can activate the IFN pathway in the endosomes of PDCs through recognition of viral dsRNA, ssRNA and dsDNA, by TLR3, TLR7 and TLR9 respectively (Fig. 1.8) (148, 149). Direct viral infection, uncoating of endocytosed viral particles, degradation of engulfed apoptotic cells can be sources of endocytosed viral dsRNA in PDCs. The mechanism of induction of IFNα is poorly characterized in leukocytes, but it is believed to feed through the interferon regulatory factor (IRF) 3 pathway. In the dsRNA arm of the pathway, binding of dsRNA leads to activation by dimerization of TLR3, causing a conformational change in the TLR dimer, bringing the Toll/IL-1 receptor-like (TIR) domains into proximity (149). This is believed to create a new signaling surface, which then recruits TIR domain-containing adaptor molecules such as the TIR domain-containing adaptor inducing IFN-β (150). The formation of the TLR3/TRIF (TIR-domain-containing adapter-inducing IFN-β) complex triggers the recruitment of the tumor necrosis factor receptor-associated factor 3 (TRAF3). In turn, TRAF3 recruits a complex formed with the TRAF3 associated NF-κB activator (TANK)-binding kinase, TBK1, the main IRF3 kinase. This forms a complex with TRAF3, which also likely involves TANK associated with the essential scaffold protein NF-κB essential modulator (NEMO). The complex is recruited to the TRIF N-terminal

region and subsequently activated in a process involving NAK-associated protein (NAP) 1. This TBK1-containing complex can both phosphorylate and activate IRF3 and IRF7. Once phosphorylated, IRF3 or IRF7 homodimerize and translocate to the nucleus where they induce INFα production in PDCs (149, 150).

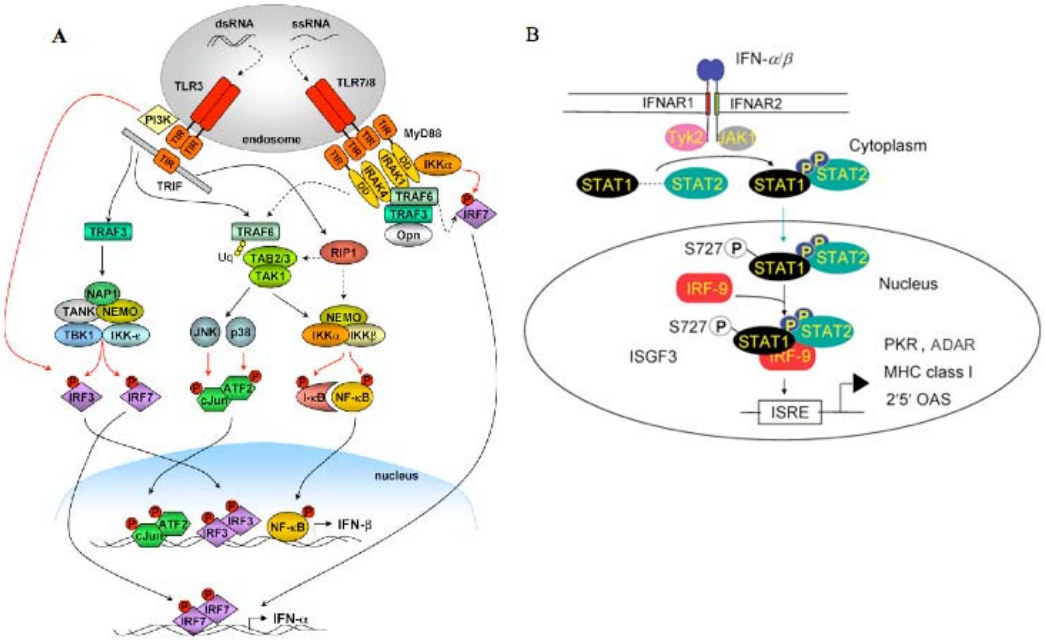


Figure 1.8. IFN signalling pathways. (A) TLR7/8- and TLR3-dependent signalling leading to IFNß expression in the endosomal compartment of DCs. See text for details. From (149) (B) IFN action on target cells harboring IFNR1 and IFNR2. See text for details. Modified from (148).

1.4.3 IFNs and HIV

IFNα binding to its receptor results in signal transducers and activators of transcription (STAT)-1/2-regulated expression of membrane Tumor Necrosis Factor (TNF)-related Apoptosis-Inducing Ligand (TRAIL) on CD4+ T cells. TRAIL mediates activated T cell apoptosis in HIV-1-infected patients (151). On CD4+ T cells, IFNα binding to its receptor results in STAT-1/2-regulated expression of membrane TRAIL. Furthermore, binding of HIV-1 to CD4 on $CD4^+$ T cells is required for expression of the TRAIL death receptor 5 (DR5). TRAIL mediates increased activation-induced cell death in both $CD4^+$ and $CD8^+$ T lymphocytes from HIV-1-infected subjects (151, 152). TRAIL mediates selective apoptosis of uninfected $CD4^+$ T cells in a human peripheral blood lymphocyte–transplanted non-obese diabetic severe combined immunodeficient (hu-PBL-NOD-SCID) mice (153). The HIV-1 Tat protein induces TRAIL production by monocytes and can result in the killing of uninfected $CD4^+$ T cells (154). In addition, soluble TRAIL is found in HIV-1–infected patients where it is believed to be responsible for the death of neurons in AIDS and may contribute to dementia (155-158). Furthermore, plasma from infected patients shows elevated TRAIL concentration, compared to healthy subjects. TRAIL plasma concentration correlates with viral load and decreases with HAART (159). Noninfectious HIV-1 particles, which represent more than 99% of the total plasma viral load in infected patients, induce TRAIL, DR5 and apoptosis in $CD4^+$ T cells by monocytes, PDCs and $CD8^+$ T cells in an IFNα-dependent manner (159-161). *In vitro* production of IFN-α by PDCs after culture with infectious or inactivated HIV-1 depends on the interaction of viral gp120 with cellular CD4. IRF-7 and the MyD88 adapter molecule *in vitro* and *in vivo* are also induced by HIV-1 particles in PDCs (162). The T cell-rich lymphoid tonsillar tissues from acutely-infected patients and patients with progressive HIV-1 disease show an increase in IFNα, TRAIL and DR5 mRNA compared with nonprogressors and healthy controls (162).

1.4.4 Signaling Responses to IFNs

Once produced, IFNα/β are secreted and initiate their biological activities through binding of the cognate type I receptor on the surrounding cells. Prior to IFN binding, the two subunits of IFNAR1 and IFNAR2 interact loosely. IFNAR1 is associated with the tyrosine kinase 2, whereas IFNAR2 is associated with tyrosine kinase JAK1 and STAT2 at their cytoplasmic tail (163). IFN binding leads to receptor dimerization, causing a conformational change that allows phosphorylation of IFNR1 by Tyk2 on tyrosine 466. IFNAR1 phosphorylation creates a strong docking site for IFNAR2-bound STAT2, allowing its phosphorylation on tyrosine 690 by Tyk2. STAT1, which is weakly bound to STAT2 prior to ligand-induced receptors dimerization, is phosphorylated by JAK1 on tyrosine 466. The phosphorylated STAT1 and STAT2 dimerize to form a stable heterodimer, leading to their translocation in the nucleus. In the nucleus, they associate with the DNA-binding transcription factor IRF9, to form the heterotrimer ISGF3 (148). ISGF3 binds to the IFN stimulatory response element (ISRE), driving the expression of several proteins that will induce a general antiviral state in the cell.

1.4.4.1 Proteins Induced: Nature of the IFN-induced antiviral state

The strength of the IFNα/β system is the induction or downregulation of several hundreds of interferon-stimulated gene (ISG) expression. These genes are likely acting in concert to allow an antiviral state aiming at restricting or prohibiting viral growth (137). The protein kinase R (PKR), 2'-5'-oligoadenylate synthetase (OAS), Mx, adenosine deaminase that acts on RNA (ADAR), are among the several enzymes induced by IFN treatment.

1.4.4.2 dsRNA-dependent protein kinase (PKR)

PKR was originally identified in humans as a 68 kDa protein kinase induced by IFN and activated by dsRNA (164, 165). Upon binding to dsRNA, PKR is activated and negatively affects cell- and virus-regulatory pathways. Activated PKR affects mainly viral mRNA translation and transcriptional events, through the phosphorylation of the eukaryotic translation factor eIF2α.

1.4.4.3 2'-5'-oligoadenylate synthetase (OAS) and RNAse L

2′,5′-OAS is the only known enzyme that catalyzes the synthesis of a 2′,5′-linked phosphodiester bond, leading to a mixture of oligoadenylates referred to as 2-5A, that activate the latent RNAse L endonuclease (166). RNAse L has been shown to degrade viral and cellular mRNAs, as well as ribosomal RNAs (167, 168). The 2'-5'-OAS pathway is activated by dsRNA and leads to cellular and viral mRNA degradation. This pathway is involved in antiviral responses, as well as cellular processes such as cell growth and differentiation, gene regulation and apoptosis.

1.4.4.4 Adenosine deaminases that act on RNA (ADARs)

ADARs are a family of RNA editing enzymes that target highly-structured double-stranded regions of nuclear-encoded and viral RNAs. They are mainly found in the nervous system where they diversify the information encoded in the genome by posttranscriptional RNA modifications. ADARs calatyze the deamination of C-6 adenosine to create inosine, thereby altering codons in some mRNAs (169). Two different ADAR genes are been characterized in human, *Adar1* which colocalizes to human chromosome 1q21.1-21.2 and *Adar2*, found on the distal region of chromosome 21 (21q22.3) (170, 171) These two genes are responsible for all the currently known Adenosine to Inosine editing events (169).

For example, ADARs-mediated deamination of different glutamate receptor subunits play an important role in the diversification of different electro-physical properties essential in mediating fast excitatory neurotransmission signal in the brain (172). Furthermore, glutamate receptor channels mediate pathology in many neurological disorders, and ADAR genes might function in several diseases with neurological symptoms, such as bipolar affective disorder and epilepsy (171).

ADAR1 is an IFN-inducible protein that maybe involved in antiviral pathway (173, 174). In 1995, Patterson and Samuel by performing a screen for interferon (IFN)-regulated cDNAs, first reported the upregulation of ADAR1 transcript, up to fivefold, following IFN treatment (175). ADAR1 protein is a multi-domain enzyme with a C-terminal deaminase catalytic domain, three centrally located dsRNA binding domains, and an N-terminal Z-DNA binding domain (175). Three naturally occurring related forms of ADAR1 are found in human cells. The first isoform is the IFN-inducible 150-kDa protein (p150) found in the cytoplasm and the nucleus. The second isoform is a constitutively expressed 110-kDa protein (p110) that lacks the first 295 N-terminal amino acids and is exclusively found in the nucleus (175). A smaller form, 80 kDa, which lacks the putative nuclear localization signal, the Z-DNA binding domain, and the entire RNA binding domain 1, has also been described (176).

In the discussion section of the 1995 report on ADAR1 transcript increase following IFN-treatment, Patterson and Samuel discussed the biological significance of an IFN-induced ADAR1. With ADAR RNA-editing activity combined with its predominantly nuclear localization, they hypothesized that the IFN-inducible cytoplasmic 150 KDa protein plays a central function in host-response against viral replication, mainly for viruses that replicate in the cytoplasm. Specifically, ADAR1 modification of viral dsRNA molecules could interfere with viral replication by irreversibly altering the sequence of their RNA or by destabilizing duplex RNA replicative structures essential in the virus replicative mechanisms (175). Possibly, these modifications could lead to the production of aberrant proteins, introducing premature translation termination

codons in viral transcripts. With the then well-established antiviral activities of IFN-inducible PKR and RNAse L, IFN-enhanced RNA editing by ADAR1 was a possible third mechanism by which IFN directly exercises its innate antiviral activities. From that moment on, following Patterson and Samuel results, ADAR1 is always presented as an IFN-inducible protein with antiviral functions, despite the lack of evidence of its presumed antiviral function.

ADAR-mediated deamination during viral infection has been reported, but none of the results involve an antiviral activity. For example, hypermutation of viral products mediated by ADAR1 has been documented in the case of infection with Measles virus, Parainfluenza virus type 3, VSV, Borna disease virus, Avian leukosis virus, and Polyomavirus infection (137, 177-181). For minus strand RNA viruses, ADAR1-mediated hypermutation might be linked to persistence, while in dsDNA polyomavirus it might be involved in the regulation of RNA transcripts (178, 182). A recent publication by Toth *et al.* reported suppression in Measles-induced apoptosis in a cell line stably expressing ADAR1, corroborating the suggestion that ADAR1 is involved in persistent infection (183). Moreover, the negative strand RNA genome of Hepatitis Delta virus (HDV) uses ADAR1-mediated adenosine deamination to target a single adenosine in the only expressed HDV open-reading frame (184). As an essential part of its life cycle, the deamination converts an amber stop codon to a tryptophan, thereby allowing the virus to make a short and a long form of the viral protein, delta antigen, HD-Ag-S, required for viral replication, and HD-Ag-L, involved in assembly, respectively (185, 186).

In support to a possible antiviral role of ADAR1, indirect studies show that three viral components inhibit its activity. Indeed, two viral antagonists of PKR, the adenovirus VAI RNA and the poxvirus E3L protein, as well as the fish betanodavirus B2 protein impair ADAR1 deaminase function (187). The small highly structured virus-associated VAI RNA of adenovirus is required for efficient translation of viral mRNAs during the late stages of infection. Lei *et al.*

examined the effect of VAI RNA on ADAR function and showed that the deaminase activity of ADAR1 is impaired in the presence of VAI RNA *in vitro* (188). Using a synthetic dsRNA substrate, Liu *et al.* showed a potent inhibitory effect of wild type vaccinia virus E3L protein on ADAR1 adenosine deaminase activity *in vitro* (189). Both proteins contain Z-DNA and dsRNA binding motifs and are likely to directly interact together. Similarly, Fenner *et al.* demonstrated that the B2 protein of fish betanodavirus, inhibits ADAR1 editing using an *in vitro* assay with long dsRNA as substrate (190). However, none of the experiments reported in the different reports were carried out *in vivo* and with a full-infectious virus. Therefore, the biological significance of those reports in a context of viral infection remains to be established.

1.4.4.5 The Myxovirus-Resistance (Mx) proteins

The Mx proteins were among the first ISGs characterized with antiviral activities (191). The Mx family of genes encodes large GTPases with unknown cellular functions besides their involvement in antiviral responses against a wide range of RNA viruses, such as Influenza, Thogoto, and Bunya viruses. The MxA protein directly binds to viral nucleocapsid proteins, causing a redistribution of viral capsid as a mechanism to inhibit viral replication (192).

1.4.5 Viral Countermeasures

To counteract IFN antiviral properties, most viruses have evolved different strategies targeting one or several steps in the IFN signaling cascade. How do viruses circumvent the IFN response? Five major strategies, which can be used alone or in combination, have been identified. Viruses can: (i) globally interfere with host cell gene expression and/or protein synthesis; (ii) minimize IFN induction; (iii) inhibit IFN signaling; (iv) inhibit IFN-induced enzymes with

antiviral activity; and (v) develop a replication strategy that is insensitive to the action of IFN (148). Viruses causing acute infection often develop evasion mechanisms aiming to interfere with cellular gene expression and/or protein synthesis. They generally inhibit cellular gene transcription, mRNA processing, export or cellular protein synthesis. For example, the Bunya virus NS protein inhibits cellular mRNA transcription by blocking the activity of RNA polymerase II (193). This strategy prevents expression of IFN, and other cytokines, as well as the induction of ISGs. However, inhibition of host-gene expression might lead to rapid cell death, limiting the time the virus has to replicate. Furthermore, by inhibiting gene expression, the IFN response prevents the virus from using the cellular machinery for its benefit, and prevents the establishment of latent or persistent infection (148). Minimizing IFN production represents a subtle means to evade the IFN response.

Most viruses produce dsRNA as a by-product of the replication, which can activate the IFN pathway, as well as latent PKR, OAS, and ADAR antiviral pathways (Table 1.2). One way to achieve minimal IFN response is through the synthesis of viral dsRNA-binding protein that will sequester the nucleic acid. Reovirus major outer capsid protein sigma3, vaccinia virus E3L protein, HSV-1 US11 protein, and Ebola virus VP35 proteins are all dsRNA-binding proteins (194-197). Viruses have also developed an impressive number of molecular mechanisms to block the pathways induced by the production of IFN. All aspects, from receptor binding to the formation and activity of IFN-induced transcription factors, of the IFN cascade are targeted. Vaccinia viruses use molecular mimicry to block IFN binding to their cognate receptor by producing secreted vIFNα/β-binding proteins (198), while HHV-8 viral products K3 and K5, target the IFNAR for degradation (198, 199). Other means to specifically inhibit IFN signaling include sequestration of STATs, upregulation of cellular phosphatases to dephosphorylate key transcription factors and interference with ISG promoter (148). Several viruses directly target one or more of the IFN-inducible enzymes. Many viral products directly or indirectly target PKR, by producing dsRNA-

binding protein to sequester dsRNA, by producing inhibiting dsRNAs or proteins, or by inhibition of eIF2α phosphorylation (200). The OAS pathway is also targeted. For example, HIV-1 and Encephalomyocarditis virus (EMCV) infected cells show elevated expression of a cellular inhibitor of the RNAse L enzyme (201, 202).

1.5 INTRINSIC OR ALTERNATIVE INNATE IMMUNITY

Host defenses against pathogen invasion traditionally imply the concerted action of the cellular arm of the immunity, restricted to a subset of specialized cells, leading to the activation of the adaptive humoral immunity. However, in recent years a more complex view of the innate immunity has arisen. It becomes apparent that, within most cells, two types of ancestral immunities participate to the fight against infection; (i) the RNA interference (RNAi) pathway and (ii) intrinsic immunity. Whether these cellular mechanisms all belong to the intrinsic immunity or if they are induced as part of the innate immunity remains to be determined.

1.5.1 RNA Interference (RNAi)

RNA silencing, or RNA interference, is a conserved phylogenetically widespread biological pathway mediated by dsRNA present in most eukaryotic cells for posttranscriptional gene silencing. It is used in the cell to control and regulate gene expression, and also mediates resistance to endogenous parasitic and exogenous dsRNA. In innate immunity, invasion of dsRNA molecules longer than 30 bp usually leads to the IFN response. However, smaller dsRNA molecules are ineffective at inducing the IFN pathway, but may mediate RNAi when complementary to an mRNA sequence (203). dsRNAs of viral origin are processed in the cytoplasm into small interfering RNA (siRNA) 19- to 21-nt fragments, by the Dicer enzyme. This enzyme, which belongs to the RNase III

family, recognizes the ends of the dsRNA molecules and excises them to form fragments with 3’ dinucleotide overhang ends (204, 205). In lower eukaryotes, the dsRNA molecule fragments unwind in an ATP-dependent process and one strand is incorporated in the RNA interference silencing complex (RISC), where it is guided to its complementary target on the viral genome. The genome is then cleaved resulting in gene silencing (206). Infection by most viruses leads to the generation of long dsRNA molecules during their life cycle that can trigger the RNAi pathway. Although this mechanism is a cell response to virus infection in plants and lower eukaryotes, evidence for the role of RNAi in most cases of mammalian virus infection is lacking. In an extensive study examining whether viral infection induces the antiviral RNAi pathway, Pfeffer *et al.* (207) cloned small RNAs from a variety of mammalian viruses following cell infection. Surprisingly, miRNAs of viral origin were recovered from herpesvirus, poliovirus, adenovirus, and ascovirus, but none from HIV or HCV (208). Another paper reported miRNAs (micro RNAs) from HIV (209). In the case of HIV, when Dicer expression was inhibited in virus-infected cells, virus production was up- or downregulated in low or high replicating cells suggesting a complex mechanism (210, 211). Nevertheless, there are reports of hijacks of the RNAi system by viruses. For example, HCV was shown to use the liver-specific microRNA-122 to aid its translation by accelerating the binding of the ribosome to its mRNAs (212). The presence of viral suppressors of interference suggests that viruses have countermeasures against RNAi, but the dual activities of TRBP in both promoting HIV replication and functioning in the RISC brings an additional complexity (209, 213, 214). Future studies will determine whether RNAi contributes to viral inhibition or to viral enhancement. They will also determine if RNAi is part of the pathway that occasionally interferes with viral replication in mammalian cells.

1.5.2 Intrinsic Immunity

The concept of intrinsic cellular inhibitors of retroviral replication has been known for some time. Reports in 1971 of a “Friend virus susceptibility” (Fv) loci that results in decreased susceptibility to murine leukemia virus (MLV) in some inbred mouse strains opened the road for intrinsic cellular inhibitors (215). During the 1990s, evidences of restriction factors to HIV replication began to accumulate. Results from heterokaryon analyses, in which HIV permissive cells were fused with non-permissive cells, were conducted. In some cases, the heterokaryons were nonpermissive, implying the existence of dominant factor therein that inhibited HIV-1 replication in nonpermissive cells (216). The apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC) 3G, the tripartite interaction motif (TRIM) 5α, and Tetherin are the three known antiretroviral restriction factors inhibiting HIV-1 replication. Their inhibitory action has been well documented (217). APOBEC3, a member of a group of cytidine deaminases, was the first HIV-1 restriction factor identified. Its cellular expression defines cells that are nonpermissive for replication of HIV-1 mutant strains lacking a functional Vif gene (218). Other members of the APOBEC family have also been shown to inhibit replication of HIV-1 and other retroviruses, such as MLV, SIV, and mouse mammary leukemia virus, in a Vif-dependent manner (219-221). In the absence of a functional Vif, APOBEC3 proteins can be incorporated in the HIV-1 and other retrovirus virions, through interaction with the genomic RNA (222-224). Following infection, the incorporated APOBEC3 enzymes catalyze the deamination of deoxycytidine during reverse transcription, leading to a hypermutated viral genome that is targeted for degradation (225, 226). Wild-type Vif protein binds APOBEC3 and targets it for proteasomal degradation, preventing its incorporation in the viral particles (227).

TRIM5α is the restriction factor responsible for resistance of old world monkey cells to HIV-1 infection (228-230). TRIM5α also plays a role in resistance of human and other primate cells to MLV, and equine infectious anemia virus (217). The fine mechanisms resulting in TRIM5α-mediated restriction are not fully understood. However, it is believed to occur after viral entry in the early phase of the replication cycle and to involve a rapid and irreversible lethal lesion in the incoming viral capsid (231). Tetherin is a membrane protein that has recently been found to inhibit the release of fully matured HIV particles from infected cells, preventing viral dissemination to uninfected cells (232, 233). It is an IFN-inducible protein, suggesting that it might play a more central role in innate antiviral mechanisms (234).

1.6 THE DSRNA-ACTIVATED PROTEIN KINASE (PKR) AND ITS REGULATION

1.6.1 PKR Structure

After its discovery, IFN was recognized for its activity on the translation of vaccinia virus (235). First recognized as a p68 protein, PKR cDNA was cloned from human and mouse libraries and the kinase was then characterized as a translational inhibitor involved in an IFN-regulated antiviral pathway following vaccinia virus infection (164, 236). The human protein was formerly referred to as p68, DAI (the dsRNA activated inhibitor), or dsI (dsRNA inhibitor), and the mouse protein as p65 or TIK, for anti-phospho*t*yrosine *i*mmunoreactive *k*inase. Both proteins were renamed PKR in 1993 (237). PKR is an important component of the IFN-induced antiviral pathway, and is one of the most studied. The human *pkr* gene spans 50 kb and is encoded at position 21-22 on the short arm of chromosome 2. The gene codes for a 551-amino acid long protein translated from a 2.5-kb mRNA. The protein consists of two domains: an amino-terminal half dsRNA-binding regulatory domain (dsRBD) and a carboxy-terminal catalytic kinase domain (238, 239). The N-terminal domain contains two dsRBDs, of approximately 65 amino acids residues each (Fig.1.9). The dsRBDs are

phylogenetically related modular domains found in a variety of proteins from all kingdoms, as well as viruses, that promote dsRNA binding and protein-protein interaction. The catalytic serine/threonine kinase domain found in the C-terminal half of PKR contains eleven conserved subdomains, with amino acids in subdomain VI (HRDLKP) consistent with recognition of serine and threonine residues by PKR on the other substrates (239, 240).

1.6.2 PKR Family

PKR belongs to a small family of protein kinases that phosphorylate the alpha subunit of the elongation Eukaryotic Initiation Factor 2 (eIF2α) in response to different stress conditions. eIF2α phosphorylation impairs its activity which results in cellular protein translation inhibition, allowing the cell to effectively manage the stress environment (239). The eIF2α kinase family includes PKR, PKR-like endoplasmic reticulum inhibitor (PERK), general control non-de-repressible 2 (GCN2), and heme-regulated inhibitor (HRI), each of which responds to different activating ligands. Amino acid starvation, which can be a consequence of viral and pathogen infections, results in an increased concentration of uncharged transfer RNAs (tRNAs) leading to GCN2 activation. PERK is activated by the accumulation of unfolded proteins in the lumen of the ER, while HRI responds to low heme concentrations. In contrast to PKR, which has only been reported in vertebrates, GCN2, PERK, and HRI are also found in lower eukaryotes. The subdomains V-VII found in PKR are shared by all the members of the family, in addition to an insert region, between subdomains IV and V, which is a distinctive feature only found in this family (241).

1.6.3 PKR Activation

PKR is constitutively expressed in mammalian cells and remains as a closed inactive monomer. The inactive state is due to the auto-inhibitory effect of PKR second dsRBD, which interacts with the insert region occluding the enzyme's kinase domain, therefore regulating the kinase activity by preventing substrate binding (242). Although mainly cytoplasmic, a small portion of the kinase has been located in the nucleus, the function of which remains obscure. PKR is activated by dsRNAs, dextran sulfate, poly(L-glutamate), heparin, pro-inflammatory stimuli, growth factors, cytokines, oxidative stress and protein activators. In the cell, PKR critically acts as a dsRNA sensor, which often signals viral or other pathogenic invasion. Activation can only be detected with dsRNAs with a minimal size of 30 base pairs (bp) size, or ssRNA with duplex region of at least 16 bp with 10-15 nucleotide-long single-stranded tails. However, optimal activation is observed with dsRNA of 85 bp-long. Recognition of dsRNA is independent of nucleotide sequence and a number of cellular dsRNA molecules activate the kinase as well. As an intrinsic mechanism believed to regulate their own expression, TNFα and IFNγ highly structured mRNAs have been reported to activate PKR. Furthermore, abnormal activation of PKR by cellular mRNAs has been reported in some diseases (243). For example, mRNAs for the mutant forms of the Huntington's and myotonic dystrophy protein kinase activate PKR (244). The kinetics of PKR activation by dsRNA exhibit a characteristic "bell-shaped" curve of activation, where low dsRNA concentrations activate the kinase function but higher concentrations inhibit it (245). The specific events leading to PKR activation and regulation by dsRNA are not fully understood. Binding to activating substrates leads to PKR homodimerization and *trans*-autophosphorylation on T446, resulting in a fully active kinase, that mediates antiviral activities, cell growth and proliferation control.

1.6.3 Cellular functions

Activated PKR subsequently phosphorylates a variety of targets, the best-characterized being eIF2α, the substrate on which PKR primarily exerts its antiviral activities through translational regulation. Phosphorylation of eIF2α on serine 51 prevents its recycling for ongoing translation, resulting in a severe inhibition of cellular, as well as viral protein synthesis. eIF2α activity is central to the cell by decreasing protein synthesis rates and promoting cell survival in stress conditions. PKR also engages in several signal transduction pathways through NF-κB. In response to other stress stimuli, PKR was shown to target adapter molecules, such as TRAF, leading to phosphorylation and activation of the IκB kinase (IKK)α/IKKβ kinase complex. Activation of IKKα/IKKβ leads to phosphorylation of NF-κB-bound inhibitor, IκB, resulting in its subsequent degradation. As a result, NF-κB is translocated in the nucleus where it promotes transcription of several genes (246). Furthermore, PKR is also highly involved in apoptosis through activation of caspase-8, which links the mitochondrial/intrinsic apoptosis pathway to the extrinsic pathway. PKR interacts with the cytosolic adaptor FADD (Fas-associated death domain-containing protein), which associates with pro-caspase-8, a major effector in the apoptosis pathway (200). PKR is also an intermediate in TLR signaling where it integrates and transmits the signal to, among others, STAT, IRFs, p53, and JNK.

1.6.4 Cellular Protein Regulators

PKR has several cellular inhibitors and activators, of which $p58^{IPK}$ was the first inhibitor to be identified. A member of the tetratricopeptide repeat family, $p58^{IPK}$ interacts directly with PKR for inhibition of the kinase activity. As part of influenza virus immune evasion, the virus partially recruits $p58^{IPK}$ and represses PKR-mediated eIF2α phosphorylation (247). Another potent PKR inhibitor, the

TAR RNA binding protein (TRBP) was identified by its ability to bind HIV TAR dsRNA (248). TRBP enhances HIV translation *in vivo* and *in vitro* by direct binding to PKR, by sequestering dsRNA and by releasing the translational block due to the TAR structure (249-252). It was recently shown to directly bind Dicer, and play a crucial role in RNA interference mechanisms (214, 253). Nuclear factor 90 (NF90), another dsRNA binding protein, also interacts with PKR and inhibits PKR phosphorylation. C114, protein induced by IL-11, HSP90 and the Fanconi anemia proteins, which regulate chromosome stability, have also been reported to bind to PKR and modulate PKR activity (254-256). As for cellular protein activators, PACT, the PKR activator, is the best described. PACT regulates PKR activity in response to stress-inducing molecules and stimuli. It contains two dsRBDs and heterodimerizes with PKR, as well as with TRBP (257-259). Some other known cellular activators of the kinase include E2F-1, the tumor suppressor interleukin-24 (Mda7), has no dsRBD domain and the nature of its interaction with PKR remains unknown (260).

1.6.5 Viral Subversion of PKR Activity

PKR plays several critical roles in the innate response to virus infection, and this is emphasized by the large number of viruses that have evolved elaborate mechanisms for its inhibition (200, 261, 262). Viral countermeasure strategies target virtually all the steps leading to eIF2α phosphorylation. These include: (i) sequestration of dsRNA by dsRBPs, (ii) RNA inhibitors, (iii) inhibition of PKR autophophosrylation by direct interaction, (iv) competitive inhibitors, (v) PKR degradation, and (vi) eIF2α dephosphorylation (Fig. 1.9; Table 1.2). Most viruses use a combination of several strategies for PKR immune evasion, and it is yet unclear if multiple mechanisms targeting PKR have redundant roles, or are necessary functions to regulate antiviral responses at different stages during the virus replication cycle (262).

1.6.5.1 Sequestration of dsRNA by dsRBPs

Overwhelming amounts of cytoplasmic dsRNA often coincide with viral replication, and signals viral infection to the cell. Therefore, several viruses have been reported to encode dsRNA-binding proteins, most of which contain the putative dsRBD to mediate PKR interaction, as a mean to avoid the wake of cellular antiviral pathways. As mentioned above, the vaccinia E3L protein binds to dsRNA and prevents PKR activation. Deletion of the E3L gene from the vaccinia genome results in a significant increase in susceptibility to IFN treatment (263). Epstein-Barr virus (EBV) and herpes simplex virus (HSV) code for the SM and Us11 proteins, respectively. Both proteins were shown to directly bind dsRNAs, as well as PKR, through an arginine and proline-rich domain, called RXP domain (264, 265).

1.6.5.2 RNA inhibitors

Several viruses encode RNA antagonists of PKR. Virus-associated RNA I and II, (VAI and VAII) are two RNA polymerase III-transcribed RNAs encoded in the adenovirus genome, that are required for viral efficient replication. Deletion of VAI is associated with diminished viral replication due, in part, to increased eIF2α phosphorylation (266, 267). VAI is highly structured and was found to bind to and inhibit PKR phosphorylation (268, 269). Epstein-Barr virus encodes two highly structured small RNAs (EBER1 and EBER2) that share several structural features with VAI, and are believed to be used as a strategy to inhibit PKR (270, 271). HCV's internal ribosome entry site (IRES) on the genomic RNA is used to escape IFN-antiviral pathway through direct inhibition of PKR (272).

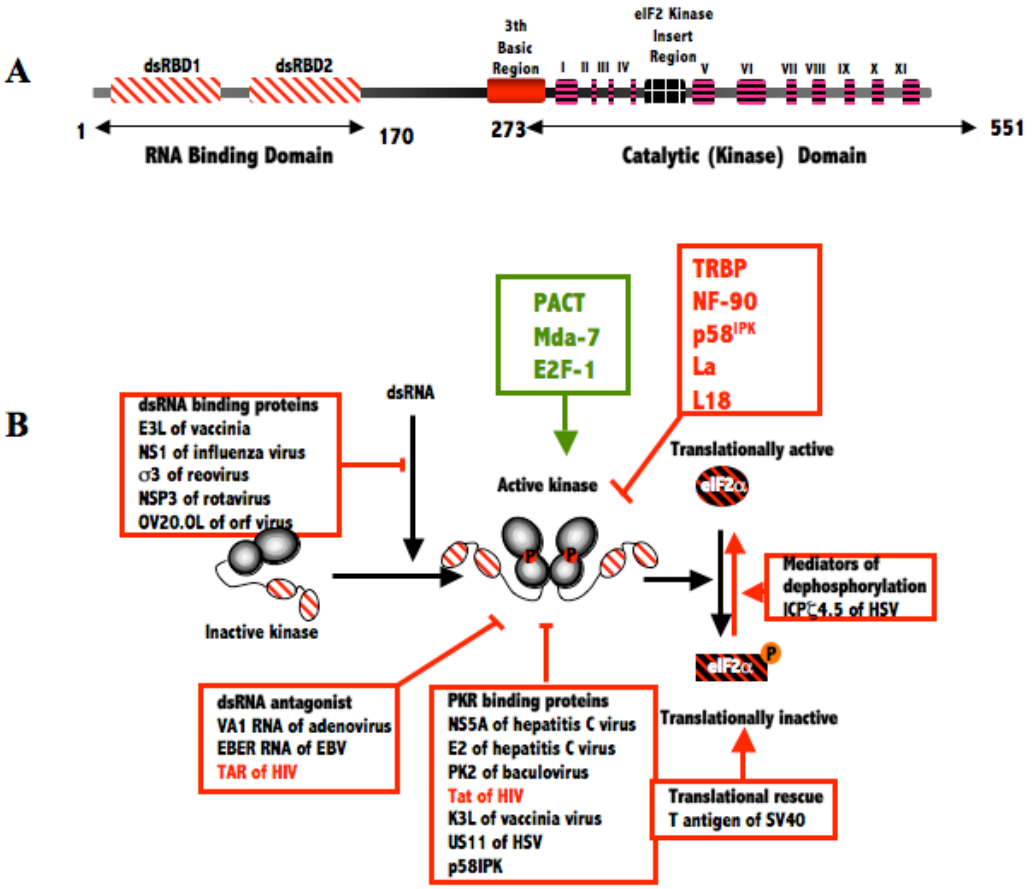


Figure 1.9. (A) Schematic representation of PKR (236, 273). PKR structural features include the two double-stranded RNA binding domains that form the N-terminal regulatory domain, and the carboxy-terminal kinase domain. The consensus subdomains (I-XI) found in all protein kinases are indicated (274, 275). (B) PKR activation following its binding to dsRNA molecules and examples of viral and cellular modulators.

1.6.5.3 Inhibition of PKR autophosphorylation by direct interaction

HCV protein NS5A directly interacts with PKR through a region termed interferon sensitivity determining region (ISDR), resulting in inhibition of PKR dimerization and activation (276). The HCV E2 protein also shows similar function on PKR (277). Human herpes virus-8 (HHV-8) encodes a homologue of interferon regulatory factors (IRFs), vIRF-2, that is capable of binding to PKR

leading to its autophosphorylation and inhibition in the presence of dsRNA (278). Negative regulation of PKR may be involved in the formation of latent herpes infections (262). Vaccinia virus K3L and the ReIF2H protein from Ranaviruses, dsDNA viruses that infect amphibians and fish, encode eIF2α homologues that serve as a pseudosubstrate for PKR (279, 280). The Tat protein of HIV also serves as a substrate for PKR (281).

1.6.5.4 PKR degradation

A few viruses were reported to target PKR for degradation as an immune evasion strategy. Poliovirus infection leads to a high PKR response and increases eIF2α phosphorylation. PKR is rapidly degraded following poliovirus infection. Although a detailed understanding of the mechanism of PKR degradation is not yet known, poliovirus' genome contains proteases that regulate cellular gene expression by different mechanisms (282).

1.6.5.5 eIF2α dephosphorylation

Finally, some viruses encode phosphatases that dephosphorylate eIF2α, thereby allowing the translation to proceed even in the presence of activated PKR. The γ134.5 gene product of HSV shares homology with cellular GADD34, a regulatory subunit for the cellular protein phosphatase 1α (PP1α) involved in eIF2α dephosphorylation (283). Human Papilloma Virus type 16 oncoprotein E6 synthesis is inhibited by phosphorylated eIF2α in response to viral infection or IFN treatment. Kazemi *et al.* showed that E6 interacts with the GADD34/PP1 holophosphatase complex and promotes dephosphorylation of eIF2α, thereby counteracting the inhibitory effect of eIF2α phosphorylation on cellular protein synthesis (284). Furthermore, in two different publications, Thimmapaya's laboratory reported that SV40 large-T antigen reverses PKR-mediated

translational inhibition at a step downstream of both PKR and eIF2α phosphorylation (285, 286).

Mechanisms	Virus	Gene product
I. Sequestration of dsRNA	Vaccinia virus Reovirus Influenza virus Rotavirus group C Rotavirus group A Herpes simple virus Epstein-Barr virus	E3L σ3 NS1 NSP3 NSP5? Us11 SM
II. RNA inhibitors	Epstein-Barr virus Adenovirus Hepatitis C virus	EBER RNA VAI RNA, VAII RNA? IRES
III. Inhibition of PKR autophosphorylation by direct interaction	Hepatitis C virus Vaccinia virus Baculovirus Herpes simplex virus-1 Epstein-Barr virus KSHV	NS5A, E2 E3L PK2 Us11 SM vIRF-2
IV. Competitive inhibitor	Vaccinia virus Ambystoma tigrinum virus HIV	K3L Rel2H Tat
V. PKR degradation	Poliovirus	Protease
VI. eIF2α dephosphorylation	Herpes simplex virus Papilloma virus SV40 KSHV	γ34.5 E6 (GADD34/PP1α Large-T antigen LANA2

Table 1.2: Regulation of PKR by viral products. Modified from (262).

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CHAPTER 2

RATIONALE, HYPOTHESIS AND OBJECTIVES

2.1 RATIONALE OF THE RESEARCH

2.1.1 IFN PRODUCTION AND HIV REPLICATION

Viral infection induces the production of IFNα from peripheral blood mononuclear cells. Two main populations of DCs are responsible for IFNα production, the myeloid DC (myDCs) and plasmacytoid DCs (PDCs), and both are profoundly deregulated during HIV infection. Plasmacytoid dendritic cells are specialized “natural IFN-producing cells” (IPCs) and are the chief IFNα producers in response to enveloped viruses, bacteria, and tumor cells (1). PDCs migrate directly from blood to the secondary lymphoid tissue. They lack hematopoietic-lineage markers, express CD4 and major histocompatibility complex (MHC) class II and are critical effector cells during antiviral and antitumour immune responses. Soumelis *et al.* showed that circulating PDCs are deficient in their capacity to produce IFN in HIV-infected subjects, producing 20-fold less IFNα compared to PBMCs from healthy donors (2). Moreover, PDCc are markedly and progressively lost during progression to AIDS compared to non-progressors HIV-1-infected patients and healthy controls. The loss of PDCs correlates with the apparition of opportunistic infections and active Kaposi sarcoma (2). Furthermore, the number of circulating IPCs is positively correlated with $CD4^+$ T cells count and negatively correlated to HIV viral load. In addition, PDCs are able to mature under certain circumstances, such as influenza challenges, into antigen-presenting cells and induce T helper (TH) 1 or TH2 responses (3, 4). These studies add to the suggestion that IPCs have a protective function in both adaptive and innate immune responses against HIV-1 infection.

2.1.2 LACK OF *IN VIVO* INHIBITION OF HIV INFECTION BY IFN TREATMENT

Early *in vitro* studies in which HIV-1-infected cells were treated with IFNα suggested that the cytokine should limit the spread of HIV-1 infection if used therapeutically. IFNα was one of the first drugs tested against HIV-1 infection, both *in vitro* and *in vivo*. During the acute phase of HIV-1 infection, high-titer viremia is present both in the plasma and in some tissues before falling to very low levels within weeks of the onset of symptoms. IFN levels in plasma directly correlate with viral load during acute viral phase (5, 6). Some clinical trials in which IFNα is administered to patients in early HIV-1 disease show significant reduction in both the level of p24 antigen in the plasma and in the frequency of AIDS-associated opportunistic infection (7, 8). IFN acts *in vivo* by preventing *de novo* cellular infection as opposed to viral production, corroborating *in vitro* findings showing IFNα greater antiviral mechanism against HIV when cells are treated prior to viral challenge (9, 10). In addition, IFN treatment was found to have some adverse effects (11). Therefore, due to its modest inherent antiviral efficacy in association with adverse toxic effects (ranging from flu-like symptoms, granulocytopenia, decrease in granular white blood cells, elevated levels of liver enzymes), and the development of HAART, IFN was abandoned as a treatment for HIV-infection (9).

2.1.3 HYPOTHESIS AND OBJECTIVES

Type I IFN function in HIV-1 pathogenesis is very complex and controversial. Much of the evidence that IFN would be effective in treating HIV-infected patients is based on *in vitro* studies. The research started as early as 1983, where IFN was shown to be sufficient to suppress viral replication in HIV-1-infected tissue cultures. Because of their antiviral activities, type I IFNs were tested for their potential to inhibit HIV-1 replication *in vitro* . Early studies of

HIV-infected primary cells, such as PBMCs monocytes/macrophages, $CD4^+$ T cells, and established T cells and monocytes lines showed that IFN treatment inhibits viral replication. This activity is observed at early steps of viral replication on DNA synthesis as well as on RNA and protein production. IFN also interferes with virion assembly and the release of newly formed virions (12). Further studies showed that IFN activity on HIV-1 replication could be in part due to ISGs 2'5' OAS dependent RNAse L, ISG15 and PKR (13-16). These studies led to several clinical trials in which type I interferon was administered to HIV-infected patients. A modest therapeutic effect was reported in some but not all trials, leaving the issue of interferon therapy for HIV-1 disease unresolved (17). In conclusion, a discrepancy exists between the findings of *in vivo* studies and *in vitro* experiments concerning the protective effects of IFN. These discrepancies highlight our poor understanding when it comes to the cellular regulation of the IFN response. These studies emphasize the necessity to better understand the various parameters that could contribute to the activity of the ISGs to elicit an antiviral response.

Our general hypothesis is that the absence of IFN effectiveness during HIV infection is due, at least in part, to the lack of activity of its downstream targets, the IFN stimulated genes. PKR is one crucial ISG that has antiviral activity.

Our hypothesis is that **PKR is not activated during HIV infection due to multiple interactions with HIV components and cellular proteins, which modulate its function.** This inhibition contributes to an increased HIV replication and to pathogenesis. Our objective is to elucidate the contribution of the ribonucleoprotein complex formed with PKR during infection to viral replication.

Our specific aims are:

1. To study the regulation of PKR activation during HIV infection.

2. To study the role of ADAR1-PKR interaction during HIV replication.

3. To study the function of PACT in the context of a ribonucleoprotein complex with PKR during HIV replication.

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CHAPTER 3

ADAR1 INTERACTS WITH PKR DURING HIV INFECTION OF LYMPHOCYTES AND CONTRIBUTES TO VIRAL REPLICATION

(This chapter is adapted from an article published in J. of Virology (Clerzius, *et al.*, 2009)(1)

3.1 INTRODUCTION

PKR is activated by HIV TAR RNA *in vitro* . The presence of TAR RNA molecules in the early stages of infection might be sufficient to activate PKR *in vivo*. Therefore, PKR may play an important role in restricting HIV replication in the early events during infection. However, PKR activation and regulation in the early stages of HIV infection has never been reported.

The adenosine deaminases that act on RNA (ADARs) are a family of RNA editing enzymes that target highly-structured double-stranded regions of nuclear-encoded and viral RNAs. They are mainly found in the central nervous system where they diversify the information encoded in the genome by posttranscriptional RNA modifications. ADARs catalyze the deamination of adenosine to create inosine, thereby altering codons in some mRNAs (2). ADAR1, an important member of the RNA-editing enzymes, is an IFN-inducible protein involved in the cellular antiviral pathway (3, 4). As mentioned previously, a report by Patterson and Samuel in 1995 where they reported a fivefold increase in ADAR1 transcript following IFN-treatment may have precipitated ADAR1 classification as an IFN-induced antiviral protein (2). Most instances where ADAR-mediated deamination during viral infection likely involved mechanisms essential to the viral life cycle, but the underlying mechanisms remain to be finely elucidated (5-7). So far, only three viral products, the adenovirus VAI RNA, the poxvirus E3L and the fish betanodavirus B2 protein, are known to impair ADAR activity directly *in vitro* (8-11). So far, identification of an active viral mechanism or viral gene product produced during infection and resulting in an altered A-to-I RNA editing of a viral or cellular substrate by direct inhibition of ADAR1 enzymatic activity has not been reported (8).

Until recently, the ADAR1-mediated deamination of HDV transcript at the amber/W site deamination, which is essential for the virus life cycle, was the only elucidated mechanism of how a virus co-opt ADAR activity for its own benefit. In recent years, studies from our laboratory and others have shed a new role for ADAR1, where the protein is involved in mechanisms used by different viruses to help their replication. In 2009, Nie *et al.* reported that expression of ADAR1 enhances replication of VSV through a mechanism that is independent of dsRNA editing. They also show that ADAR binds to and inhibits PKR activation, leading to the suppression of eIF2α phosphorylation (12). Similarly, Li *et al.* reported this year that decreased in ADAR1 expression leads to a lower in vesicular stomatitis replication following IFN treatment and resulted to an increase in PKR activity (13). A recent paper by Doria *et al.* linked ADAR1-mediating edition of HIV-1 transcripts to an increase in the virus replication (14). In this section of the thesis, we present our findings regarding the regulation of PKR activation during HIV replication, and its regulation by ADAR1. The results were published in the Journal of Virology in 2009 (1).

3.2 PAPER: ADAR1 INTERACTS WITH PKR DURING HIV INFECTION OF LYMPHOCYTES AND CONTRIBUTES TO VIRAL REPLICATION

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3.3 ABSTRACT

The interferon-induced protein kinase RNA-activated (PKR) is activated after virus infection. This activation is only transient during human immunodeficiency virus type 1 (HIV-1) infection of lymphocytes and the protein is not activated at the peak of infection. We observed that the interferon-induced Adenosine deaminase acting on RNA (ADAR)1-p150 and ADAR1-p110 expression increases while the virus replicates actively. Furthermore, both forms of ADAR1 show enhanced interactions with PKR at the peak of HIV infection suggesting a role of this protein in the regulation of PKR activation. We observed that ADAR1-p150, like previously shown for the TAR RNA binding protein, TRBP, reverses PKR inhibition of HIV expression and production in HEK 293T cells. This activity requires the Z-DNA binding motif and the three double-stranded RNA binding domains, but not the catalytic domain. In astrocytic cells, ADAR1-p150 increased HIV expression and production to a similar extent as TRBP. Small interfering RNAs against ADAR1-p150 decreased HIV production moderately. These results indicate that two interferon-induced proteins, ADAR1 and PKR have antagonistic functions on HIV production. They suggest that ADAR1, TRBP and the viral Tat belong to a multi-protein complex that inhibits PKR during HIV infection of lymphocytes.

Keywords: HIV, PKR, ADAR1, TRBP, lymphocytes, astrocytes,

3.4 BACKGROUND

Treatment of human cells by interferon induces the expression of hundreds of IFN-stimulated genes (ISGs), some of them having an antiviral activity. These genes include the 2'-5'-oligoadenylate synthetase, Adenosine Deaminase acting on RNA 1 (ADAR1), Mx GTPases, Major Histocompatibility Complex class I and II, protein kinase RNA-activated (PKR) and many others (15). Among the ISGs, PKR is a key serine/threonine kinase that has antiviral and antigrowth activities (16, 17). PKR is activated by dimerization after binding to low level of dsRNA through its two double-stranded RNA binding domains (dsRBDs) (18). Once active, PKR phosphorylates several substrates, including the alpha subunit of the translation initiation factor2 (eIF-2α), which alters the efficiency and rate of translational initiation.

PKR activation is a critical component of antiviral and cell growth pathways (19) and its importance is illustrated by numerous cellular and antiviral mechanisms aiming to counteract its response. Viral mechanisms include the expression of competitive inhibitory RNAs or viral proteins that act either by direct inhibition of PKR, by sequestration of dsRNA, as competitive substrates or as translational rescuers by dephosphorylating eIF2α (19, 20). Cells also control PKR activation to limit the translational repression induced by the protein and to control cell growth. For example, the ribosomal protein L18, the TAR RNA binding protein (TRBP) and $p58^{IPK}$ sequester dsRNA or prevent PKR phosphorylation (20). Inhibition by protein-protein interactions also occurs with TRBP, hDUS2 and ADAR1, which bind PKR through their dsRBDs (12, 21, 22). In contrast, dsRNA, heparin and cellular proteins MDA7, PKR activator (PACT) and E2F-1 activate PKR (23-28). Viruses have also adapted to the cell in which they replicate by using cellular factors to regulate PKR activation. For example, Influenza virus activates $p58^{IPK}$ (29), Herpes virus US11 inhibits PACT (30), HIV

TAR RNA recruits TRBP in proximity of PKR (21, 31, 32), and Vesicular Stomatitis virus (VSV) uses ADAR1 to inhibit PKR (12).

ADARs are RNA-editing enzymes that modify nuclear and viral RNAs by deamination that convert adenosines to inosines (2). Full-length ADAR1 enzymes possess two N-terminal Z-DNA binding domains, three central dsRBDs, the first of which is responsible for PKR binding, and a C-terminal deaminase domain. Three immunologically related isoforms of ADAR1 are found in human cells: the IFN-inducible cytoplasmic 150-kDa protein and constitutively expressed 110-kDa and 80-kDa proteins, which lack the first and both Z-domains respectively (33). The 150kDa form of ADAR1 was recently shown to bind to and inhibit PKR and to increase susceptibility to VSV infection (12). Whether ADAR1 plays a role as a PKR inhibitor in other viral infections has not been explored.

HIV expression is controlled at the transcriptional, post-transcriptional and translational levels (34-36). HIV-infected cells treated with IFN show a decreased production of HIV proteins and a reduced HIV production mainly ascribed to PKR activation (37). The HIV-1 Tat protein was shown to inhibit PKR activity by acting as a competitive substrate (38). Astrocytic cells represent an example of naturally HIV-resistant cells with high PKR activation. In these cells, TRBP is expressed in very low amounts and cannot counteract PKR activation induced by the virus (32, 39, 40). Therefore PKR activation can become a barrier to HIV replication, but the status of PKR phosphorylation has not been studied during viral infection of lymphocytes.

In this paper, we show that PKR is only transiently activated during HIV infection of lymphocytic cells. The analysis of cellular factors that interact with PKR during HIV infection show that ADAR1 plays an important role to inhibit the kinase function during active replication.

3.5 MATERIALS AND METHODS

3.5.1 PLASMID CONSTRUCTIONS AND SIRNA SYNTHESIS.

pCMV-ADAR1 plasmid, containing ADAR1 mRNA (1 to 4058), GenBank accession # NM_001111.3, was obtained from Dr. K. Nishikura (41). This plasmid was used as a template to generate a cloning intermediate plasmid, ADAR1-p150 (1 to 3678), with a XhoI cleavage site added to the 3' site to facilitate cloning. ADAR1-p150 fragment was cleaved with HindIII and XhoI and subcloned into the pcDNA3.1_V5 vector (Invitrogen). This construct was used to generate the different variants ADAR-p110 (888 to 3678), ADAR p80 (1869 to 3678), ADAR p70 (1 to 1869), and ADAR Dcat (1 to 2475). All constructs were verified by sequencing. pGL2-LTR-Luc, pcDNA3-TRBP2, pcDNA1-PKR were previously described (21, 42, 43). Sequences of siRNAs used in this study were: NS (31), siA (12) or si4 (Qiagen SI00292320). They were all synthesized by Qiagen.

3.5.2 CELLS AND TRANSFECTIONS.

Astrocytoma cell line U251MG (40) and HEK 293T (ATCC CRL-11268) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen). HEK 293T cells express adenovirus sequences and SV40 Large T antigen (44). Jurkat T cells (ATCC TIB-152) and Jurkat-CCR5 (45) obtained from Dr. K. Peden were maintained in RPMI-1640 (Invitrogen) supplemented similarly and with 0.4 mg/ml G418 (Multicell) for Jurkat-CCR5. For transfection of HEK 293T and astrocytes, cells were plated at 50% confluence 24 h prior to transfection using TransIT-LTi Transfection Reagent following manufacturer's protocol (Mirus). Transfection of HEK 293T cells with siRNAs was performed in six-well plates using Lipofectamine 2000 (Invitrogen) as previously (31) 24 h prior to transfection with

pNL4-3 using TransIT-LT (Mirus). Cells were lysed 48 h posttransfection for immunoblotting or luciferase analysis.

3.5.3 TRANSFECTION OF HIV CLONES AND REVERSE TRANSCRIPTASE (RT) ASSAY.

For transfection of HIV provirus, HEK 293T were transfected as above with pNL4-3 or pMAL proviral DNA. Cell supernatants were collected 48 h post transfection and assayed for standard RT assay (46) except that reaction was spotted onto DEAE filtermat (PerkinElmer). After 5 washes in 2X SSC, and twice in 95% ethanol, the filtermat was air-dried and read using Microbeta Scintillation counter (PerkinElmer). These supernatants were used for infection of Jurkat or Jurkat-CCR5.

3.5.4 HIV-1 VIRAL INFECTION.

For each infection, 10^7 Jurkat or Jurkat-CCR5 cells were infected with HIV cell supernatant corresponding to 2.5 x 10^6 cpm measured by standard RT assay in a final volume of 5 ml RPMI (Invitrogen), supplemented as above, and incubated for 2 h at 37^{o}C with mixing every 30 min. 10 ml of RPMI was then added to the cell-virus mixture, transferred to a T75 flask and incubated overnight at 37°C. Another 15 ml of medium was added and the cell culture was maintained at 37°C for 25 days. The cells were fed every other day (or three days when their growth was not sufficient) by replacing 12 ml of supernatant with fresh medium and maintaining the cell density between 2.5 x 10^6 and 1 x 10^7 cells/ml. Supernatant and cell samples were collected at different times and assayed for RT activity, immunoblotting and immunoprecipitation (IP).

3.5.5 IMMUNOBLOTTING

HEK 293T, U251MG or Jurkat T cells extracts were prepared, separated and transferred for immunoblotting as previously described (47). Membranes were blocked for 1 h in 5% nonfat dry milk and Tris-buffered saline-0.1% Tween 20 (TBST) or 5% BSA and 0.1% TBST for anti- PKR-pT^{451} antibody (Biosource). Membranes were incubated overnight at 4°C with the primary antibody. After five washes in TBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibody (GE Healthcare). Anti-P-PKR was used first in 3% BSA/TBST and the membranes were washed overnight in TBST and reused to detect other proteins. The bands were visualized using ECL (GE Healthcare). Primary antibodies used for immunoblotting in 5% milk/TBST were monoclonal anti-PKR 71-10 (48) obtained from Dr. A. Hovanessian, anti-Actin (Chemicon) at a 1/500 dilution, anti-GAPDH (Santa Cruz) at a 1/2500 dilution, anti-HIVp24 183-H12-5C (49), anti-V5 (Invitrogen) at a 1/5000 dilution, polyclonal anti-P-PKR, anti-human ADAR1 (a kind gift from Dr. B. Bass) at a 1/1000 dilution, and anti-TRBPjbx as previously (50). Actin or GAPDH was probed on each separate blot. Where indicated, the bands where quantified by densitometry analysis as described (51).

3.5.6 IMMUNOPRECIPITATION

HIV-infected and mock-infected Jurkat T cells were washed twice with ice-cold PBS and lysed in the cold lysis buffer with protease inhibitors. For each IP, 50 µl of protein G agarose fast flow compact beads (Sigma) were washed with ice-cold PBS and left rotating at 4°C for 4 h with 8 µg anti-PKR 70-10 or 5 µg anti-human ADAR1 antibodies. 2.5 mg of cell extracts were added to the beads for overnight incubation at 4°C. The beads were washed 5 times with 1 ml of ice-cold PBS and resuspended in SDS loading dye. Bound proteins were eluted by boiling the beads for 5 min and fractionated by 10% SDS-PAGE. The

immunoprecipitates were analyzed by Western blot analysis using appropriate antibodies.

3.6 RESULTS

3.6.1 PKR ACTIVATION IS INHIBITED DURING ACTIVE HIV REPLICATION.

PKR becomes activated after transfection of HIV molecular clones in the non-productive astrocytes, but not in the productive HeLa cells (32). To determine if PKR becomes activated in lymphocytes, we analyzed its phosphorylation during viral infection. Jurkat cells were infected by HIV NL4-3, an X4 virus that uses CXCR4 as a coreceptor (52, 53) and viral kinetics were followed by RT assay on the culture media over 25 days (Fig. 3.1A). Cell extracts were analyzed for PKR expression and phosphorylation (Fig. 3.1B). We observed that PKR was transiently phosphorylated up to day 6. This phosphorylation decreased at day 8-10 and was no longer observed after day 12. This PKR activation correlated with RT activity that became visible at day 10 and showed a peak at day 11 to 15, corresponding to active viral expression and production as shown by immunoblotting against HIV p24 antibody revealing $p55^{gag}$ expression (Fig. 3.1B). This correlation suggests that PKR activation is inhibited during active viral replication.

Because TRBP has been demonstrated to be a strong inhibitor of PKR in the context of HIV expression (21, 31, 32, 37), we verified if an increase of its expression could explain the lack of PKR activation but no correlation between TRBP levels and PKR activation was observed (Fig. 3.1B). ADAR1 is also a PKR inhibitor in HEK 293T cells and during VSV infection (12) and therefore its expression was verified on the same extracts. Surprisingly, a strong increase of both ADAR1 p110 and p150 forms correlated with the appearance of HIV $p55^{gag}$ expression and the decrease of P-PKR (Fig. 3.1B). Cell extract analysis from a mock infection of Jurkat cells performed in the same conditions showed no PKR

activation, indicating that besides HIV infection, no other parameter has influenced PKR activation (Fig. 3.1C).

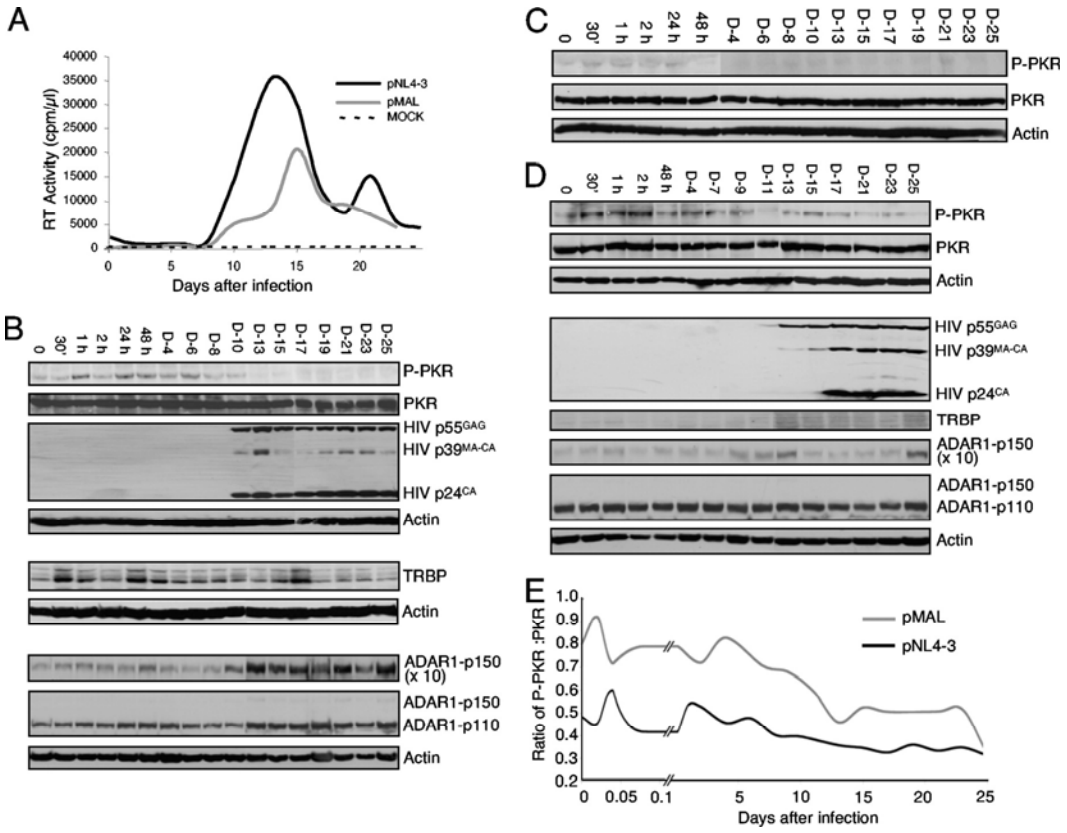


Figure 3.1: PKR is transiently activated after HIV infection and inhibited during active HIV replication. A) HIV NL4-3 and pMAL infection kinetics. Jurkat cells were infected with none (dotted line) or HIV NL4-3 (large black line). Jurkat-CCR5 cells were infected with HIV-1 MAL (thin grey line). Aliquots of cell supernatant were collected at different times and assayed for RT activity. **B) Protein expression of pNL4-3-infected Jurkat cells.** (upper part) 250 µg of whole-cell extracts from pNL4-3-infected Jurkat cells were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR, anti-HIV-p24 and anti-actin antibodies as indicated. (middle part) 250 µg of the same extracts were subjected to a similar SDS PAGE and blotted with anti-TRBPjbx and anti-actin antibodies

as indicated. (lower part) 250 μg of the same extracts were subjected to a 7.5% SDS PAGE and blotted with anti-ADAR1 and anti-actin antibodies as indicated. Exposure was 10 times longer for ADAR1-p150 than for ADAR1-p110 + p150 when indicated. **C) Protein expression of mock-infected Jurkat cells.** 250 μg of whole-cell extracts from mock-infected Jurkat cells were subjected to a 10% SDS PAGE and blotted as in B with the indicated antibodies. **D) Protein expression of pMAL-infected Jurkat-CCR5 cells.** 250 μg of whole-cell extracts from pMAL-infected Jurkat-CCR5 cells were subjected to a 10% SDS PAGE and blotted as in B with the indicated antibodies. **E) Ratio of phosphorylated PKR versus PKR during HIV infection.** P-PKR/PKR ratio was calculated from the shown bands in B for pNL4-3 and in D for pMAL.

pMAL is an R5 HIV strain that uses CCR5 as coreceptor, and can replicate in lymphocytes that express the appropriate coreceptor (53, 54). We infected Jurkat-CCR5 cells (45) with pMAL and followed viral kinetics (Fig. 3.1A). RT assays show an overall lower activity compared to pNL4-3 in this setting. Viral production peaked at day 15 and was half the production of that of pNL4-3. pMAL infected Jurkat cell extracts analysis showed that PKR was phosphorylated up to day 9, then remained weakly activated throughout the infection (Fig. 3.1D). In this infection, TRBP levels was moderately increased after day 13 suggesting a contribution of protein. As for ADAR1, its expression increases at during days 7 through 13, and again at day 26. The calculated P-PKR/PKR ratio measured during the infection by pNL4-3 and pMAL reflected an overall decrease of PKR activation during active HIV replication (Fig. 3.1E)

3.6.2 HIV-INFECTION OF LYMPHOCYTES INCREASES ADAR1-PKR INTERACTIONS.

Because we observed an increase in ADAR1-p150 and p110 expression during HIV infection of Jurkat cells we wanted to determine if the interaction of these proteins with PKR reflected these modifications. Cell lysates from mock-infected and HIV NL4-3 infected Jurkat T-cell at the peak of infection were immunoprecipitated with an anti-PKR antibody and the associated proteins were analyzed using antibodies against PKR, ADAR and TRBP (Fig. 3.2). Whereas TRBP-PKR interactions were not changed by viral infection, we observed a dramatic increase in binding of both the cytoplasmic full-length and the nuclear spliced form of ADAR1 to PKR in the presence of replicating HIV. This result suggested a role of the protein in enhancing HIV replication by controlling PKR activation. Furthermore, the reverse IP with the ADAR antibody showed that both ADAR1 variants, PKR and TRBP were immunoprecipitated and ADAR-PKR interactions were also increased in the presence of HIV (Fig. 3.2, upper right panel).

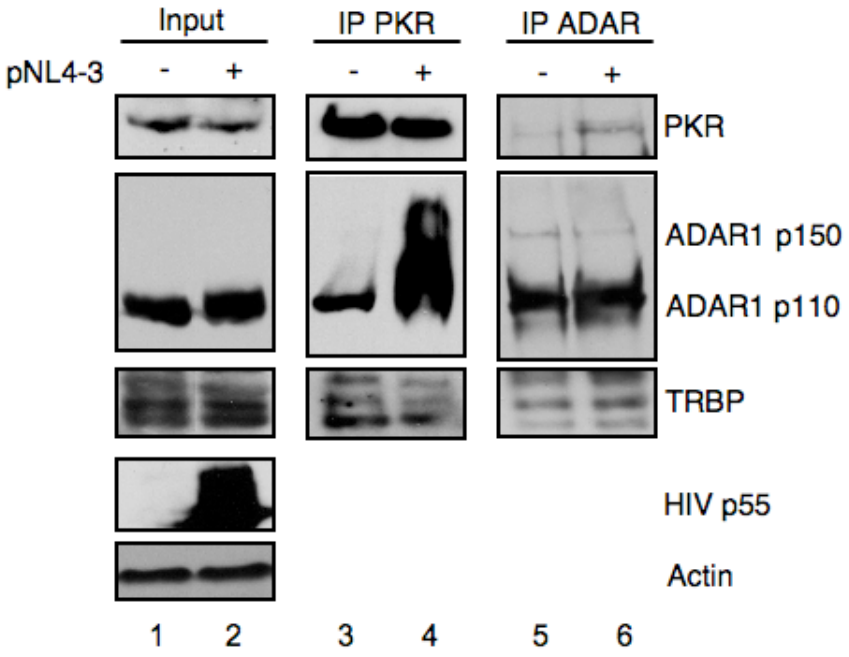


Figure 3.2: ADAR1-PKR interaction increase during HIV-1 infection. Jurkat cells were mock infected or infected with HIV NL4-3. Cell lysates collected at day 15 (peak of infection) were immunoprecipitated with anti-PKR or anti-ADAR1. 250 μg of proteins from each lysate (input; lanes 1-2) and the PKR (lanes 3-4) or ADAR (lanes 5-6) immunoprecipitated complexes were run on a 12% SDS-PAGE and blotted using anti-PKR, anti-ADAR1, anti-TRBPjbx, anti-HIV-p24 and anti-actin.

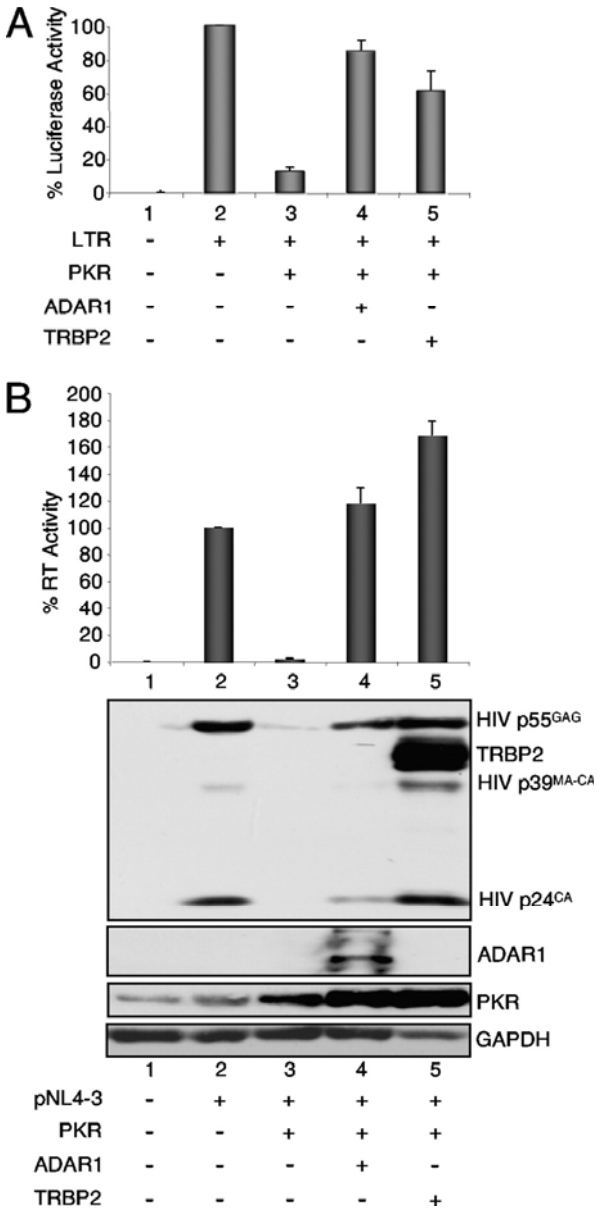


Figure 3.3: ADAR1 p150 and TRBP2 reverse PKR inhibition of HIV expression and virus production. A) ADAR1 p150 and TRBP2 reverse PKR inhibition of HIV LTR-driven expression. HEK 293T cells were transfected with 0.05 µg of pGL2-LTR-Luc (lanes 2-4), 0.5 µg of pcDNA1-PKR (lanes 3-4), and with 1 µg pCMV-ADAR1-V5 p150 (lane 4) or 1µg pcDNA3-TRBP2 (lane 5). Empty plasmids pcDNA1 and pcDNA3.1_V5 or pcDNA3 were added to reach the same amount of transfected DNA. % Luciferase activity is the ratio between the luciferase level in the presence of PKR and either ADAR1 or TRBP2 versus LTR-Luc alone. Shown is the average of 4 independent transfections ± SEM. **B) ADAR1 and TRBP reverse PKR-inhibited HIV-1 expression.** HEK 293T cells were transfected with 2 µg pNL4-3 (lanes 2-5), 0.25 µg pcDNA1-PKR (lanes 3-5) and 1.5 µg of pCMV-ADAR1 (lane 4) or pcDNA3-TRBP2 (lane 5). (Top) RT assay from cell supernatants normalized to 100% in the absence of PKR or dsRBPs. (Bottom) 250 µg of each cell extract was analyzed by immunoblot against HIV p24, ADAR1, TRBP, PKR, or GAPDH as indicated. TRBP was blotted before HIV p24 and appears on the same blot. GAPDH was used instead of actin that runs close to TRBP.

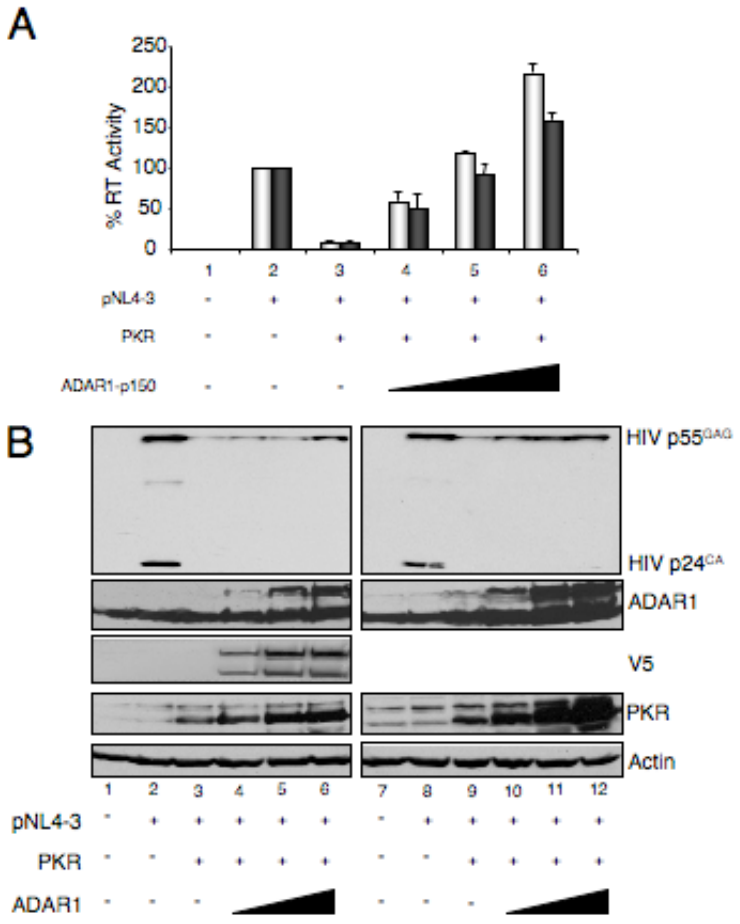
3.6.3 ADAR1p150 REVERSES PKR-INHIBITION OF HIV-1 LTR-DRIVEN EXPRESSION AND VIRAL PRODUCTION

Because ADAR1 has been shown to inhibit PKR activation (12) and because it may have a similar role during HIV replication, we verified if the protein was able to reverse PKR inhibition of HIV long terminal repeat (LTR) expression. A similar role has been previously attributed to TRBP (21) and we therefore compared ADAR1 and TRBP in the same assay (Fig. 3.3A). In this context, both ADAR1 and TRBP reversed the PKR-mediated inhibition of HIV-1 LTR-driven Luciferase expression, suggesting a similar activity. ADAR1 was also compared to TRBP in the context of HIV-1 production (Fig. 3.3B). As previously, transfection of a PKR expressing vector inhibited HIV expression in HEK 293T cells and TRBP reversed this effect (32). In this assay, ADAR1 had the same activity as TRBP, strongly suggesting that ADAR1 is also a cellular inhibitor of PKR during HIV replication.

3.6.4 ADAR1-p150 INCREASES HIV PRODUCTION IN THE PRESENCE AND IN THE ABSENCE OF OVEREXPRESSED PKR

To monitor adequately the expression and the activity of ADAR1-p150 and compare it with mutant forms, we constructed a plasmid expressing a tagged protein with V5 in its C-terminus. We first determined if this tagged protein had the same activity as the untagged form for PKR inhibition in the context of active HIV production. HEK 293T cells were transfected with PKR and pCMV-ADAR-p150 or pcDNA3-ADAR-p150-V5 (Fig. 3.4). Cell culture supernatants and lysates were collected and assayed for RT assay and immunoblotting analysis respectively. In a context of ten-fold inhibition of HIV expression by PKR, the addition of either form of ADAR-p150 reversed this inhibition completely and added an additional two-fold increase above the original level. This result indicates that the long form of ADAR1 is a powerful PKR inhibitor, whether it is

tagged or not (Fig. 3.4A). The expression of HIV $p55^{gag}$ protein confirmed the restoration of viral protein production with ADAR1 (Fig. 3.4B). Unexpectedly, although we transfected the same amount of PKR expressing vector, PKR expression increased with ADAR1 plasmid transfection. To determine if part of the increased HIV production could be ascribed to a PKR-independent activity, the same experiment was performed with ADAR1-V5 in the absence of exogenous PKR. In this case, a maximum of a twofold increase in HIV production was observed with increasing amounts of ADAR1-p150-V5, with a slight decrease at the highest concentration (Fig. 3.4C). We noted that increased ADAR1 concentrations had no effect on endogenous PKR expression.



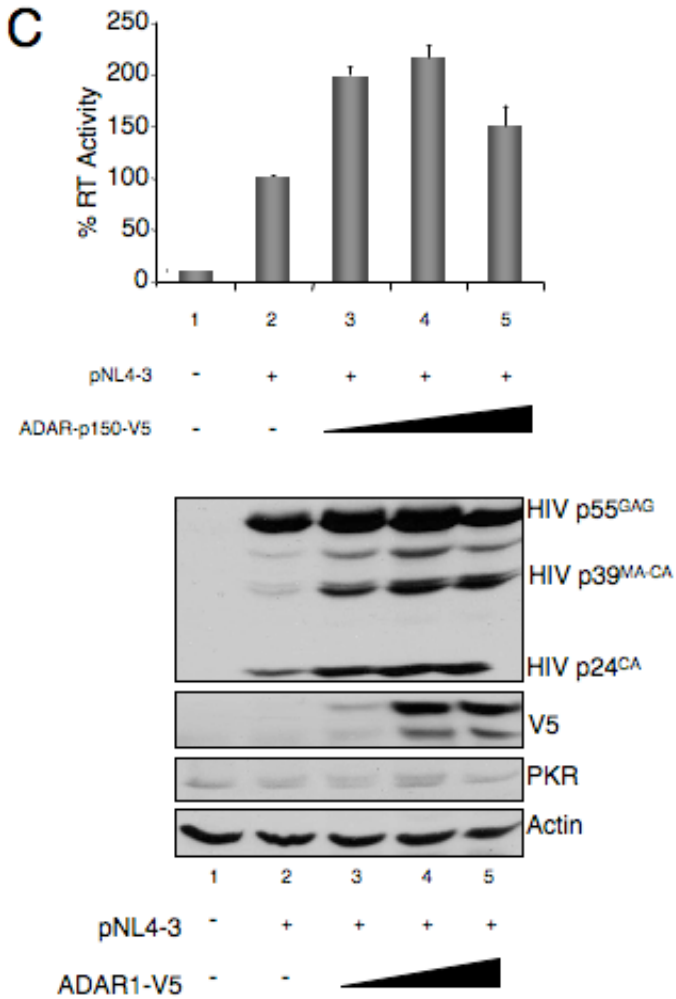


Figure 3.4: ADAR1-p150 increases HIV production in the presence and in the absence of overexpressed PKR. A) ADAR1 and ADAR1-V5 activity on PKR-inhibited virus production. HEK 293T cells were transfected with none (lane 1), 2 µg pNL4-3 (lanes 2-6), 0.5 µg pcDNA1-PKR (lanes 3-6), 0.5 µg (lane 4), 1.0 µg (lane 5) or 1.5 µg (lane 6) of ADAR1-p150-V5 (light grey) or ADAR1-p150 (dark grey). 48 h posttransfection, supernatants were collected for RT assay and cell lysates were generated. **B) ADAR1 and ADAR1-V5 activity on PKR-inhibited HIV protein expression.** 200 µg of cell extracts produced in A)

without and with ADAR1 p150-V5 (lanes 1-6) or ADAR1 p150 (lanes 7-12) were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-V5, anti-PKR and anti-actin antibodies as indicated. **C) ADAR1-V5 activity on HIV expression and virus production.** HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lanes 2-5), 0.5 μg (lane 3), 1.0 μg (lane 4) or 1.5 μg (lane 5) of ADAR1-p150-V5. 48 h posttransfection, supernatants were collected for RT assay (top) and cell lysates were generated. 200 μg of cell extracts were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-V5 and anti-actin antibodies as indicated.

3.6.5 ADAR1 INHIBITION OF PKR REQUIRES THE THREE DSRBDS AND IS NOT DUE TO DEAMINASE FUNCTION

To test which part of ADAR1 is responsible for inhibition of PKR, full-length ADAR1 (p150), the truncated variants p110 and p80 (33), a mutant deleted in the catalytic deaminase function (Dcat) and a mutant with only the Z-DNA and dsRBD1 (p70) were tagged with the V5 epitope at their C-termini and were transiently transfected into HEK 293T cells (Fig. 3.5A). To determine the ability of the ADAR1 variants and mutants to reverse PKR activity, they were first assayed in a HIV1-LTR luciferase assay as in Fig 3A (Fig. 3.5B). In conditions where PKR inhibited the LTR activity fivefold, ADAR1-p150 restored this activity almost completely, Dcat increased it threefold relative to PKR only, p110 increased it by less than twofold relative to PKR only, but ADAR p80 and p70 mutants failed to restore luciferase expression. The wild-type and mutants were then tested on the restoration of HIV production inhibited by PKR (Fig. 3.5C). In this case, HIV production was fully restored by ADAR1-p150 whereas a threefold and twofold increase relative to PKR only was observed with Dcat and p110 respectively. Similar to the above results, p80 and p70 had no or very mild activity in this context. These results were confirmed by Western blot against HIV

p24 that shows a complete restoration of expression with ADAR1 p150 and a partial one with Dcat and p110. The expression of the different ADAR1 variants and mutants show that they were all expressed, but they exhibit some variations in their level of expression. Although the full-length p150 form was weakly expressed, it had the strongest activity, suggesting that its real activity is much more potent than the one seen in the luciferase and RT assays. Overall, these results suggest that the deaminase function is not required for PKR inhibition, but that the integrity of the three dsRBDs is necessary.

It was recently suggested that overexpression of ADAR1 or ADAR2 increases HIV production in the absence of PKR transfection and this activity was ascribed to the deaminase function (55). To determine if it is also the case in our assay, we verified the activity of our constructs in the absence of transfected PKR (Fig. 3.5D). We observed a twofold increase in HIV $p55^{gag}$ expression and only a mild increase (30-40%) in RT activity with wild-type and mutants ADAR1. This increase occurred also with mutants in the catalytic domain, suggesting that the deaminase function does not affect HIV production in our assay. As in Figure 3.3 and 3.4, transfection of the various ADAR1-expressing plasmids increased the expression of transfected PKR (Fig. 3.5C), but not endogenous PKR (Fig. 3.5D).

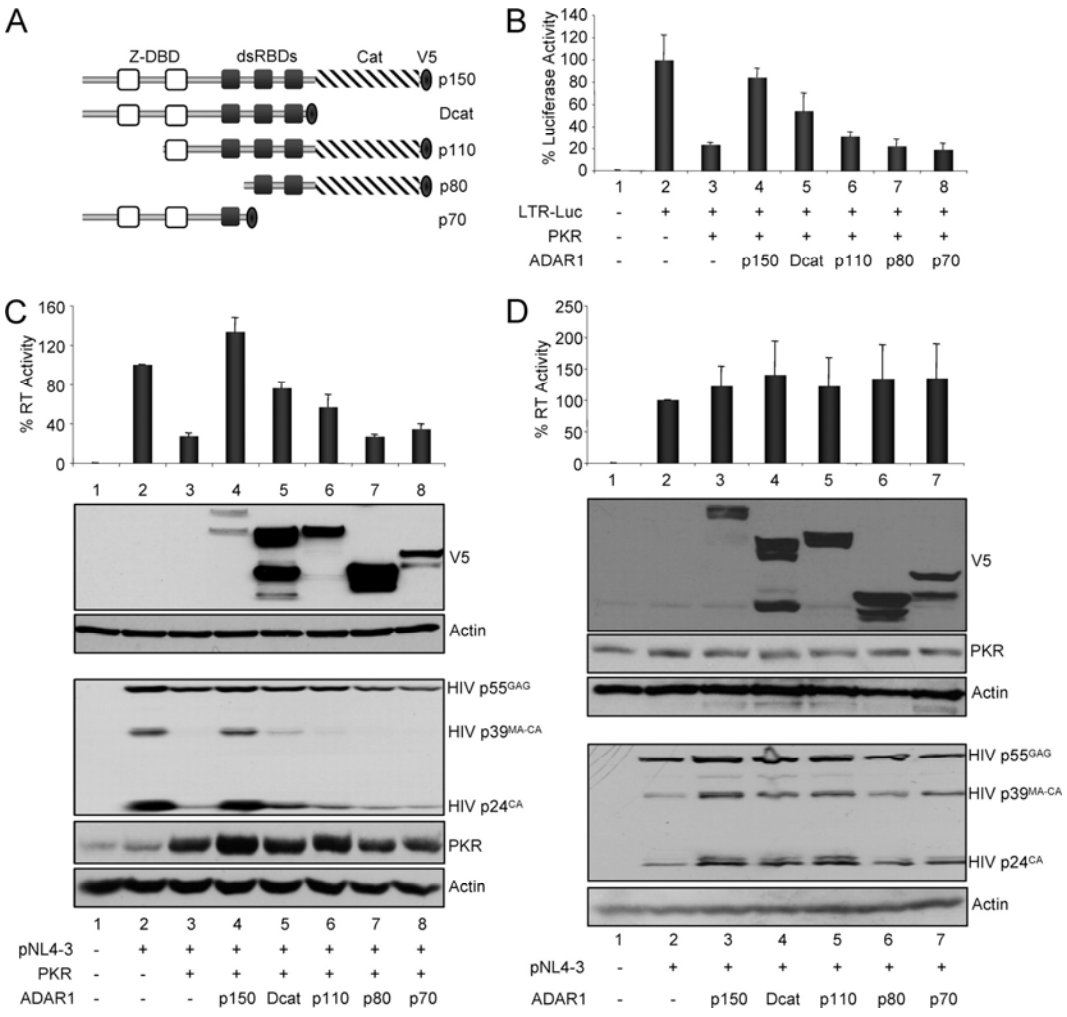


Figure 3.5: ADAR1 inhibition of PKR requires the dsRBDs but not the deaminase function. A) Schematic of naturally existing variants and mutant forms of ADAR1 tagged with V5. ADAR1-p150 is the full-length protein (aa 1 to 3678). Mutants and variants are ADAR1 Dcat (aa 1 to 2475), p110 (888 to 3678), p80 (1869 to 3678) and p70 (1 to 1869). The DNA-binding domain (Z-DBD), dsRBDs, the catalytic domain (Cat) and the V5 tag are indicated. **B) Activity of ADAR1 and ADAR1 mutants on PKR inhibited HIV-1 LTR-driven expression.** HEK 293T cells were transfected with 0.10 μg of pGL2-LTR-Luc (lanes 2-8), 0.10 μg of pcDNA1-PKR (lanes 3-8), and with 1 μg ADAR1 p150 (lane 4), Dcat (lane 5), p110 (lane 6), p80 (lane 7) and p70 (lane 8). Empty

plasmids pcDNA1 and pcDNA3.1_V5 were added to reach the same amount of transfected DNA. % Luciferase activity is the ratio between the luciferase level in the presence of PKR and different ADAR1 mutants versus LTR-Luc alone. Shown is the average of 3 independent transfections ± SEM. **C) Activity of ADAR1 and ADAR1 mutants on PKR inhibited HIV-1 production.** HEK 293T cells were transfected with 2.0 μg of pNL4-3 (lanes 2-8), 0.50 μg of pcDNA1-PKR (lanes 3-8) and with 1.5 μg ADAR1 p150 (lane 4), Dcat (lane 5), p110 (lane 6), p80 (lane 7) and p70 (lane 8). Empty plasmids pcDNA1 and pcDNA3.1_V5 were added to reach the same amount of transfected DNA. (Top) % RT activity is the ratio between the RT level in the presence of PKR and different ADAR1 variants versus pNL4-3 alone. Shown is the average of 5 independent transfections ± SEM. (Bottom) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against V5, HIV p24, PKR and actin. **D) Activity of ADAR1 and ADAR1 mutants on HIV-1 production.** HEK 293T cells were transfected with 2.0 μg of pNL4-3 (lanes 2-7) and with 1.5 μg ADAR1-p150 (lane 3), Dcat (lane 4), p110 (lane 5), p80 (lane 6) and p70 (lane 7). Empty plasmids pcDNA1 and pcDNA3.1_V5 were added to reach the same amount of transfected DNA. (Top) % RT activity is the ratio between the RT level in the presence of different ADAR1 variants versus pNL4-3 alone. Shown is the average of 3 independent transfections ± SEM. (Bottom) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against V5, PKR, HIV p24, and actin.

3.6.6 ADAR1 INCREASES HIV-1 PRODUCTION IN ASTROCYTES

Astrocytes are a model in which HIV replication is very low due, in part, to a high PKR activation that prevents viral translation (32, 56). This high PKR activation is due to the weak activity of the TRBP promoter. This induces the production of only very small amounts of TRBP, which are unable to counteract PKR activation (39, 40). To determine if ADAR1 also contributes to PKR inhibition in this cellular context, we analyzed the activity of ADAR1-p150-V5 compared to TRBP on HIV expression and production in the U251MG astrocytic cells (Fig. 3.6). In these cells, ADAR1 and TRBP induced up to threefold increase in HIV production and a similar increase in $p55^{gag}$ expression. This result is compatible with ADAR1 activity as a PKR inhibitor.

3.6.7 INHIBITION OF ADAR1-P150 EXPRESSION DECREASES HIV EXPRESSION

A previous study showed that a siRNA against ADAR1-p150 only (siA) decreased the long form of the protein and increased VSV production correlated with increased PKR activation (12). Another study showed that a siRNA against both ADAR1-p150 and p110 decreased HIV expression but was not correlated to PKR activation (55). To further determine the role of ADAR1 in HIV replication, we used siRNAs targeting only ADAR1-p150 (siA) and a siRNA targeting both forms (si4, Qiagen), and analyze their effect on viral expression and production (Fig. 3.7). The activity of siA mildly decreased ADAR1-p150, whereas si4 decreased both forms. Despite the mild activity of siA, virus expression was significantly decreased in cells and virus production was decreased by 30%. In contrast, si4 reduced ADAR1-p110 significantly, HIV gag expression moderately and HIV virus production very weakly.

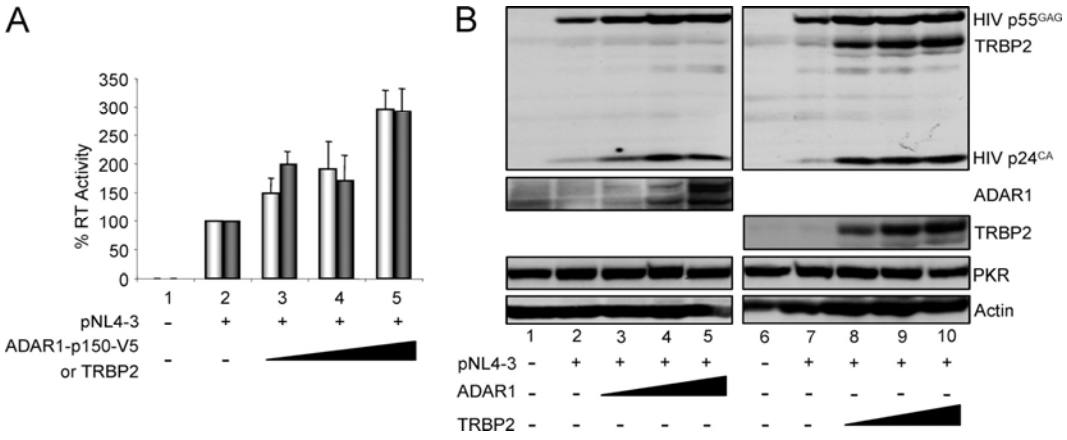


Figure 3.6: ADAR1 and TRBP increase HIV-1 virus production in astrocytes. A) ADAR1 and TRBP increase pNL4-3 virus production in astrocytes. U251MG cells were transfected with none (lane 1), 2 μg pNL4-3 (lanes 2-5) and 0.5 μg (lane 3), 1.0 μg (lane 4) or 1.5 μg (lane 5) of pCMV-ADAR1-p150-V5 (light grey) or pcDNA3-TRBP2 (dark grey). Empty corresponding plasmids were added to reach the same amount of transfected DNA. % RT activity is the ratio between the activity in the presence of pNL4-3 and ADAR1 or TRBP versus pNL4-3 alone in the cell supernatant. **B) ADAR1 and TRBP increase HIV protein expression in astrocytes.** 200 μg of cell extracts produced in A) without and with ADAR1-p150-V5 (lanes 1-5) or TRBP2 (lanes 6-10) were subjected to 10% SDS PAGE and blotted with anti-HIV-p24, anti-ADAR1, anti-TRBP, anti-PKR and anti-actin antibodies as indicated.

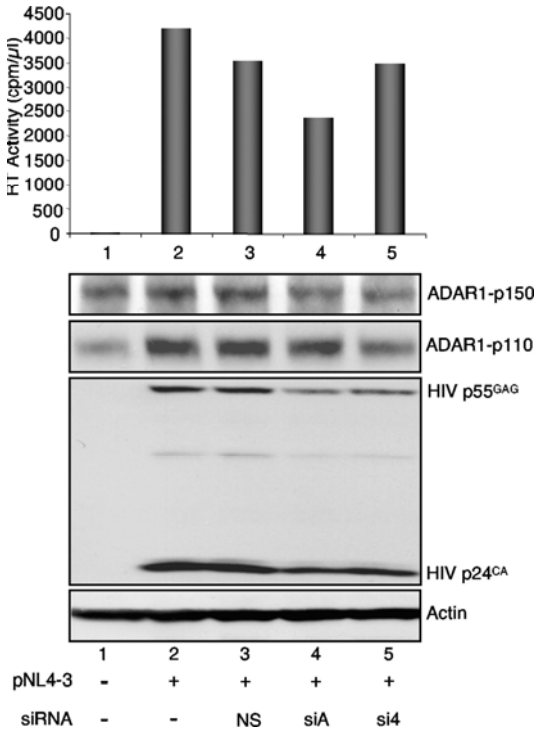


Figure 3.7: Decrease of ADAR1 p150 expression affects HIV production. HEK 293T cells were not transfected (lane 1) or transfected with none (lane 2), 14 nM of sins (si Non silencing) (lane 3), siA (lane 4) or si4 (lane 5). They were transfected 24 hr later with 2.0 μg of pNL4-3 (lanes 2-5). (Top) RT activity of the cell supernatant is represented on the graph. (Bottom) Immunoblot of 150 μg of cell extracts from the same transfected cells using antibodies against ADAR1, HIV p24, and actin.

3.7 DISCUSSION

Although IFN is able to inhibit HIV production in cell culture (37), the IFN produced in plasmocytoid dendritic cells during HIV infection does not eliminate the virus in patients and, in long-term, contributes to pathogenesis. (57-60). This *in vivo* inefficacy could have its origin in the inadequate innate immune response during the first days of infection, but the activity of the ISGs on virus replication in lymphocytes during this time frame has been poorly investigated. Because PKR is one of the main ISGs that can inhibit HIV production in cell culture, we wanted to determine if HIV infection of lymphocytes occurs with or without PKR activation. We found that PKR becomes phosphorylated soon after HIV infection. This is followed by an inactivation of PKR, which correlates with HIV replication (Fig. 3.1). These results suggest that the innate immune response mediated by PKR is fully functional but only transiently active. Because in astrocytes PKR activation is an important barrier to HIV expression and replication (32), we thought that HIV might specifically replicate in cells where PKR activation is repressed.

We studied the expression of ISGs and the role of various PKR inhibitors, and observed that the expression of ADAR1-p150 and p110 forms is enhanced during HIV infection, which correlates with increased HIV replication and increased PKR binding (Fig 1 and 2). Because ADAR1 is an ISG, one could expect that this protein contribute to a cell response against virus replication. While these results were obtained, ADAR1 was isolated in a two-hybrid screen using PKR as bait (M. Bonnet and E. Meurs, data not shown) and ADAR1 was shown as a PKR inhibitor in the context of VSV infection (12). Both studies showed that the first dsRBD in ADAR1 is the domain that binds PKR. Results of the two-hybrid screen showed that the five isolated clones all express amino acids 503 to 556 within dsRBD1. Similarly to TRBP, ADAR1 was able to reverse PKR inhibition in cells expressing only HIV LTR or with active HIV replication indicating that its increased binding to PKR at the peak of HIV infection

contributes to the enhancement of HIV replication in lymphocytes (Fig. 3.3 and 3.4A). The analysis of ADAR variants' and mutants' activity further demonstrates that the integrity of the three dsRBDs and at least one Z-DNA binding domain, but not the catalytic domain, are necessary for PKR inhibition (Fig. 3.5).

Because in HIV-replicating cells PKR is not expressed from an overexpressed plasmid, we also asked the question of ADAR1 activity in the absence of exogenous PKR. In this context, ADAR1 p150 increased HIV expression and production but this effect was about twofold (Fig. 3.4B and 3.5D). A similar activity was recently observed in an independent study and showed that the deaminase function was responsible for this increased HIV production (55). However, the similarity between our Dcat mutant and ADAR1 p150 in the absence of PKR does not allow us to reach the same conclusion (Fig. 3.5D). In addition, the activity of the Dcat in the presence of exogenous PKR (Fig. 3.5B and C) suggests a main activity by inactivating also endogenous PKR. The discrepancy between their results and ours is currently unexplained but could be due to a difference in experimental settings. Similar to their study, siRNAs that decreased both ADAR1-p110 and p150 decreased HIV expression, but this effect was stronger with a specific inhibition of the p150 form alone (siA in Fig. 3.7).

Astrocytes are a model of low HIV replication due to a high PKR activation that cannot be counteracted by TRBP expressed at low level in these cells (32, 39, 40). The similar activity between ADAR1 and TRBP in increasing viral production in astrocytes further suggests that ADAR1 could replace TRBP in regard to PKR inhibition and enhancement of HIV production in astrocytes (Fig. 3.6). The similar effect of siRNAs against ADAR1 (Fig. 3.7) and siRNAs against TRBP (31) confirms this hypothesis. Taken together, our results suggest that the IFN-induced ADAR1 p150 isoform has a main activity to counteract PKR inhibition of HIV expression. They indicate that two IFN-induced proteins can have opposite effects, which ultimately contributes to the enhancement of HIV replication in lymphocytes.

Previous results have shown that the virus itself can counteract PKR activation with its Tat protein that acts as a competitive substrate (38, 61, 62). Taken together, previous and present results suggest that PKR inhibition during HIV replication is mediated both by the viral Tat protein and by host factors ADAR1 and TRBP (Fig. 3.8). We have also shown recently that in addition to direct PKR inhibition, TRBP binds to and inhibits the PKR activator PACT (47, 50). This latter function could further contribute to PKR inhibition during HIV replication. It is also possible that in the experiments that use HEK 293T cells, the presence of VAI RNA and SV40 large T antigen also contributes to PKR inhibition (Fig. 3.3-3.5, 3.7), but this presence did not prevent the PKR and ADAR1 activities observed here. Overall, HIV uses at least three different mechanisms to counteract PKR activation: i) it produces the Tat protein during the early steps of its replication, ii) it has evolved to replicate specifically in cells that express high levels of TRBP and iii) it increases directly or indirectly through IFN induction the synthesis of ADAR1-p150 isoform. Further studies of PKR-binding factors during HIV infection may reveal additional proteins that could contribute to PKR inactivation and enhanced virus replication.

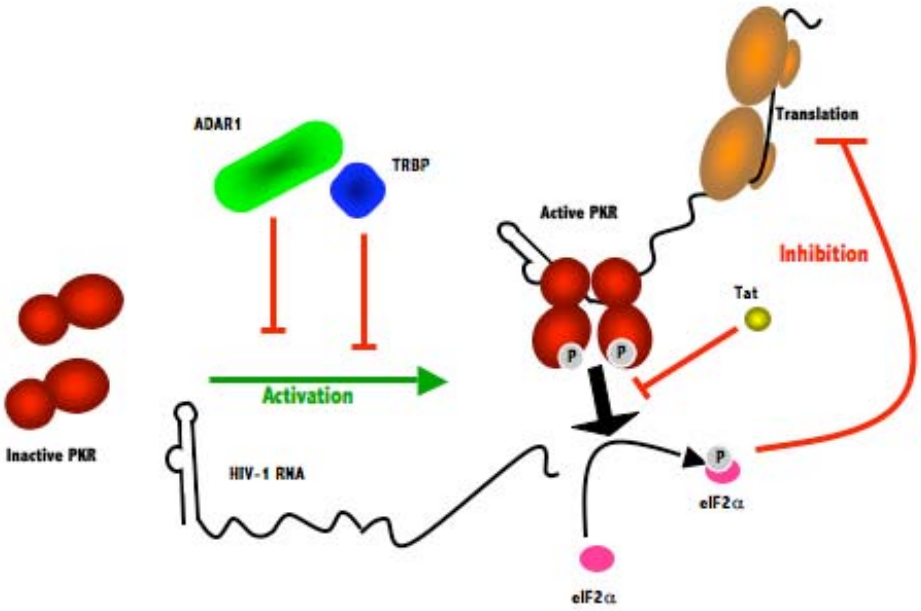


Figure 3.8: Schematic representation of the regulation of HIV translation by PKR and the contribution of host and viral factors. The viral TAR RNA contributes to PKR activation by phosphorylation, which in turn phosphorylates eIF2α and consequently inhibits HIV translation. During HIV replication, the cellular proteins TRBP and ADAR1 prevent or inhibit PKR phosphorylation whereas the viral protein Tat prevents eIF2α phosphorylation. All three proteins contribute to increase HIV mRNA translation.

3.8 ACKNOWLEDGEMENTS

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CHAPTER 4

THE PKR ACTIVATOR, PACT, IS A PKR INHIBITOR DURING HIV REPLICATION

(This chapter is adapted from a manuscript that will be submitted shortly)

4.1 INTRODUCTION

The PKR activator, or PACT, is one of the only known cellular proteins capable of activating PKR (1). Other known cellular activators of PKR include the melanoma differentiation-associated gene 7 (MDA7), the trascription factor E2F-1, which play a role in PKR-mediated apoptosis (2). Caspases also activate PKR by a mechanism that involves its proteolytic cleavage. In response to apoptosis, activated caspase-3, caspase-7 or caspase-8 cleave PKR to remove the first N-terminal 251 amino acids, corresponding to the inhibitory amino-terminal RBD (3). The truncated kinase is capable of auto- and trans-phosphorylation of PKR at different sites, as well as eIF2α *in vivo*. However, the truncated PKR does not dimerize and functions as a constitutively active monomer (3). PACT is a cellular protein that heterodimerizes with PKR through its two dsRBDs and its C terminus, the Medipal domain, which is essential for PKR activation and TRBP binding (4-8). It activates PKR *in vitro* and *in vivo* independently of the presence of dsRNA. PACT activation of PKR is set in motion only upon induction of cellular stresses such as treatment with a low dose of actinomycin D, peroxide, arsenite, thapsigargin, tunicamycin, or growth factor withdrawal (9-11). PACT is first activated by phosphorylation in the stressed cells, where it subsequently activates PKR and PKR-dependent apoptosis in response to the stress (12, 13). PACT and TRBP share 40% similarity and interact with each other through their dsRBDs and their Medipal domains (1, 4, 11). In this section of the thesis, we look at another facet of the PACT/PKR relationship. In the absence of any stress during HIV-1 infection, we analyzed the role of PACT/PKR interaction. We found that PACT can be immunoprecipitated with a PKR or an ADAR1 antibody and that PKR-PACT interaction is increased during HIV infection of lymphocytes. We found that PACT acts as a PKR inhibitor similarly to both ADAR and TRBP, highlighting yet another level of PKR regulation during HIV-1 replication.

4.2 MANUSCRIPT: THE PKR ACTIVATOR, PACT, IS A PKR INHIBITOR DURING HIV REPLICATION

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4.3 ABSTRACT

Background: Human immunodeficiency virus (HIV)-1 translation is modulated by the activation of the interferon-inducible Protein Kinase R (PKR), which phosphorylates its downstream target the alpha subunit of the Eukaryotic Initiation Factor 2 (eIF2α). The consequence of this activation is an inhibition of viral replication. In lymphocytic cell lines, HIV-1 activates PKR only transiently and the kinase is not activated in cells replicating the virus at high levels. The regulation of this activation is due to a combination of viral and cellular factors that have been only partially identified.

Results: PKR is transiently activated in peripheral blood mononuclear cells after HIV infection and little phosphorylated form can be observed during intense replication. In the lymphocytic Jurkat cells infected by HIV, we identified a ribonucleoprotein complex around PKR, which contains the double-stranded RNA binding proteins (dsRBPs), adenosine deaminase acting on RNA (ADAR)1, TAR RNA Binding Protein (TRBP) and PKR Activator (PACT). In HEK 293T cells transfected with an HIV molecular clone, PACT inhibited PKR and eIF2α phosphorylation and increased HIV-1 protein production and virion production in the presence or absence of overexpressed PKR. In the same cells, siRNAs against PACT decreased HIV protein expression. The comparison between different dsRBPs showed that ADAR1, TRBP and PACT inhibit PKR and eIF2α phosphorylation in HIV-infected cells, whereas Staufen did not.

Conclusion: In contrast to its previously described activity, PACT contributes to PKR inactivation during HIV replication. It belongs to a ribonucleoprotein complex, which includes PKR, TRBP and ADAR1. HIV has evolved to replicate in cells that express PKR inhibitors and induces mechanisms that enhance or change the activity of proteins to inhibit PKR for increased replication. This process contributes to HIV pathogenicity.

4.4 BACKGROUND

Human immunodeficiency virus (HIV) mRNA expression is controlled at the transcriptional, processing and translational levels (14-16). The main translational mechanism is a cap-mediated scanning from its 5' end but additional mechanisms occur including the use of an internal ribosome entry site in gag, programmed -1 ribosomal frameshift to produce Gag-Pol and discontinuous ribosome scanning to translate Env (17-19). HIV translation is modulated by viral components, like the Trans-Activation Response element (TAR) RNA (19-21) and by cellular factors including translation factors, Protein Kinase RNA activated (PKR), TAR RNA Binding Protein (TRBP), PKR ACTivator (PACT), the La autoantigen, Staufen1 and ADAR1 (8, 11, 21, 22). The positive factors act by releasing the block due to the TAR structure, by inhibiting PKR or by inhibiting PACT.

The interferon (IFN)-inducible PKR is a key double-stranded RNA-binding protein (dsRBP), which has a serine/threonine kinase activity mediated in large part by the phosphorylation of its downstream target, the alpha subunit of the translation initiation factor (eIF2α). Phosphorylated eIF2α prevents translational initiation of viral and cellular mRNAs. PKR is central in the host innate defense strategies with strong antiviral and antigrowth activities (23-25). PKR is extremely effective in restricting HIV expression and replication *in vitro* (8, 26-29). Despite this observed activity, HIV replicates efficiently in many permissive cell lines and primary cells, suggesting that the activity of PKR in natural infection of lymphocytes and monocytes is highly regulated.

Many viruses that replicate efficiently have mechanisms to inactivate PKR, and the HIV Tat protein is one of these countermeasures (23, 30, 31). Cells also avoid PKR activation using dsRNA sequestration or protein-protein interactions, likely as a normal process to allow their growth. Examples of direct interaction include the $p58^{IPK}$, which binds to PKR and prevents its dimerization, TRBP and the Adenosine

Deaminase Acting on RNA (ADAR)1, which bind through their dsRBDs and exert a strong inhibitory activity (23, 30, 32). Besides dsRNA and heparin, cellular proteins PACT, MDA7 and the transcription factor E2F-1 induce PKR activation (30). Astrocytic cells represent an example of naturally HIV-resistant cells with high PKR activation. In these cells, TRBP is expressed at very low amounts and cannot counteract PKR activation induced by the virus (28, 33, 34). In HIV-infected lymphocytes, PKR activation is reduced when the virus reaches high concentrations and this is due in part to an increased ADAR1 expression that inhibits PKR activation (8, 35).

PACT and its murine homolog, RAX, are stress-inducible PKR activators (1, 9, 10). They are proapoptotic proteins that induce apoptosis by PKR activation (10). PACT has two dsRBDs and a C-terminus called Medipal domain by homology with TRBP (1, 4). All three domains in PACT bind PKR and TRBP. The Medipal domain mediates activation of PKR or inhibition by TRBP (4-7, 11). Stresses dissociate TRBP-PACT interactions and allow PACT activation of PKR. Therefore, PACT acts as a PKR activator in cells with low TRBP concentration or after stress induction, whereas it acts as a PKR inhibitor in cells with high TRBP content (4, 11, 36). Its activity has not been tested in HIV-infected cells. Here, we show that PACT binds to PKR during HIV infection and that it inhibits its activation.

4.5 RESULTS:

4.5.1 PKR IS ACTIVATED IN PBMCS AT THE BEGINNING OF HIV INFECTION AND INHIBITED DURING ACTIVE HIV REPLICATION.

We have shown in previous results that HIV infection of the lymphocytic Jurkat T cell line induces PKR activation during the first days, followed by an inactivation during high HIV replication (8). To determine if this regulation is also true in primary cells, we infected Peripheral Blood Mononuclear Cells (PBMCs) from healthy donors with pNL4-3 HIV clone (Fig. 4.1). The viral kinetics in PBMCs from two donors was very similar, but faster than the one observed in Jurkat cells, with the peaks of infection at days 6 and 9 (Fig. 4.1A). Because PBMCs do not expand as much as cell lines, it was not possible to gather cell samples every two days. Three samples were chosen representing the mock (day 0), the beginning of the infection (day 2) and full replication just after the peak of reverse transcriptase (RT) (day 11). Activation of PKR was monitored in these samples (Fig. 4.1B). We observed that PKR is not activated in the absence of infection, it is activated at day 2 and is no longer activated at day 11, indicating that, similarly to the infection in Jurkat cells, PKR is only transiently activated in primary lymphocytes and monocytes.

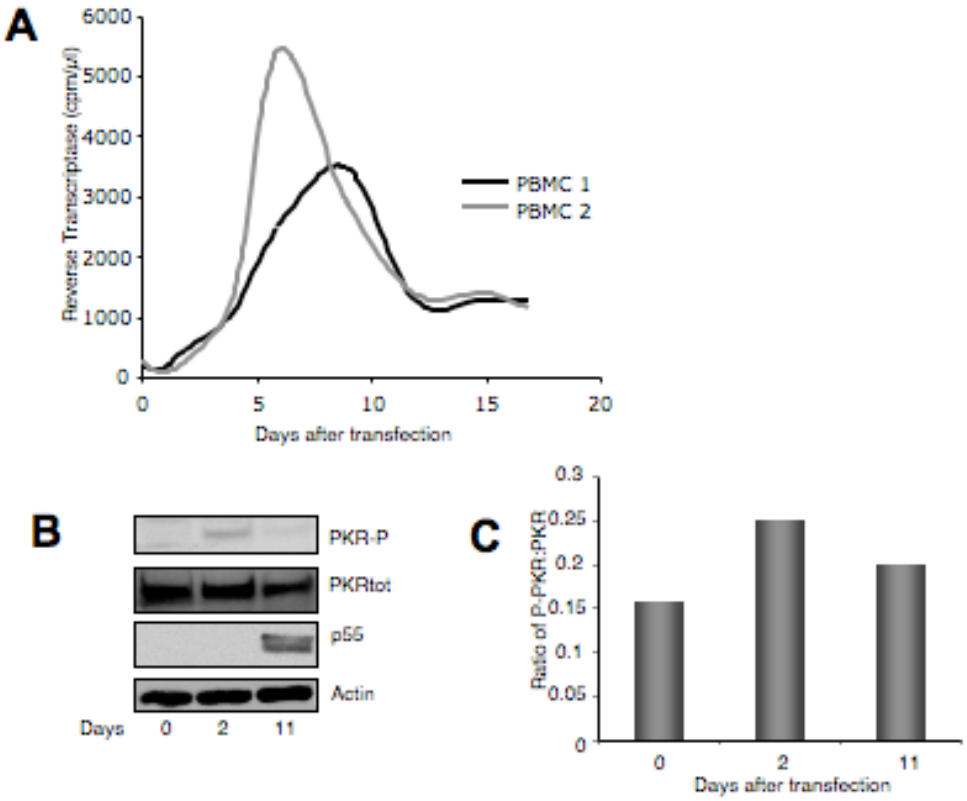

Figure 4.1: PKR is activated after HIV infection and inhibited during active HIV replication. A) HIV NL4-3 kinetics from infected PBMCs. 7 x 10^7 PBMCs from two healthy donors were infected separately with HIV NL4-3. Aliquots of cell supernatant were collected at different times and assayed for reverse transcriptase (RT) activity. **B) Protein expression of pNL4-3-infected PBMCs.** 50 µg of whole-cell extracts from pNL4-3-infected PBMCs from different times were subjected to a 10% SDS PAGE and blotted with anti-P-PKR, anti-PKR, anti-HIV-p24, and anti-actin antibodies as indicated. **C) Ratio of phosphorylated PKR versus PKR during HIV infection.** The band intensity was digitalized using Adobe Photoshop software from the bands shown in B. P-PKR/PKR ratio was calculated by dividing the P-PKR intensity by the total PKR intensity of each band.

4.5.2 PACT BELONGS TO A MULTIPROTEIN COMPLEX FORMED AROUND PKR DURING HIV INFECTION

Many viral and cellular factors prevent PKR activation, resulting in active viral infections and cell growth (30). In the case of HIV infection, the viral Tat protein, large amounts of TAR RNA, cellular proteins TRBP and ADAR1 all contribute to PKR inhibition (8, 31, 34, 35, 37). Because cells also express PKR activators, we questioned whether they could contribute to PKR activation to enhance cell response and balance its inhibition by other factors. We have previously demonstrated that PACT is an activator or an inhibitor of PKR depending on TRBP expression in the cell and stressed or non-stressed conditions (4, 11). We first observed that PACT expression is slightly increased at the peak of infection (Fig. 4.2). We next determined if PACT was present in the complex formed around PKR during HIV infection of lymphocytes. By immunoprecipitation (IP) with a PKR antibody, we observed that PACT interaction with PKR is increased at the peak of infection. This result resembles the previously observed increase in ADAR1 production and interaction with PKR (8). By IP with an ADAR antibody, we also found that PACT is in the same complex as ADAR, therefore suggesting a multiprotein complex around PKR in HIV-infected cells.

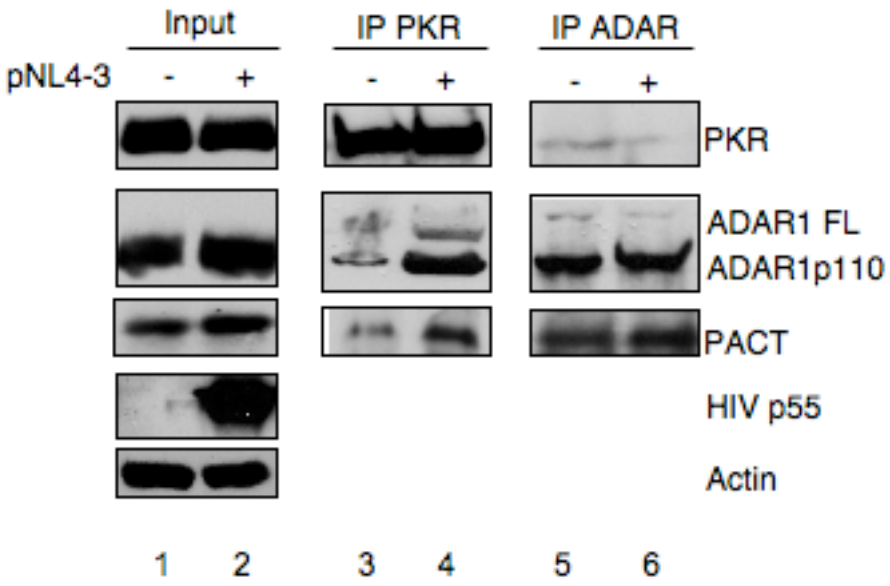

Figure 4.2: Increase of ADAR1-PKR and PACT-PKR interactions during HIV-1 infection. Jurkat cells were mock-infected or infected with HIV NL4-3. Cell lysates were collected at day 15, which correspond to the peak of infection for the pNL4-3-infected Jurkat, were immunoprecipitated with anti-PKR or anti-ADAR1. 50 μg of proteins from each lysate (input; lanes 1-2) and the PKR (lanes 3-4) or ADAR (lanes 5-6) immunoprecipitated complexes were run on a 10% SDS-PAGE and blotted using anti-PKR, anti-ADAR1, anti-PACTeg2, anti-HIV-p24 and anti-actin.

4.5.3 PACT IS A PKR INHIBITOR IN HIV-INFECTED HEK 293T CELLS

We next questioned whether the role of PACT in a complex with PKR during HIV infection would be as an activator or an inhibitor. To determine this role in a single viral replication cycle, we transfected HEK 293T cells with pNL4-3 in the presence or absence of transfected PKR and evaluated the activity of a PACT expressing vector (Fig. 4.3). Transfection of the HIV molecular clone induced PKR and eIF2α phosphorylation (Fig. 4.3A and B, lane 2). As previously observed (8, 34), transfected PKR reduced the expression of HIV proteins and viral production and we show here that this is due to the concomitant increase in the ratio between P-PKR and PKR (Fig. 4.3A, lane 3). Surprisingly, increasing amounts of PACT restored viral protein expression and virion production. The large amount of PKR did not allow for an accurate quantification of any differences in the P-PKR/PKR ratio, but the P-eIF2α/eIF2α ratio clearly indicate that low amounts of PACT prevent the phosphorylation of eIF2α and increasing amounts restore HIV protein expression and virion production (Fig. 4.3A).

When cells were transfected with pNL4-3 and PACT in the absence of overexpressed PKR, PACT was also able to increase HIV protein expression and virion production (Fig. 4.3B). In this case, increasing amounts of PACT clearly prevented PKR and eIF2α phosphorylation, indicating that the protein acts as a PKR inhibitor and contributes to the enhancement of HIV translation and consequently to the increased virion production.

4.5.4 siRNAS AGAINST PACT INHIBIT HIV-1 EXPRESSION

To further determine the role of PACT on HIV-1 expression, we synthesized small interfering (si) RNAs against PACT mRNA to decrease its expression (Fig. 4.4). Cotransfection of HEK 293T cells with these siRNAs decreased the expression of PACT compared to non-silencing siRNAs (Fig. 4.4, lane 4 compared to 3). The

decrease in PACT expression induced a decrease in HIV-1 protein expression. In agreement with the above data these results suggest that in these cells, PACT contributes to the enhanced HIV protein production.

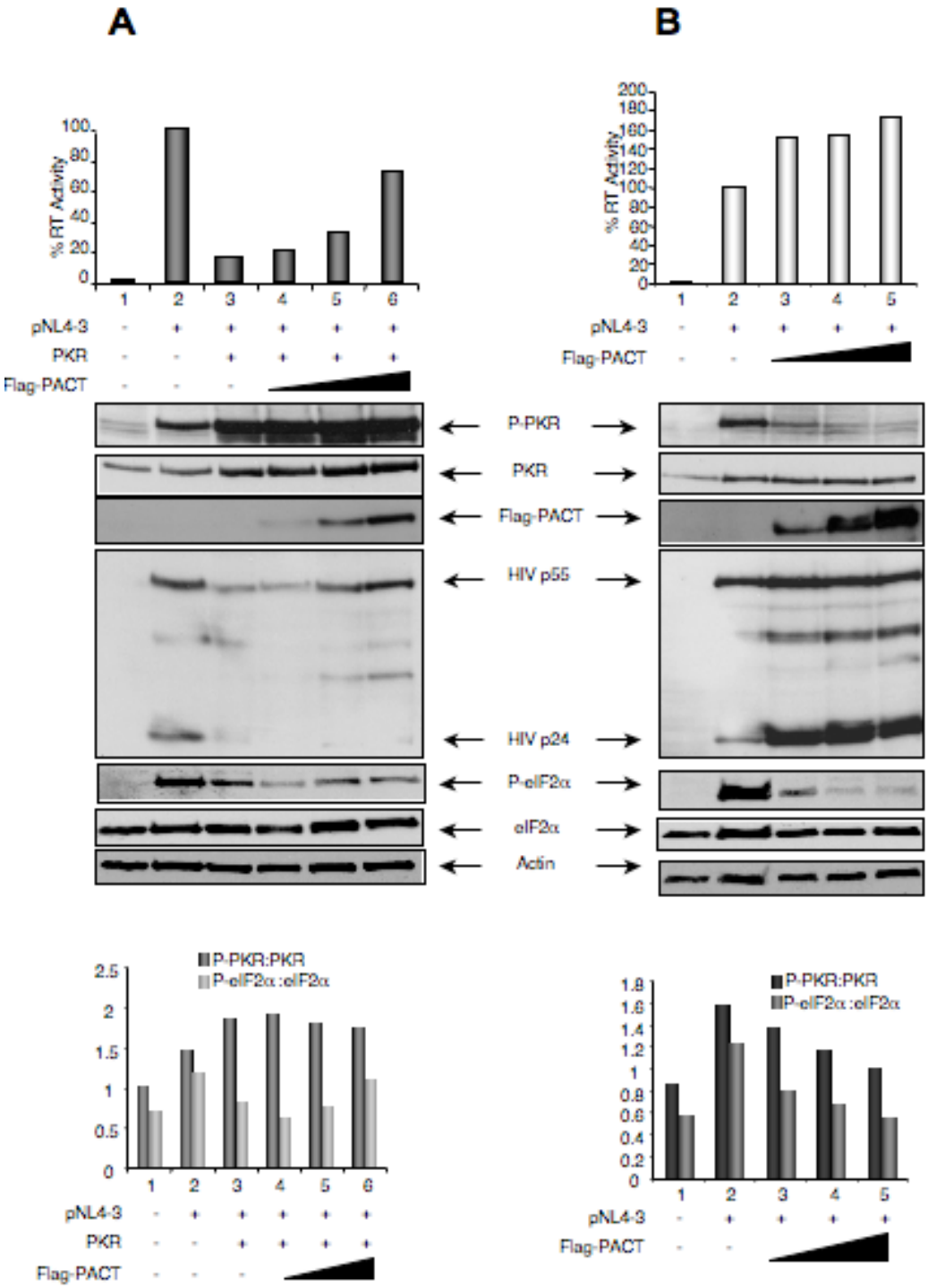

Figure 4.3: PACT increases HIV-1 protein expression and virion production in HEK 293T cells by PKR and eIF2α inhibition. A) PACT reverses PKR inhibition of pNL4-3. HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lanes 2-6), 0.5 μg pcDNA1-PKR (lanes 3-6), 0.5 μg (lane 4), 1.0 μg (lane 5) or 1.5 μg (lane 6) of pCMV2-Flag-PACT. Empty corresponding plasmids were added to reach the same amount of transfected DNA. (Top) Fold activation of RT activity is the ratio between the RT level in the presence of PKR and PACT versus pNL4-3 alone. Shown is the average of 4 independent transfections. (Middle) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against P-PKR, PKR, Flag, HIV p24, P-eIF2α, eIF2α and actin. (Bottom) Ratio of phosphorylated PKR versus PKR during HIV infection. The band intensity was calculated as in Fig. 1. **B) PACT increases pNL4-3 expression and virus production.** HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lanes 2-5) and 0.5 μg (lane 3), 1.0 μg (lane 4) or 1.5 μg (lane 5) of pCMV2-Flag-PACT. pCMV2 was added to reach the same amount of transfected DNA. (Top) Fold activation of RT activity is calculated as in A). Shown is the average of 3 independent transfections. (Middle) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against P-PKR, PKR, Flag, HIV p24, P-eIF2α, eIF2α and actin. (Bottom) Ratio of phosphorylated PKR versus PKR during HIV infection. The band intensity was calculated as in Fig. 4.1.

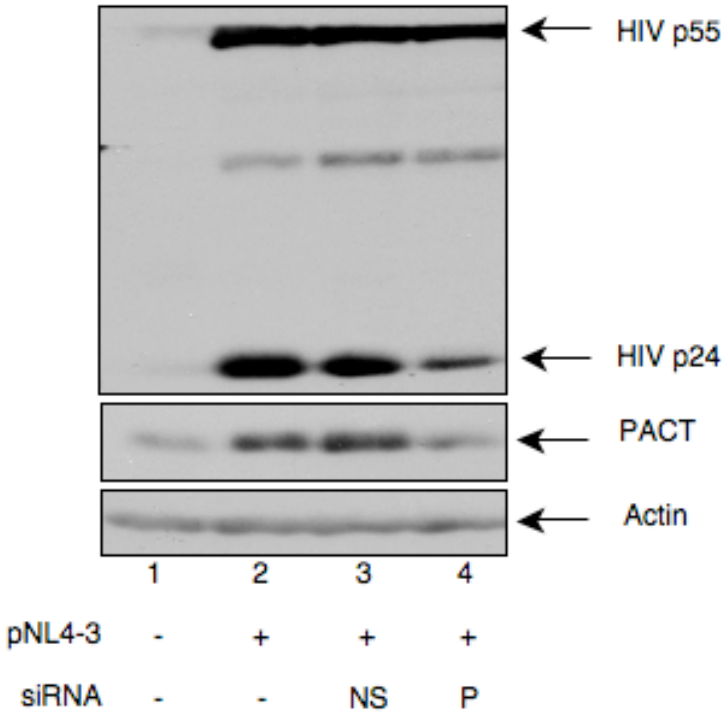

Figure 4.4: siRNAs against PACT inhibit HIV-1 expression. HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lane 2-4), and 14 nM of siRNAs NS (lane 3) or siPACT (lane 4). 200 μg of cell lysates were resolved by SDS-PAGE and analyzed by immunoblotting using antibodies against HIV p24, PACT and actin.

4.5.5 TRBP, ADAR AND PACT CONTRIBUTE TO PKR AND EIF2ALPHA INHIBITION

To compare the activity of the different dsRBPs that contribute to HIV expression and may inhibit PKR activation in HIV-infected cells, we next expressed ADAR1, PACT, TRBP and Staufen with pNL4-3 in the presence or absence of transfected PKR (Fig. 4.5). As previously, ADAR1, PACT and TRBP restored PKR-inhibited HIV expression and production, but Staufen did not and therefore was used

as a negative control (Fig. 4.5A). ADAR1, TRBP and, to a lesser extent, PACT increased the expression of the transfected PKR. This increase is currently unexplained, but was already observed with ADAR1 (8). The phosphorylated form of PKR also increased, but the overall ratio was difficult to evaluate. In contrast, Staufen did not increase the level of overexpressed PKR and its phosphorylated form was slightly increased. Overall, Staufen increased the P-PKR/PKR ratio showing a strong difference with the previous dsRBPs. Similarly, eIF2α phosphorylation decreased strongly with TRBP and mildly with ADAR1 and PACT. Staufen maintained its opposite activity with an increase of the P-eIF2α form. When PKR was not overexpressed, all four proteins reduced PKR and eIF2α phosphorylation with a weak effect of Staufen. They also induced a mild increase in protein expression and virion production (Fig. 4.5B).

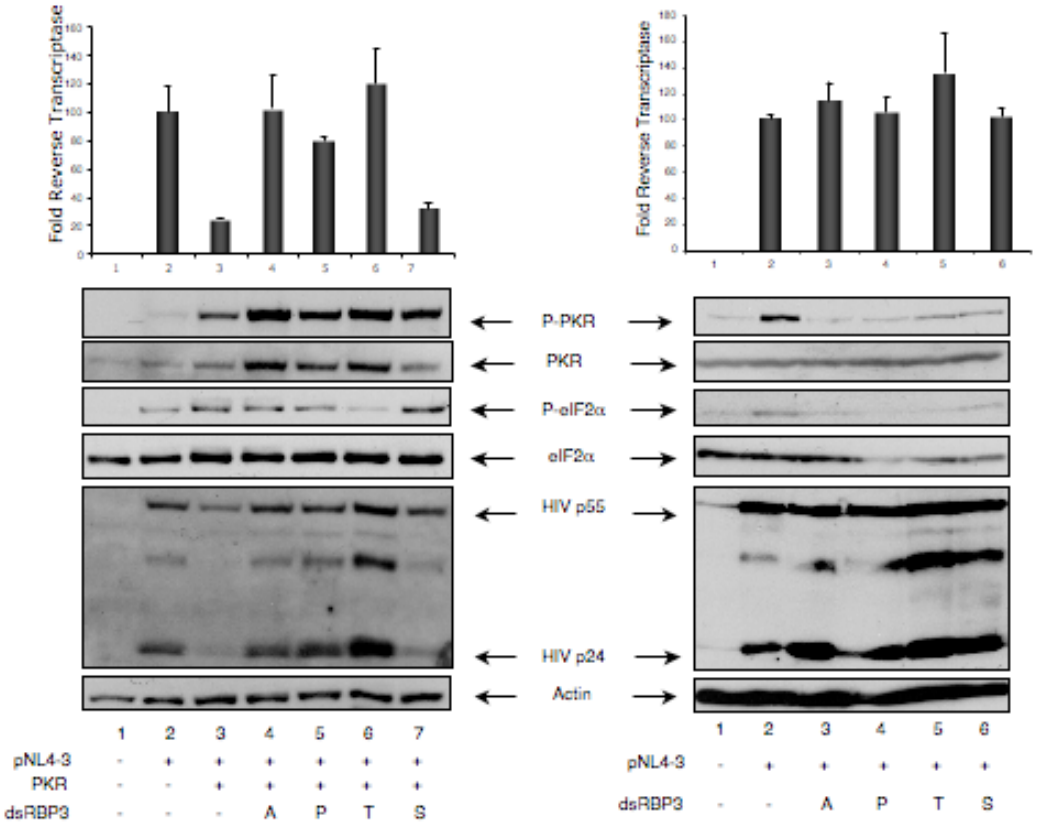

Figure 4.5: ADAR1, PACT and TRBP contribute to PKR inhibition during HIV infection. (A) ADAR1, PACT and TRBP reverse PKR-inhibited HIV-1 expression. HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lane 2-7), 0.5 μg pcDNA1-PKR (lanes 3-7) and 1.5 μg of pcDNA3.1-ADARp150_V5 (lane 4), pCMV2-Flag-PACT (lane 5), pcDNA3-TRBP2 (lane 6) or pCMV-RSV-Staufen-HA (lane 7). (Top) RT assay from cell supernatants normalized to 100% in the absence of PKR or dsRBPs. (Bottom) 250 μg of each cell extract was analyzed by immunoblot against P-PKR, PKR, P-eIF2α, eIF2α, HIV p24 and actin as indicated. Shown is the average of 3 independent transfections ± SEM. **B) ADAR, PACT, TRBP and Staufen mildly increase pNL4-3 expression and virus production.** HEK 293T cells were transfected with none (lane 1), 2 μg pNL4-3 (lanes 2-6), and 1.5 μg of pcDNA3.1-ADARp150_V5 (lane 3), pCMV2-Flag-PACT (lane 4), pcDNA3-TRBP2 (lane 5) or pCMV-RSV-Staufen-HA (lane 6). pcDNA3 was added to reach the same amount of transfected DNA. (Top) Fold activation of RT activity is calculated as in A). Shown is the average of 3 independent transfections ± SEM. (Bottom) Immunoblot of cell extracts of a representative experiment from the same transfected cells using antibodies against P-PKR, PKR, P-eIF2α, eIF2α, HIV p24 and actin as indicated. A: ADAR1. P: PACT. T: TRBP. S: Staufen.

4.6 DISCUSSION:

During HIV infection, IFN is produced mainly by plasmocytoid dendritic cells and will act on HIV-infected cells, but this cell response is not sufficient to clear the virus in patients (38). Although IFN can have adverse effects (39), a poor cell response to this cytokine will also contribute to a higher HIV replication rate. The transient activation of PKR followed by an absence of activation during HIV infection of PBMCs (Fig. 4.1) resembles what is observed with lymphocytic cell lines infected with X4 or R5 HIV strains (8). This observation suggests that this part of the innate immune response is active but is also highly regulated during the infection of primary lymphocytes and monocytes in patients. This controlled cell response likely contributes to active viral replication in permissive cells and to viral pathogenesis.

The regulation of PKR activation is the result of the action of activators and inhibitors and the equilibrium reached after a viral infection will contribute to a high or poor cell response that will either block viral replication or let the virus proceed (30). In the case of HIV infection, the TAR RNA is likely one of the main activators of PKR at the beginning of the infection, but may become an inhibitor if produced in large amounts in the cell (37). The HIV Tat protein is also an inhibitor of PKR acting by substrate competition (31, 40, 41).

Besides direct viral countermeasures, viruses have also evolved to replicate in cells that have the appropriate cellular components to allow their replication (42). Viruses can also induce the production of cellular proteins that will counteract an antiviral cell response. A cell that expresses high amounts of PKR inhibitors will certainly favor HIV replication. We have previously shown that HIV replicates in cells that express a large amount of TRBP that will inhibit PKR (34). HIV also induces ADAR1 production that contributes to PKR inhibition and RNA editing and favors viral replication (8, 35, 43). Because TRBP not only acts on PKR, but also prevents PACT activity on PKR (4, 11), we originally thought that PACT may

activate PKR and that the end result of the PKR status will be a balance between PKR activators and PKR inhibitors. The identification of PACT in the ribonucleoprotein complex with PKR, TRBP and ADAR during HIV infection supported the idea that PACT has a function in regulating PKR, but raised the question of what its role within this complex may be (Fig. 4.2).

When overexpressed in HIV-expressing cells, PACT inhibited PKR and eIF2α phosphorylation and consequently increased HIV expression (Fig. 4.3 and 4.5). This inhibition was observed whether PKR was overexpressed or not. Furthermore, PACT inhibition by siRNAs decreased HIV protein expression, reaching the same conclusion that PACT is a PKR inhibitor during HIV replication. There are several possible explanations for this activity: i) TRBP is present in high amounts in HEK 293T cells and forms heterodimers with all PACT molecules, which prevents PKR activation; ii) the large amount of ADAR1 induced by HIV binds to PACT, inhibits its activity and reverses its function; or iii) an HIV component or an HIV-induced component changes the role of PACT from being an activator to being an inhibitor of PKR. Further experiments will determine which mechanism is involved. Interestingly, it is well established that PACT activation of PKR is stress dependent. This process is most likely link to the formation of stress granules in the stressed cells, which are transient large abortive translation initiation complexes that are in dynamic equilibrium with translating polysomes and that accumulate upon inactivation of eIF2α by specific kinases (44, 45). Recently, Abrahamyan *et al.* show that HIV-1 infection prevents the formation of stress granules (44). This could be a possible mechanism by which HIV-1 prevent the phosphorylation and activation of PACT, and subsequent activation of PKR. However, this mechanism does not explain the inhibitory activity of PACT on PKR during HIV replication.

Finally, our results show that three cellular proteins, TRBP, ADAR1 and PACT contribute to the inhibition of PKR and eIF2α phosphorylation observed in HIV-infected cells (Fig. 4.5). All of them are dsRBPs, which raises the possibility that all proteins of this family act similarly. Staufen was used as another dsRBP that has a

positive activity on the virus by binding to Gag and by increasing translation from TAR-containing RNAs (21, 46). In agreement with its PKR-independent mechanism on translation (21), we found that Staufen did not inhibit PKR activation. This result indicates a specific mechanism mediating PKR inhibition for TRBP, ADAR1 and PACT. Further studies will determine if PKR forms a large ribonucleoprotein complex with TAR RNA, Tat, TRBP, ADAR1 and PACT to inhibit PKR simultaneously or if several complexes act at different times or locations in HIV-infected cells.

4.7 CONCLUSIONS:

Previous results have characterized PACT as a PKR activator, but with the native protein, this activity occurs only in stress conditions. In contrast, we show here that PACT contributes to PKR inactivation during HIV replication. PACT belongs to a ribonucleoprotein complex formed around PKR, including TRBP and ADAR1, which also contribute to PKR inhibition. HIV has evolved to replicate in cells that express cellular PKR inhibitors. It also induces mechanisms that enhance or change the activity of proteins to inhibit PKR for increased replication. This process contributes to HIV pathogenicity.

4.8 METHODS:

4.8.1 CELLS AND TRANSFECTIONS.

HEK 293T (ATCC CRL-11268) were maintained at 37°C in 5% CO_2 in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (HyClone), 2 mM L-glutamine, and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). HEK 293T cells express adenovirus sequences and SV40 Large T antigen (47). Jurkat T cells (ATCC TIB-152) were maintained in RPMI-1640 (Invitrogen) supplemented similarly.

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors previously selected to be negative for HIV, HTLV-I and II, HCV, CMV and syphilis. Blood sample collection was approved by the ethic review board of McGill University.

For transfection of HEK 293T with plasmids, cells were plated at 50% confluence 24 h prior to transfection using polyethylenimine (PEI) following manufacturer's protocol (Polysciences, Inc Warrington, PA). Transfection of HEK 293T cells with siRNAs was performed in six-well plates using Lipofectamine 2000 (Invitrogen) as previously (48) 24 h prior to transfection with pNL4-3 using TransIT-LT (Mirus). Cells were lysed 48 h posttransfection for immunoblotting analysis.

4.8.2 PLASMIDS AND SIRNA SYNTHESIS.

HIV-1 clone pNL4-3, pcDNA1-PKR, pCMV2-Flag-PACT, pcDNA3-TRBP2, pcDNA3.1-ADARp150-V5, and pcDNA3-RSV-Staufen-HA were previously described (8, 49).

The small interfering RNAs (siRNAs) used in this study were NS (48) and siPACT (Qiagen, cat. SI00288085; 5' TAAGTATGATTGATTGTTAAA 3').

4.8.3 TRANSFECTION OF HIV CLONES AND REVERSE TRANSCRIPTASE (RT) ASSAY.

For transfection of HIV provirus, HEK 293T were transfected as above with pNL4-3 proviral DNA. Cell supernatants were collected 48 h post transfection and viral production assayed for standard reverse transcriptase (RT) assay. RT assay as previously described (8, 50) with the following modifications. 5 µl of supernatant of HIV-incubated cells were incubated at 37°C in 25 µl of a NP40-modified RT cocktail [60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 1.1% Nonidet P-40, 1.04 mM EDTA, poly(A) (5 µg/ml), oligo(dT) (0.16 µg/ml)] for 30 min in the Confinement laboratory level 3 to inactivate the virus. 25 µl of the RT cocktail [60 mM Tris-HCl (pH 7.8), 75 mM KCl, 5 mM $MgCl_2$, 0.1% Nonidet P-40, 1.04 mM EDTA, poly(A) (5 µg/ml), oligo(dT) (0.16 µg/ml)] supplemented with 16 µl of 0.5 M DTT and 5 µl of [α-^{32}P]dTTP (PerkinElmer, Waltham, MA, USA) per ml was then added and incubated at 37°C for an additional hr. Next, 5 µl of the reaction mixture mixture was then spotted onto DEAE filtermat (PerkinElmer), washed and read as previously (8). Supernatants from transfected HEK293T cells alone were used for infection of Jurkat or PBMCs.

4.8.4 HIV-1 VIRAL INFECTION OF JURKAT CELLS AND PBMCS.

Jurkat cell infection was previously described (8). For PBMCs HIV-1 infection, approximately with 7 x 10^7 cells were stimulated for four days in supplemented RPMI (Invitrogen, Carlsbad, CA, USA) with 20U/ml recombinant Human interleukin-2 (R&D Systems, cat # 202-IL) and 5 μg/ml phytohemagglutinin (Sigma cat. 12646). The cells were infected with HIV cell supernatant corresponding to 2.5 x 10^5 cpm measured by standard RT assay in a final volume of 1 ml supplemented RPMI in polypropylene round-bottom tube, and incubated for 2 h at 37°C with mixing every 30 min. RPMI was then added to the cell-virus mixture, transferred to a T25 flask and incubated at 37°C. The cells were fed on average every other day with fresh medium. Supernatant and cell samples were collected at different times and assayed for RT activity, immunoblotting and immunoprecipitation when indicated.

4.8.5 IMMUNOPRECIPITATION AND IMMUNOBLOTTING

Jurkat immunoprecipitation was previously described (8). For immunoblotting, HEK 293T, or Jurkat T cells extracts were prepared, separated and transferred for immunoblotting as previously described (4). Membranes were blocked for 1 h in 5% nonfat dry milk and Tris-buffered saline-0.1% Tween 20 (TBST). Membranes were incubated overnight at 4°C with the primary antibody. After five washes in TBST, membranes were incubated with Horseradish Peroxidase-conjugated secondary goat anti-rabbit or goat anti-mouse antibody (GE Healthcare). Anti-P-PKR (Abcam) and anti-P-eIF2α (Invitrogen) was blotted in 3% BSA/TBST overnight. After an immunoblotting with an antibody, the membranes were washed in TBST overnight or stripped and reused to detect other proteins. The bands were visualized using ECL (GE Healthcare). Primary antibodies used for immunoblotting were monoclonal anti-PKR 71-10 (51) obtained from Dr. A. Hovanessian, anti-Actin (Chemicon) at a 1/500 dilution, anti-HIVp24 183-H12-5C (52), at a 1/1000 dilution,

polyclonal anti-P-PKR (Abcam), at a 1/1000 dilution, anti-PACTeg2 (11), anti-P-eIF2α (Invitrogen), at a 1/1000 dilution, and anti-eIF2α (Cell signaling), at a 1/1000 dilution. Where indicated, the bands where quantified by densitometry analysis as described (53).

4.9 LIST OF ABBREVIATION:

HIV-1: Human immunodeficiency virus type 1; PKR: Protein Kinase RNA-activated; eIF2α: alpha subunit of the Eukaryotic Initiation Factor 2; IFN: Interferon; dsRBP: double-stranded RNA binding protein; dsRBD: double-stranded RNA binding domain; ADAR1: adenosine deaminase acting on RNA 1; TRBP: TAR RNA Binding Protein; PACT: PKR Activator; PBMCs: Peripheral Blood Mononuclear Cells; siRNA: small interfering RNA; RT: reverse transcriptase; IP: immunoprecipitation.

4.10 COMPETING INTERESTS

The authors declare that they have no competing interests.

4.11 ACKNOWLEDGEMENTS:

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CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 Discussion

5.1.1 HIV Replication and the IFN pathway

Type I IFN induction during HIV-1 disease is a double-edged sword. A discrepancy exists between *in vitro* studies regarding IFN's benefits on HIV replication and *in vivo* in the treatment of AIDS patients. Results in cell culture show that IFN is a potent HIV inhibitor, while clinical data show very mixed effects. Moreover, plasma level of IFNα in HIV-1 infected patients in the late stages of progression to AIDS correlates with poor prognosis.

5.1.1.1 Beneficial effects of IFN

In macrophages from healthy subjects pretreated with IFNα, β or γ before HIV-1 infection, Meylan *et al.* showed reduced viral DNA signal by PCR and abolition of spliced mRNA signal and viral production during single replication cycles (1). In cells with established productive infection, the same authors also showed that the addition of IFN does not significantly affect the levels of HIV spliced transcripts and there is no intracellular accumulation of viral proteins. They conclude that the major effect of IFNs was at an early step of the virus life cycle and resulted in a reduced viral DNA synthesis. IFNs also inhibit HIV-1 replication by acting at the post-translational level (1). Similarly, Shirazi and Pitha demonstrated that IFNα markedly inhibits HIV-1 replication in the CEM-174 lymphocytic cell line during a single replication cycle. The IFN-mediated block was later shown to be at the level of HIV-1 provirus formation both *in vivo* and *in vitro* using CEM-174 cytoplasmic extracts (2). The antiviral mechanism of IFN inhibition of HIV-1 replication has also been suggested to result from the prevention of the association of viral mRNA to polyribosomes and inhibition of virions assembly through posttranslational modification of viral proteins (3). In

addition, IFN interferes with HIV-1 in the later stages of the virus replication by interfering with viral budding and release. By quantitation of gp120 by immunogold labeling analysis of HIV-1 virion analysis from IFN-treated and control HIV-infected cells, Hansen *et al.* revealed a marked depletion of envelope glycoprotein in virions released from IFN-treated cells compared to the control suggesting that IFNα treatment interferes with HIV virion assembly (4). Furthermore, the defect in gp120 assembly into mature viral particles correlated with a loss of infectivity, a fact corroborated by a study showing that IFN treatment prevents cell infection (5). Another proposed activity of IFN was that it may interfere with the release of newly formed virions (6). Therefore, early studies concluded that IFN action against HIV-1 occurs at multiple levels (7).

5.1.1.2 Deleterious effects of IFN

HIV disease progression is characterized by the loss of $CD4^+$ T cells and inhibition of immune cell responses leading to cytokine deregulation (8). These events are the result of infected cell lysis following immune activation, with the subsequent release of virions, and they lead to HIV-1-induced immune disorders associated with cytokine deregulation (9, 10). Deleterious effects of high concentration of type I IFN have been reported in some studies during AIDS progression and are associated with inhibition of T cell proliferation and activated T cells apoptosis (11-13). High levels of IFNα in sera are predictive of disease progression in HIV-1-infected subjects and in macaque monkeys (Macaca mulatta) with AIDS-like disease (14).

Direct cytopathic effect of the virus on $CD4^+$ T cells accounts for the major mechanism leading to memory $CD4^+$ T cell depletion (15). Moreover, viral infection causes a widespread activation and apoptosis of both infected and uninfected bystander $CD4^+$ and $CD8^+$ T cells in both lymphoid tissues and peripheral blood (16). Apoptosis occurs predominantly in bystander cells rather than in productively infected cells of both HIV-1- and SIV-infected lymph nodes

(16). TRAIL was shown to mediate the apoptosis of activated T cells from HIV-1-infected patients (17). On CD4+ T cells, IFNα binding to its receptor results in STAT-1/2-regulated expression of membrane TRAIL. TRAIL mediates increased activation-induced cell death in both $CD4^+$ and $CD8^+$ T lymphocytes from HIV-1-infected subjects (17, 18). These findings suggest that IFNα produced by PDCs contributes to HIV-1 pathogenesis by inducing expression of soluble and membrane-bound TRAIL and other death molecules in the lymph node, which bind to HIV-induced DR5 expressed on $CD4^+$ T cells, leading to their apoptosis during progression to AIDS (19).

In addition to contributing to pathogenesis, IFN response through the ISGs might not be effective but this had not been tested at the beginning of this study.

5.1.2 PKR IS TRANSIENTLY ACTIVATED DURING HIV-1 INFECTION

Type I IFN through the induction of PKR is central to the inhibition of HIV-1 replication and spread. *In vivo* data with cell culture show that the kinase is highly regulated during HIV-1 challenge. During the course of the work described in this thesis, we presented the first evidence that HIV-1 infection of lymphocytes activates PKR in the early stages but, as the infection progresses, PKR activation declined (20). Similarly, PKR is also only transiently activated in the early stages of HIV-1 infection of PBMCs, which might better resemble infection *in vivo* (Chapter 4). These results suggest that the cellular innate antiviral pathway is activated at the early stages of HIV infection followed by inhibition at the later stages of HIV infection. PKR activation might be a result of the few TAR RNA and viral mRNA molecules present at the beginning of the infection. However, as more viral mRNAs and Tat proteins are being produced, PKR activation is inhibited.

5.1.3 Multiple controls of PKR activation during HIV replication

The large number of viruses that have evolved elaborate mechanisms for PKR inhibition emphasizes its critical role in the innate response to virus infection (21). In the case of HIV, the viral trans-activator Tat and large amounts of the viral TAR RNA contribute to PKR inhibition (22-25). In addition to viral components, our results show that activation of PKR during HIV-1 replication can be counteracted by the expression of cellular dsRBPs such as TRBP, ADAR, and PACT, but not Staufen. These proteins likely inhibit PKR activity by direct binding and by sequestration of activating dsRNA molecules.

5.1.3.1 Activation and inhibition of PKR by HIV TAR RNA

The 5'-untranslated region (5'-UTR) present at the 5' terminus of all HIV-1 transcripts is responsible for both transcriptional and translational regulation of HIV-1. The 5'-UTR also plays a central role in PKR regulation during infection by direct binding of the kinase on the TAR RNA. Specifically, chemical shift mapping showed that PKR's dsRBDs binding site spans from the upper bulge to lower stem of the TAR RNA. High affinity binding requires both the bulge and the loop of TAR RNA and the kinase dsRBD1, with 1:1 stoichiometry. This leads to the activation of the kinase and subsequent inhibition of protein synthesis and HIV-1 replication (25-27). HIV-1 TAR RNA structure mediates PKR phosphorylation by direct binding, which leads to translational and viral replication inhibition as part of the cellular antiviral response to HIV infection. This antiviral mechanism is likely to be effective during the early stages of infection, prior to the synthesis of Tat and highly productive HIV-1 mRNA transcription. PKR typically shows a characteristic bell-shaped activation curve with low concentrations of dsRNA activating the kinase activity and higher concentrations inhibiting it (28, 29). The assembly of a homodimer structure may be favored at low concentrations of dsRNA on a single dsRNA, whereas PKR

monomers binding on separate molecules of dsRNA would prevail at higher dsRNA concentrations (28, 30). Therefore, the high concentrations of TAR RNA that occur during the late stages of HIV replication could possibly act as a viral countermeasure against cellular antiviral pathway by inhibiting PKR activation, thereby allowing translational processes to occur.

5.1.3.2 Inhibition of PKR by HIV-1 Tat

HIV-1 Tat protein binds to the TAR RNA structure and plays a primordial function in HIV transcription (31). PKR binds to and phosphorylates Tat on serine and threonine residues in the vicinity of the arginine-rich region important for TAR RNA binding and Tat function (23). The extent of Tat phosphorylation highly depends on the prior activation of PKR by dsRNA, establishing Tat as a substrate for PKR. Tat inhibits the activation and activity of the kinase by both RNA-dependent and RNA-independent mechanisms, blocks PKR autophosphorylation triggered by dsRNA, and competes with its substrate, eIF2α (22-24). Furthermore, PKR levels are reduced in HeLa cells stably expressing HIV-1 wild-type Tat compared to cells expressing a mutated Tat or control cells, suggesting that Tat downmodulates endogenous PKR expression (32, 33). Using a synthetic Tat peptide encompassing the basic region, Judware *et al.* showed that the peptide was sufficient to prevent the activation and autophosphorylation of PKR by TAR RNA at low concentrations (34). However, higher TAR RNA concentrations overcome the inhibitory effect of the Tat peptide suggesting that Tat effect on PKR is via binding to TAR RNA and preventing its interaction with the kinase.

Overall, the Tat-PKR interaction may play a major role in HIV-1's ability to circumvent the host innate immune responses by blocking the activity of PKR at various steps using both RNA-dependent and RNA-independent mechanisms: (i) By sequestration of activating dsRNA; (ii) By binding to and preventing PKR

activation *via* autophosphorylation; (iii) By preventing inhibition of PKR autophosphorylation by direct interaction, (iv) By acting as a competitive inhibitor. Moreover, once Tat is phosphorylated by PKR, it binds to TAR RNA faster and with higher affinity compared to unphosphorylated. Phosphorylation of Tat also correlates to enhanced Tat transactivation (35). This is another method by which HIV-1 hijacks the natural innate cell response to further its own replication.

5.1.3.3 TRBP inhibits PKR in HIV-1-infected cells

TRBP enhances HIV-1 expression and replication in cell culture (36). TRBP strongly inhibits PKR activation through direct binding of their respective dsRBDs and sequestration of dsRNA molecules (37, 38). Experiments in HEK293T cells show that expression of TRBP is sufficient to completely restore PKR-inhibiting HIV-1 expression and replication (20, 39). Astrocytic cells that naturally express low levels of TRBP have a high PKR activation upon HIV transfection and consequently restrict viral production. Complementation by TRBP rescues viral protein expression and enhances virion production (39-41). Furthermore, downregulation of TRBP expression by siRNA greatly reduces HIV-1 expression and replication, likely as a result of both a heightened PKR activation and TRBP's role in RNA interference (42, 43). Therefore, endogenous TRBP strongly regulates PKR activity during viral expression and replication.

5.1.3.4 ADAR inhibits PKR during HIV-1 replication

ADAR1 is an adenosine deaminase that can edit viral RNA and therefore may induce proviral or antiviral activities. Our results show an ADAR1-enhancing impact on HIV replication, with no or little other specific effects on HIV replication as shown by mutagenesis (20). While our experiments were being conducted, Nie *et al.* showed that ADAR1 enhances VSV replication due to PKR inhibition (44). Therefore, our and theirs results reach similar conclusions on two

different viruses. In contrast, another study on HIV showed an enhancement of HIV replication by ADAR1 and ADAR2 due to the editing activity of the enzyme (45). At the time our data were published, Doria *et al.* reported evidence showing that ADAR1 stimulates HIV-1 replication by a dual mechanism involving both editing-dependent and PKR-dependent mechanisms (46). They also reported that HIV-1 virions generated in the presence of over-expressed ADAR1 are released more efficiently and display enhanced infectivity compared to an editing-inactive ADAR1 mutant. The consensus from our studies and others is that ADAR1 contributes to HIV replication by two independent mechanisms: RNA editing and PKR inhibition. One mechanism or the other may be predominant in different experimental settings. Finally, ADAR enzymes seem to be highly involved in the spread of HIV-1 and other viruses, which is an unexpected function for an ISG.

5.1.3.5 PACT is a PKR inhibitor in HIV infected cells

PACT is mainly known in the literature as a stress-dependent PKR activator. However, in this report we show that PACT inhibits PKR in HIV-1-infected cells (Clerzius *et al.*, in preparation). Interestingly, results from our laboratory showed that PACT acts both as a PKR inhibitor and a PKR activator (47). PACT homodimerizes with itself and heterodimerizes with TRBP through the two proteins' double-stranded RNA binding domains (dsRBDs) and their Medipal domains (48). PACT binding to TRBP influences its activity on PKR. In a cellular environment where PACT homodimer formation is favored, such as in low TRBP concentration, in the presence of stress, which dissociates TRBP-PACT interaction, or with PACT overexpression, PACT induces PKR activation. Therefore, in the absence of stress, TRBP controls PACT activation of PKR. An important experiment that is currently underway is to determine the effects of PACT on HIV-1 replication in astrocytes, which are a natural cellular system expressing very low levels of TRBP. As a result, HIV-1 replicates poorly in astrocytes due in part to a heightened PKR response. Viral replication can be

partially rescued by expressing low amounts of TRBP. In this context, TRBP prevents PKR activation, restores the production of viral proteins and consequently enhances HIV replication (39).

Whether or not PACT expression partially rescues HIV-1 replication in astrocytes will shed light on the nature and importance of TRBP regulation of PACT activity on PKR during HIV replication. If we observe an inhibition of HIV production by PACT in astrocytes, our interpretation will be that in HEK293T cells, PACT is mainly bound to TRBP and inhibits PKR activation. If we observe an activation of HIV production, the increase will not be due to TRBP-PACT heterodimers but to a change of PACT activity induced by the virus. It could be a viral component or a pathway induced by the virus. The observed increase of ADAR1 expression during HIV infection could also mediate such a pathway. Figure 5.1 shows a summary of the different levels of regulation of PKR during HIV-1 replication.

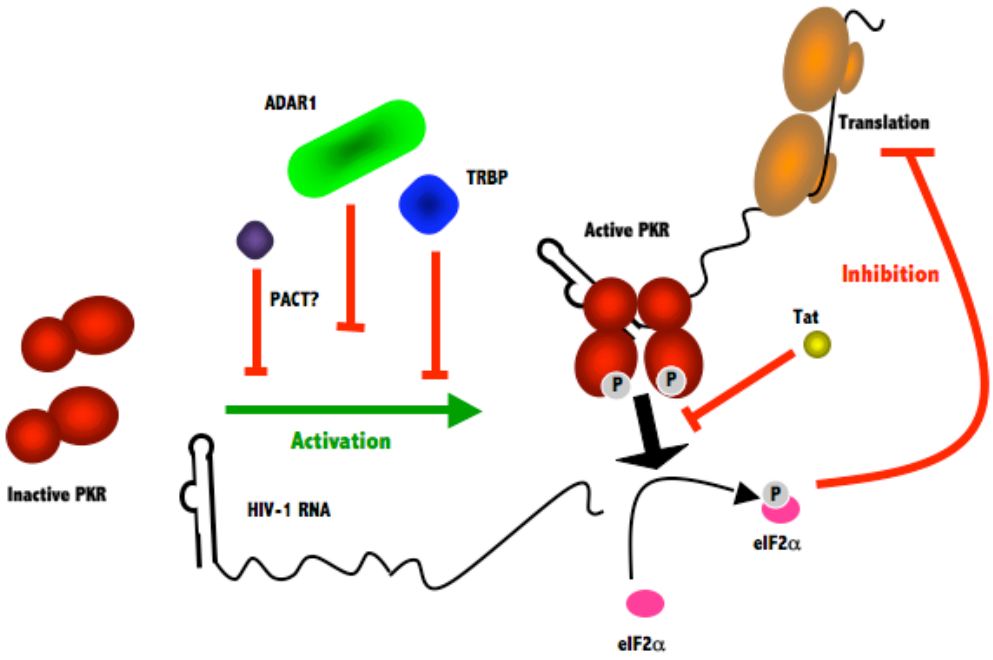

Figure 5.1: An updated schematic representation of the regulation of HIV translation by PKR and the contribution of host and viral factors. The schematic is derived from figure 3.8 showing the different levels of regulation of PKR by ADAR1, TRBP, Tat during HIV-1 replication. The figure also depicts the possible role of PACT as a PKR inhibitor during HIV-1 replication.

5.2 CONCLUSION

HIV-1 infection in the human population is rarely contained and so far has never been shown to be cleared. However, several lines of evidence show a very active host innate immunity against HIV-1 infection during primary infection. Our data show that PKR, a protein central to the IFN-mediated antiviral pathway, is activated during HIV-1 infection and highly regulated at multiple levels. The IFN-antiviral pathway alone is unlikely the sole mechanism responsible for the failure of the innate immunity to counteract HIV-1 infection. Interestingly, TRBP, ADAR and PACT, all of which regulate PKR during HIV replication, are involved in RNA interference. RNAi may serve as an antiviral immune response in mammalian cells against pathogens and viral invasions (49). Thus far, viral miRNAs have been isolated from cells infected by a wide range of viruses, including Epstein-Barr virus (EBV), but not HIV (50). Furthermore, some cellular miRNAs have been suggested to be implicated in the viral life cycle, or have been shown to restrict viral replication, as exemplified by the primate foamy virus (51). However, despite some data showing that HIV-1 Tat protein acts as an RNAi suppressor in the pathway mediated by shRNAs, and inhibits the ribonuclease type III Dicer, some controversy currently exists regarding the involvement of RNAi in HIV replication (52, 53). Furthermore , TRBP binds to Dicer and is a major component of the RNAi pathway (54). TRBP also favors HIV replication, and therefore its role in RNAi and HIV-1 replication is unclear (42, 43). PACT function in RNAi is similar to that of TRBP (55). ADAR1 and ADAR2 have been shown to affect RNA interference (RNAi) and microRNA processing by deamination of specific adenosines to inosine (56, 57).

HIV-1 efficiently replicates in the face of an active host innate immunity. Clearly, a crosstalk exists in the two arms of the innate immunity and PKR might again play a leading role. The events underlying the failure of the immune system

against HIV-1 infection are complex and require a thorough understanding of several pathways.

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APPENDICES

A-1 CONTRIBUTION OF AUTHORS

I have substantially contributed to both papers included in this thesis.

A-1.1 ADAR1 Interacts with PKR during human immunodeficiency virus infection of lymphocytes and contributes to viral replication

For figure 1.1, I was responsible for Jean-François Gélinas' training, who was a new Master student at that time. We did the Jurkat infection, collected supernatants and cell pellets together for about month. However, I did all the analysis, including all the RT assays and Western blot analysis.

Jean-François Gélinas did all the luciferase analysis alone, which are presented in figures 3.3A and 3.5B. Jean-François Gélinas and myself also did figure 3.6 together, but I contributed to most of the results. With the exception of figure 3.7, which I did with Aïcha Daher, I worked on all the remaining results alone.

A-1.2 The PKR activator, PACT, is a PKR inhibitor during HIV replication

With the exception of figure 4.4, which Jean-François Gélinas and Aïcha Daher did together, I am the sole contributor to all the remaining figures.

A-2 ACKNOWLEDGEMENTS

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A-2.2 Doctoral defense jury

In addition, I am grateful to all the members of my PhD doctoral defense committee who took the time to read and correct my thesis, were present at the defense and eventually awarded me the title of Doctor of Philosophy.

These members were:

- Dr Anne Gatignol, my supervisor.
- Dr Michael Laughrea, the Internal examiner.
- A special mention for Dr Léa Brakier-Gingras, the External examiner who was not allowed to be present for the defense but whose corrections were the most insightful.
- Dr Andrew Mouland, representing the Chairman of the Department.
- Dr Mark Wainberg.
- Dr Guy Lemay.
- Dr Benoit Barbeau.

Thanks also to Dr Louis Z.G. Touyz from the Faculty of Dentistry, the Pro-Dean who chaired the oral and was appointed by the Graduate and Postdoctoral Studies Office.

A-2.3 Department of Medicine, Division of Experimental Medicine, McGill University

Finally, I am grateful to the Department of Medicine, Division of Experimental Medicine, McGill University. I would personally like to thank Dr Hugh P.J. Bennett, Ms. Dominique Besso, the Student Affairs Officer, and Ms. Marylin Linhares, the Student Affairs Coordinator, from the Experimental Medicine Department for their constant help, guidance and professionalism.

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