IFITM proteins inhibit HIV-1 replication

Master's Thesis

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Abstract (English)

Viral infection triggers production of interferon (IFN) that in turn leads to the expression of genes known as IFN-stimulated genes (ISGs), some of which possess antiviral activities. Previous studies have shown that IFN suppresses the replication of human immunodeficiency virus type I (HIV-1). While several ISGs have been linked to this specific antiviral activity with well-defined inhibitory mechanisms, others remain to be investigated. With the purpose of identifying novel ISGs capable of inhibiting HIV-1 replication, we have performed a shRNA screen of the genes upregulated by IFN in SupT1 cells. This study reports three ISGs, known as interferon-induced transmembrane proteins 1, 2 and 3 (IFITM1, 2 and 3), that substantially inhibit HIV-1 replication in SupT1 cells. Further studies suggest that HIV-1 entry is impaired. Collectively, these findings identify a small family of cellular restriction factors that serve as a barrier to HIV-1 entry into the host cell.

Abstract (French)

Suite à une infection virale, les interférons (IFNs) sont produites et servent à induire l'expression de certains gènes, appelés gènes stimulés par l'interféron (ISGs), dont certains possèdent des effets antivirales. Plusieurs études ont démontré que l'IFN possède la capacité d'inhiber la réplication virale du virus de l'immunodéficience humaine de type I (VIH-1). Tandis que certains ISGs ont été associés à une activité antivirale spécifique avec un mécanisme d'action bien défini, d'autres ISGs sont moins bien caractérisés. Dans le but d'identifier de nouveaux ISGs responsables d'inhiber la réplication virale du VIH-1, nous avons réalisé un criblage par shRNA des gènes régulés par l'IFN dans les cellules SupT1. Cette étude rapporte trois ISGs, appelés «interferon-induced transmembrane proteins 1, 2 et 3» (IFITM1, 2, et 3), dont l'expression dans les cellules SupT1 peut inhiber la réplication virale du VIH-1 de façon significative. Les résultats indiquent que ces protéines agissent au niveau de l'entrée du virus dans la cellule. Collectivement, cette étude a identifié une famille de facteur de restriction cellulaire qui agit comme barrière pour prévenir l'entrée du VIH-1 dans la cellule hôte.

Publications

1. Manuscript submitted: **Lu, J.**, Q. Pan, S.L. Liu, and C. Liang. 2010. IFITM proteins inhibit HIV-1 infection.

2. Rong, L., J. Zhang, **J. Lu**, Q. Pan, R.P. Lorgeoux, C. Aloysius, F. Guo, S.L. Liu, M.A. Wainberg, and C. Liang. 2009. The transmembrane domain of BST-2 determines its sensitivity to down-modulation by human immunodeficiency virus type 1 Vpu. J. Virol. **83**:7536-46.

3. Ma, J., L. Rong, Y. Zhou, B. B. Roy, **J. Lu**, L. Abrahamyan, A. J. Mouland, Q. Pan, and C. Liang. 2008. The requirement of the DEAD-box protein DDX24 for the packaging of human immunodeficiency virus type 1 RNA. Virology **375**:253-264.

4. Zhou, Y., L. Rong, **J. Lu**, Q. Pan, and C. Liang. 2008. The insulin-like growth factor II mRNA binding protein 1 associates with the Gag protein of human immunodeficiency virus type 1 and its overexpression affects virus assembly. J. Virol. **82**:5683-5692.

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There are two very important people in my life who have followed me through my ups and downs since I was born and who have never stopped believing in me and these

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Abbreviations

ADAR1	Adenosine deaminase acting on RNA
AIDS	Acquired immune deficiency syndrome
APOBEC	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like
ART	Antiretroviral treatment
BlaM	β-lactamase
BST-2	Bone marrow stromal cell antigen 2
CA	Capsid
CBMC	Cord blood mononuclear cells
CCR5	C-C chemokine receptor type 5
CCR3	C-C chemokine receptor type 3
CD4	Cluster of differentiation 4
cDNA	Complementary DNA
CMV	Cytomegalovirus
CPM	Counts per minute
CXCR4	CXC chemokine receptor 4
DNA	Deoxyribonucleic acid
Dox	Doxycycline
ECL	Enhanced chemiluminescence
Env	Envelope
EPSTI1	Epithelial stromal interaction 1
ESCRT-I	Endosomal sorting complexes required for transport I
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
GAS	IFN-γ-activated site
gp	Glycoprotein
HAART	Highly Active Antiretroviral Therapy
HCV	Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
hr	Hour
IFI44	Interferon-alpha-inducible protein 44
IFITM	Interferon induced transmembrane
IFN	Interferon
IL-2	Interleukin 2
IN	Integrase
IRF9	Interferon-regulatory factor 9
ISG	Interferon stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISG15	IFN-stimulated protein of 15 kDa
ISRE	IFN-stimulated response element
JAK1	Janus activated kinase 1
kDa	Kilodaltons
LTR	Long terminal repeat
µCi	Microcurie
µg	Microgram
µl	Microliter

M	Molar
MA	Matrix
mg	Milligram
mL	Milliliter
mM	Millimolar
mRNA	Messenger RNA
Mx	Myxovirus resistance
NC	Nucleocapsid
Nef	Negative-regulation factor
ng	Nanogram
NLS	Nuclear localization signal
nm	Nanometer
NNRTIs	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NS	Nonstructural
OAS	2',5'-oligoadenylate synthetase
PAMP	Pathogen-associated molecular pattern
PB	Pacific blue
PBMC	Peripheral blood mononuclear cell
PBS	Primer-binding site
pDC	Plasmacytoid dendritic cell
PE	R-Phycoerythrin
PIs	Protease Inhibitors
PIC	Pre-integration complex
PKR	dsRNA-dependent protein kinase

Pol	Polymerase
PPT	Polypurine tract
PR	Protease
PRR	Pattern-recognition receptor
$Pr55^{Gag}$	Group-specific antigen
P/S	Penicillin/Streptomycin
Rev	Regulator of viral expression
RIG-I	Retinoic acid inducible gene I
RLU	Relative light units
RNA	Ribonucleic acid
RNase L	Ribonuclease L
rpm	Revolutions per minute
RRE	Rev responsive element
RT	Reverse transcriptase
RTC	Reverse transcription complex
RTP4	Receptor (chemosensory) transporter protein 4
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus
SP	Spacer peptide
STAT	Signal transducers and activators of transcription
SU	Surface
TAR	Transactivation response region
Tat	Transactivator
TCA	Trichloroacetic acid

TDRD7	Tudor domain containing 7
TLR	Toll-like receptor
TM	Transmembrane
TRIM	Tripartite-motif-containing
TYK2	Tyrosine kinase 2
U	Unit
UN	United Nations
Vif	Viral infectivity
VIH-1	Virus de l'immunodéficience humaine de type I
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Vesicular stomatitis virus glycoprotein
WHO	World Health Organization
YFP	Yellow fluorescent protein

Chapter 1-Introduction

1.1 Epidemiology

The year 2010 marks the 27th commemoration of the discovery of the human immunodeficiency virus type 1 (HIV-1). The HIV pandemic is one of the most serious health crises the world faces today. In 2007, the joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that 33.4 million were living with HIV/AIDS worldwide, among them 2.7 million were newly infected that year and 2.1 million were children under the age of 15. In that year alone, 2 million HIV-related deaths were estimated to have occurred. Sub-Saharan Africa remains the most affected region, with 67% of the global burden and 90% of the global pediatric burden. There has been an epidemiological shift since 2003, the rate of infection has stabilized or begun to decrease in sub-Saharan Africa but new infections have increased in other countries. Moreover, there is a discrepancy in the major mode of transmission between sub-Saharan Africa and all other regions. Heterosexual transmission mainly affects sub-Saharan countries whereas intravenous drug users, men having sex with men, and sex workers are mainly affected in other countries. Globally, the percentage of people living with HIV who are women has remained stable at 50% [121].

Despite the grim statistics, great efforts have been deployed to treat and prevent this disease. From 2002 to 2008, the access to antiretroviral treatments (ART) in developing countries increased ten-fold to cover 4 million of those in needs [133]. Mother-to-child transmission has decreased ever since ART have been made available to HIV-positive pregnant women [121]. Efforts continue to be made to reach those in need.

1.2 Disease and transmission

1.2.1 Discovery of the virus that causes AIDS

The first cases of acquired immune deficiency syndrome (AIDS) were reported in 1981 [19,35]. These patients presented with Kaposi's sarcoma and/or opportunistic infections such as pneumocystis pneumonia and cytomegalovirus (CMV) infection. Similar cases started to be diagnosed around the world and yet the causing agent was not known. In 1983, Dr. Françoise Barré-Sinoussi was the first one to have isolated a retrovirus from the lymph nodes of a patient that showed signs of AIDS [8]. In 1984, Dr. Robert Gallo made similar discoveries and confirmed that a virus is the etiologic agent of AIDS [47,100]. Following this, similar isolates have been found by other researchers thus different names were given to this virus. It is not until 1986 that the AIDS-virus has been officially designated the human immunodeficiency virus (HIV) [23].

1.2.2 Disease transmission

HIV is transmitted by sexual contact with an infected person, by sharing needles with an infected person, transfusion with infected blood, although this last route of transmission has been eliminated in developed countries where blood is screened for the presence of HIV. HIV-positive mothers may transmit the virus to their children at birth or through breast-feeding.

1.2.3 Disease progression

The typical course of untreated HIV infection is illustrated in figure 1. The first few weeks following primary infection are characterized by flu-like symptoms in up to

80% of cases, a dramatic decrease in CD4 T-cell count and a sharp increase in HIV RNA in plasma. Once the adaptive immune system has kicked in, the CD4 T-cell count is restored at a certain level and the level of HIV RNA in plasma drops and stays relatively low. This phase may persist between 2 to 15 years with an average of 10 years. As infected individuals in this phase feel relatively healthy, it is called the asymptomatic phase or clinical latency. The adaptive immune response is capable of containing the virus but not eliminating it. As the CD4 T-cell count continues to drop, the individual becomes more at risk of opportunistic infections and developing cancer. Symptoms start to appear when the CD4 T-cell count reaches 500 cells/µl and an individual is said to have AIDS when the CD4 T-cell count drops below 200 cells/µl [66,94].

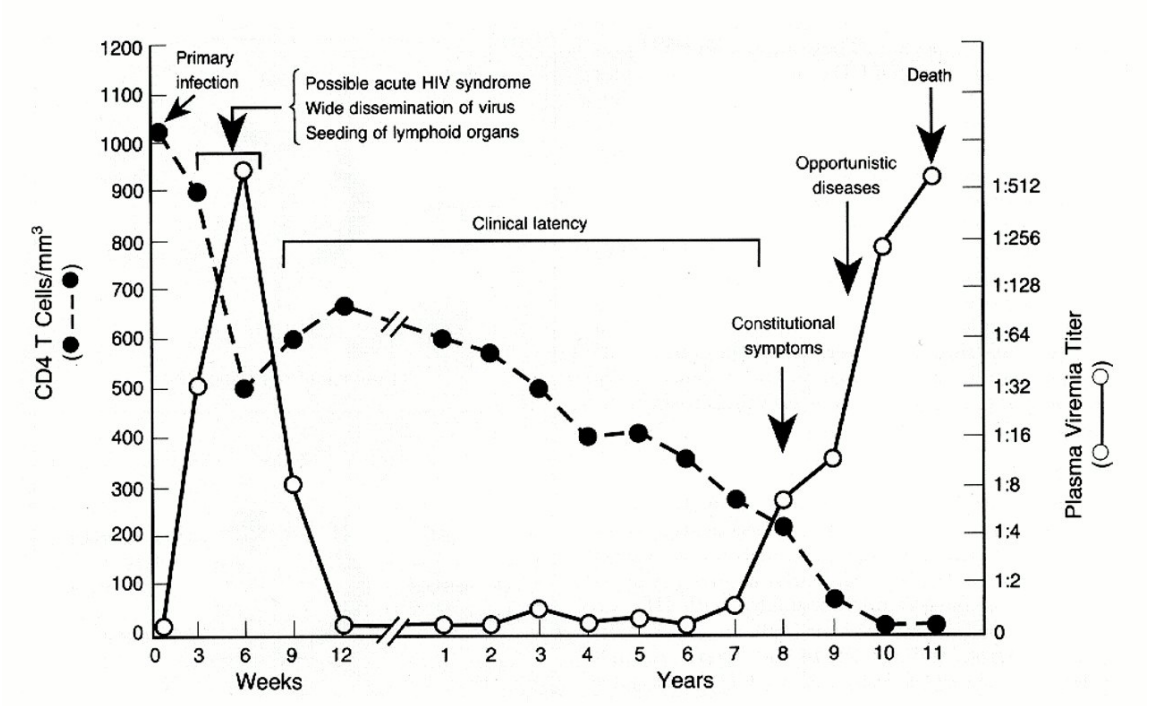


Figure 1: The typical course of an infection by HIV [94]. The few weeks following primary infection, there is a sharp increase in viral load and a dramatic decrease in CD4 T-cells. Once the adaptive immune response is stimulated, the viral load drops and the

CD4 T-cell count climbs back up. Shortly after, the CD4 T-cell count continues to slowly decrease over several years. Eventually, the person becomes at risk of death by opportunistic infections and cancers.

1.3 Antiretroviral drugs used in the treatment of HIV infection

Since the discovery of HIV in 1983, intensive efforts have been put into the research and development of antiretroviral therapies. Up to now, 25 individual antiretroviral drugs classified into six categories have been approved for use by the Food and Drug Administration (FDA) (Table 1) [44]. Some drugs are manufactured as combination of multiple drugs in a single pill so that pill burden is decreased. Early drug regimens consisted of a single or two drugs and often led to treatment failure. It is not until 1995 that the use of at least three agents, a regimen known as highly active antiretroviral therapy (HAART), became the standard of care [95]. The advantages of HAART include: slower disease progression, decreased viral loads, increased CD4 T-cell counts and decreased in probability of resistant virus from arising [95]. In overall, the quality of life of the patient is improved. The decision of when to begin treatment depends on CD4 T-cell count, viral load and symptoms of HIV-related disease. Specific guidelines vary from one country to another and remain a matter of debate among health care providers and those who establish the guidelines. Early treatment and deferred treatment both have their risks and benefits. Numerous side effects are associated with the use of ART, such as gastrointestinal effects, lipodystrophy, liver failure, renal failure, anemia and many more [95].

In 2008, the World Health Organization (WHO) estimated that 43% of people in need of treatment in low- and middle-income countries had access to ART. Among those, the coverage for children under the age of 15 was 38% [133].

Class of drug	Mechanism of action	Generic name
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Analogs of deoxyribonucleosides lacking a 3'-OH group that incorporate to newly synthesized viral DNA thus preventing elongation during reverse transcription. Act as a chain terminator [53].	• Lamivudine, 3TC • Zidovudine, ZDV, AZT • Emtricitabine, FTC • Abacavir, ABC • Tenofovir disoproxil fumarate, TDF • Didanosine, ddI • Stavudine, d4T • Zalcitabine, ddC
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Bind outside of the RT active pocket and cause conformational change so that function is inhibited [114].	• Etravirine • Delavirdine, DLV • Efavirenz, EFV • Nevirapine, NVP
Protease Inhibitors (PIs)	Inhibit the activity of protease by binding at the active site so that cleavage of viral polyproteins necessary for virion assembly is prevented [3].	• Amprenavir, APV • Tipranavir, TPV • Indinavir, IDV • Saquinavir Mesylate, SQV • Lopinavir, LPV • Fosamprenavir Calcium, FOS-APV • Ritonavir, RTV • Darunavir, DRV • Atazanavir sulfate, ATV

		• Nelfinavir mesylate, NFV
Fusion Inhibitor	Synthetic oligopeptide mimicking part of the gp41 envelope protein and inhibiting fusion with the cell membrane [131].	• Enfuvirtide, T-20
Entry Inhibitor/CCR5 co-receptor antagonist	Binds to CCR5 chemokine receptor causing a conformational change that will prevent binding by gp120 [4].	• Maraviroc, MRV
Integrase Inhibitor	Prevents the integration of viral DNA into the host genome [93].	• Raltegravir

Table 1: Classes of antiretroviral drugs approved by the FDA. Twenty-five different antiretroviral drugs are approved by the FDA. Many anti-HIV drugs on the market are manufactured as combinations of those drugs into a single pill. Adapted from the FDA website [44].

1.4 Classification and origins

1.4.1 Virus Classification

HIV belongs to the *Lentivirus* genus which is a member of the *Retroviridae* family [24]. Retroviruses have a linear and single-stranded ribonucleic acid (RNA) genome of positive polarity. This family is particular as it requires reverse transcription of viral genomic RNA into linear double-stranded deoxyribonucleic acid (DNA) and its subsequent integration into the host genome. The HIV genome is complex as it encodes

regulatory proteins in addition to the three major coding domains which encode the structural proteins also found in simple retroviruses.

1.4.2 Origins, evolution and distribution of HIV

Two species of HIV are known to exist: HIV-1 and HIV-2. It is widely accepted that HIV is derived from cross-species transmission from non-human primates to humans. Evidences for this include: (1) similarities of HIV genomic organization with simian immunodeficiency virus (SIV); (2) phylogenetically related viruses found in many species of non-human primates; (3) prevalence of SIV in the natural host; and (4) geographic location of HIV epidemics coincides with SIV pool [49,109]. Although HIV-1 and HIV-2 derive from SIV, they have different origins, global distribution and characteristics.

HIV-1 arises from the transmission of SIV from chimpanzee (SIV_{cpz}), more specifically from the *Pan troglodytes troglodytes* (*P. t. troglodytes*) subspecies found in West-central Africa [49,130]. HIV-1 is divided into three major groups based on viral sequences: M (main), N (non-M, non-O) and O (outlier) (Figure 2). It is believed that three different transmission events from *P. t. troglodytes* gave rise to these groups [49]. The M group is the pathogenic one responsible for the pandemic, with group N and O causing a minority of infections in central Africa [58]. The group M itself is divided into several subtypes according to their genetic sequences, also known as clades, which are designated by the letters: A, B, C, D, F, G, H, J and K [58]. In addition, there are numerous recombinant viruses in circulation which are made up of components from more than one subtype [132]. The distribution of HIV-1 group M subtypes around the world is

disproportionate (Figure 3). Subtype B is mostly found in the Americas, Western Europe and East Asia; whereas subtype C is mostly found in Southern Africa, East Africa, and India and its surrounding countries. Subtypes F, H, J and K cause a relatively small number of infections. Globally, subtype C is the predominant one causing 52% of all infections.

HIV-2 arises from the transmission of SIV from sooty mangabeys (SIV_{sm}), more specifically from the *Cercocebus torquatus atys* subspecies found in West Africa [60]. Eight transmission events gave rise to eight groups designated A through H. Only group A and B have shown evidence of human-to-human transmission; whereas group C through H failed to establish such transmission chain [26]. Unlike HIV-1, HIV-2 causes AIDS in only 20-25% of infected individuals and most people are asymptomatic, meaning that they have an undetectable viral load and a normal CD4 T cell count [27]. It is unclear why HIV-2 does not cause disease in the remainder 75-80% of infected people. Cases of HIV-2 infection are limited to Guinea-Bissau, a small country located in West Africa (Figure 3).

Due to the genetic diversity among the different subtypes, treating HIV is a challenge. Certain subtypes are naturally less susceptible or completely resistant to some of the currently used ART. The type of resistant mutations and the time it takes for those mutations to emerge once treatment has started may vary among subtypes [84]. In addition, viral genetic diversity creates an even greater challenge for the development of a preventive vaccine against HIV.

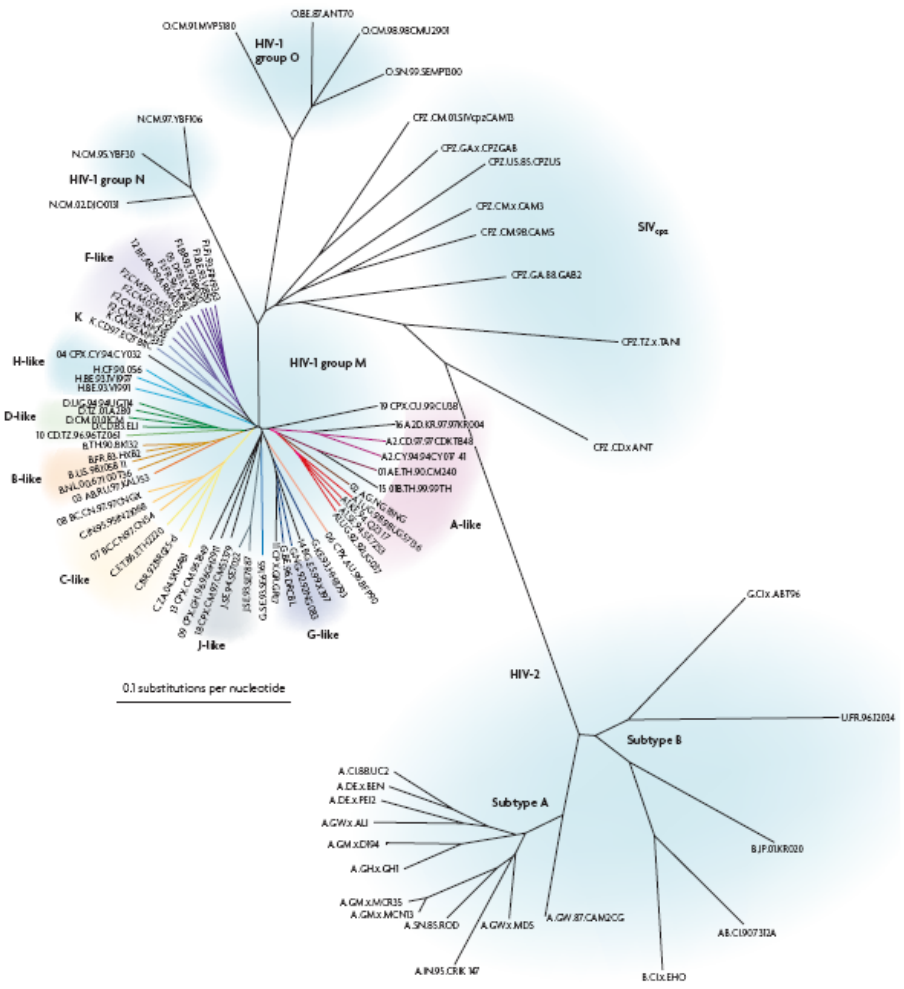


Figure 2: Phylogenetic tree of human and simian lentiviruses [6]**.** Similarities between HIV-1, HIV-2 and SIV_{cpz} at the nucleotide level were compared by aligning their full genome sequences. HIV-1 and HIV-2 share 50-60% sequence identity. HIV-1 group M is divided into numerous subtypes identified by letters from A to K. Only subtypes A and B from HIV-2 are shown. M, main; N, non-M non-O; O, outlier.

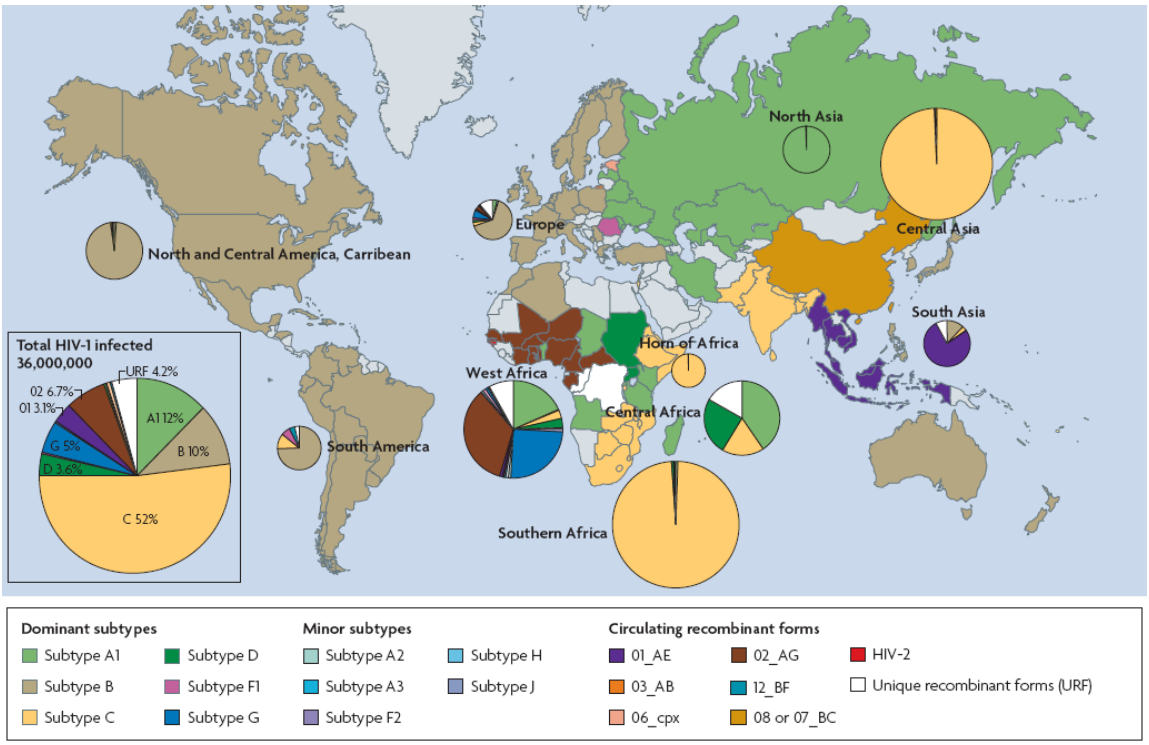


Figure 3: Distribution of HIV in the world according to subtypes and recombinants [6]. The countries are given a color based on the dominant HIV-1 group M subtype. The pie charts show the proportion of each subtype or recombinant form in each geographical region. The size of the pie chart is proportional to the number of infected individuals.

1.5 Virology

1.5.1 Viral genes and proteins of HIV-1

The HIV-1 genome consists of nine genes flanked by long terminal repeat sequences (LTR) (Figure 4). The LTRs are essential for the integration of the provirus into the host cell DNA and they contain binding sites for transcription factors to control the expression of viral genes. HIV has the three major genes, group-specific antigen (*gag*), polymerase (*pol*), and envelope (*env*), found in all retroviruses. The *gag* gene

encodes the structural proteins – matrix (MA or p17), capsid (CA or p24) and nucleocapsid (NC or p7) – that make up the viral core along with three peptides – p6, spacer peptide 1 (SP1) and spacer peptide 2 (SP2). The *pol* gene encodes the enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes the viral envelope glycoproteins gp120 and gp40/41. The *gag* and *pol* mRNAs are translated as polyproteins, $Pr55^{Gag}$ and $Pr160^{GagPol}$ respectively, and require cleavage by viral PR to give functional proteins. The $Pr160^{GagPol}$ is synthesized as a result of a ribosomal frameshift during the $Pr55^{Gag}$ translation. The *env* mRNA is translated as gp160 and also requires cleavage by a cellular protease during trafficking to the cell surface to give surface (SU) gp120 and transmembrane (TM) gp40/41. In addition to the three major domains, HIV-1 encodes two regulatory genes, transactivator (*tat*) and regulator of viral expression (*rev*), and four accessory genes, viral infectivity (*vif*), viral protein R (*vpr*), viral protein U (*vpu*) and negative-regulation factor (*nef*) [45,66]. Many genes overlap in different reading frames so that many proteins can be encoded in a small genome.

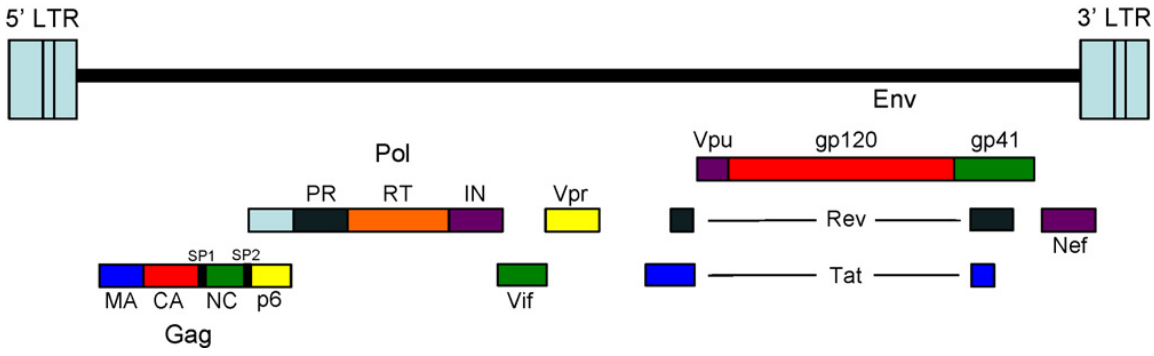


Figure 4: Organization of the HIV-1 genome [1]. This schematic representation shows the gene products encoded by HIV-1. There are three major genes – *gag*, *pol* and *env* – and six smaller genes – *tat*, *rev*, *vif*, *vpr*, *vpu* and *nef*.

1.5.2 Structure of HIV-1

HIV-1 is an enveloped retrovirus where its membrane is a lipid bilayer acquired from the host cell as the newly made virion buds out. It is spherical in shape and measures approximately 130 nm in diameter (Figure 5) [48]. The viral Env proteins are embedded throughout the membrane and they form spikes on the surface. gp120 consists of the cap and is found at the surface; whereas gp40/41 consists of the stem and is protruding through the membrane. Three molecules of Env protein associate non-covalently via their gp40/41 portion to form one glycoprotein spike. MA is found underneath the viral envelope. The viral core is conical in shape and is made up of CA proteins. Within the core are enclosed two copies of the viral genomic RNA which are protected by NC proteins. In addition, PR, RT and IN are enclosed and are necessary for the maturation and the early steps in the next replication cycle.

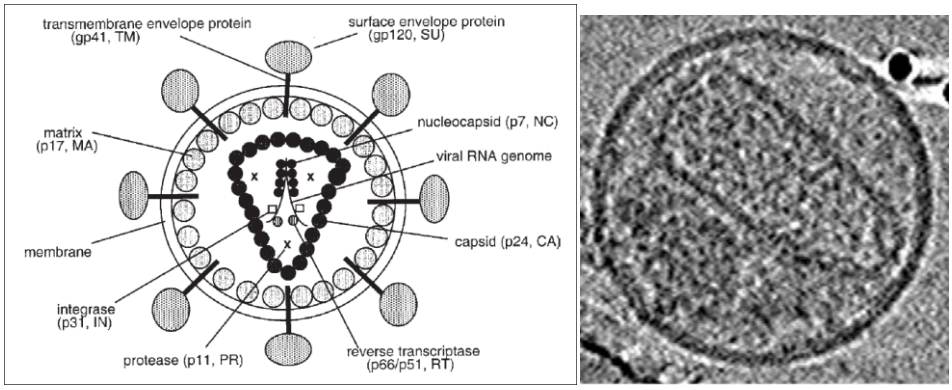


Figure 5: Structure of a mature HIV-1 particle. Schematic representation [45] (left) and electron cryotomography derived picture [48] (right).

1.5.3 HIV-1 Replication cycle

The HIV-1 replication cycle proceeds in a series of events that can be divided into an early phase and a late phase. The early phase comprises of the entry step until viral DNA integration into the host cell genome. The late phase ranges from transcription of the viral DNA to maturation of the virion (Figure 6). Although not mentioned in the figure below, the HIV-1 replication cycle is highly dependent on cellular factors.

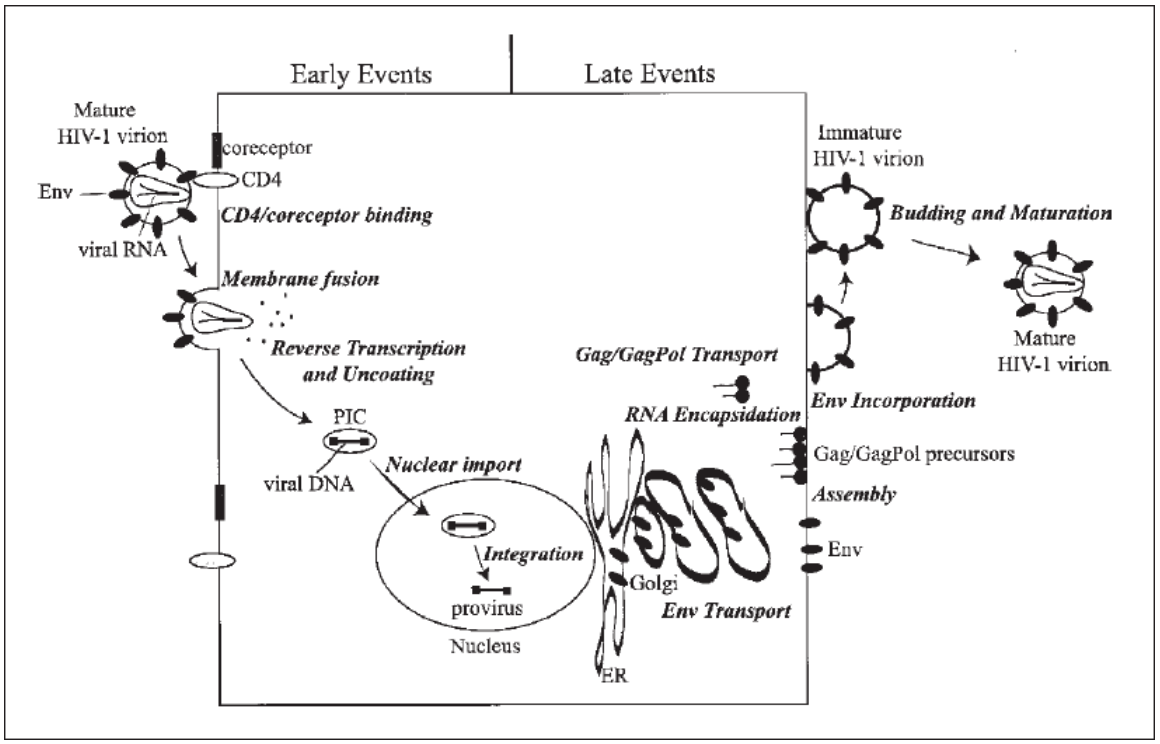


Figure 6: Schematic representation of the HIV-1 replication cycle [45]. The replication cycle is divided into an early phase and a late phase. The main events are highlighted in the figure (refer to text for details).

1.5.3.1 Viral entry

Replication begins with viral attachment followed by entry into the target cell, which are CD4 expressing cells such as CD4 T-cells, macrophages and dendritic cells. First, the virus attaches to the cell when gp120 binds to the CD4 receptor with high-affinity. Then, gp120 undergoes a conformational change to expose the binding sites which would interact with the coreceptor [10]. Two coreceptors, CCR5 and CXCR4, are mainly used by HIV-1 [34,40]. Virus strains using CCR5 are named R5 tropic, those using CXCR4 are X4 tropic and those utilizing both coreceptors are termed R5X4 tropic or dual-tropic [45]. The coreceptor usage determines which cell type the virus can infect. T-cell lines typically express CXCR4, primary T-lymphocytes express both CXCR4 and CCR5, and macrophages express CCR5 [10,45]. HIV-1 mainly uses CCR5 or CXCR4 as coreceptors but other chemokine receptors, such as CCR3, may also act as coreceptors [21]. The V3 loop of gp120 is mainly responsible for determining coreceptor usage [90]. Coreceptor binding triggers another conformational change in both gp120 and gp40/41 so that the fusion peptide within gp40/41 is exposed to trigger fusion between the viral membrane and the target cell plasma membrane [10]. In addition, there are evidences suggesting that HIV-1 may first enter the cell via macropinocytosis [79] and pH-independent endocytosis [86] followed by fusion with macropinosomes and endosomes respectively.

The fusion event delivers the viral core into the cytoplasm of the cell. The core disassembles and is rearranged to form the reverse transcription complex (RTC) which is responsible for reverse transcribing single-stranded viral RNA into double-stranded DNA [126]. Details of the rearrangement process and the viral and cellular factors involved

remain unclear. Once reverse transcription has been completed, the complex is then known as the pre-integration complex (PIC) [125]. As the HIV-1 replication cycle requires integration of its viral DNA into the host cell DNA, the RTC/PIC needs to be transported to the nucleus. There is evidence that the RTC/PIC utilizes actin filaments and microtubules for transport to the nuclear periphery [5].

1.5.3.2 Reverse transcription

Reverse transcription consists of transcribing the single-stranded viral RNA into double-stranded DNA using the RT enzyme. HIV-1 RT is composed of two subunits, p66 and p51. The p66 subunit contains two active sites, one is the DNA polymerase, which can copy either a DNA or a RNA template, and the other one is RNaseH, which degrades RNA only when found in a RNA/DNA duplex [106].

The process of reverse transcription occurs in a sequence of multiple steps (Figure 7). Briefly, reverse transcription is initiated with $tRNA_{lys3}$ binding to the primer-binding site (PBS) on the viral RNA [67]. As the negative-strand DNA is being synthesized until the 5' end of the viral RNA, RNaseH degrades the template RNA in parallel. The 5' and 3' ends, marked by an R, of the viral genomic RNA are identical in sequence. Strand transfer occurs where the newly synthesized DNA "jumps" and anneals to the complementary 3' end of the viral RNA. Negative-strand DNA synthesis and RNA degradation resume. The polypurine tract (PPT) serves as primer for the positive-strand DNA synthesis. RNaseH finally degrades the PPT and a second strand transfer occurs. Both DNA strands resume and complete synthesis creating a linear double-stranded DNA with LTRs at both ends [24,45].

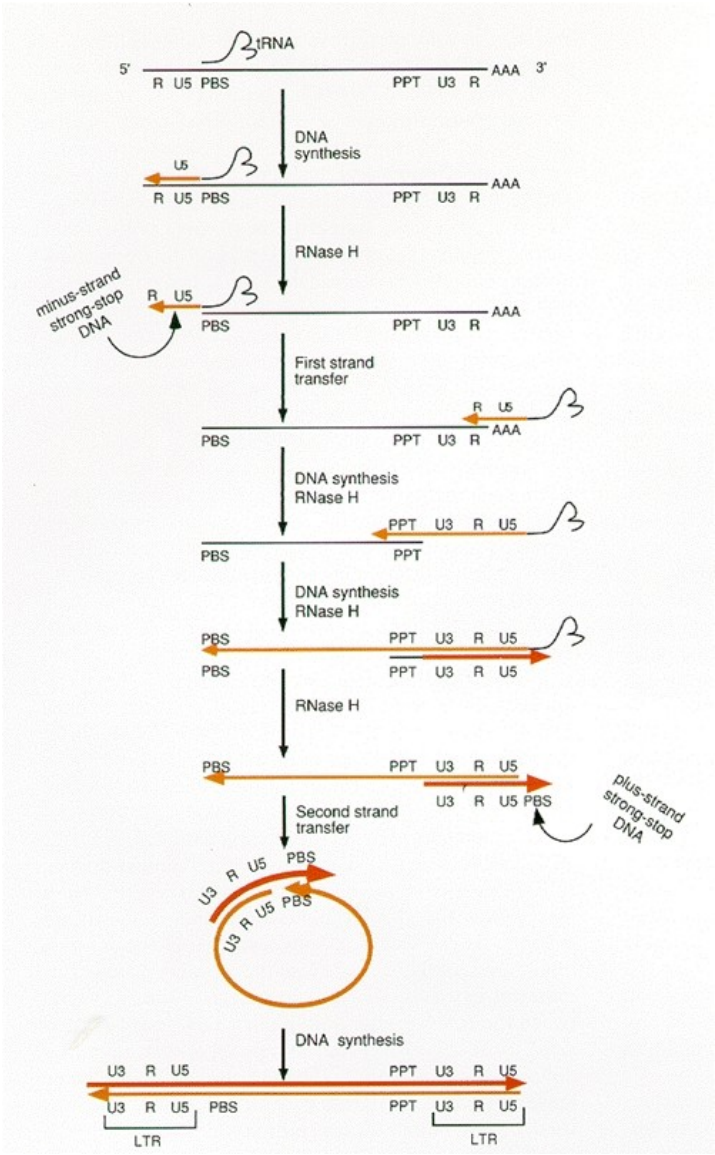


Figure 7: Process of reverse transcription of HIV-1 genome [24]. RNA (black line); negative-strand DNA (light orange); positive-strand DNA (dark orange). Refer to text for details.

HIV-1 replication is error prone as the RT enzyme lacks proof-reading activity. It has been estimated that the mutation rate is 3 x 10^{-5} mutations per base pair per cycle of replication [78]. Consequently, HIV-1 is capable of evading the immune system and drug resistant mutants will eventually develop.

1.5.3.3 Nuclear import and integration

Once reverse transcription has been completed, the PIC is transported to the nucleus via a mechanism that is not well characterized yet [2]. Some retroviruses require cell division to enter the cell nucleus [102]. During each cycle of cell division, the nuclear envelope disassembles temporarily so that exchange between the nucleus and the cytoplasm occurs directly. Because HIV-1 can actively transport its PIC to the nucleus, it can also infect non-dividing cells such as terminally differentiated macrophages [17,128].

Following nuclear transport of the PIC, the IN enzyme stably integrates the viral DNA into the host cell chromosome via a sequence of multiple steps [24,31]. Briefly, IN cleaves off two bases from each 3'-OH end of the viral DNA, a process known as 3'end processing [25]. IN also removes four to six bases from the cellular DNA to give 5'overhangs [25]. The sticky ends from viral DNA are joined to the cleaved ends of cellular DNA, a process known as strand transfer. Cellular DNA repair enzymes fill in the gaps between integrated viral DNA and cellular DNA [16]. Once integrated, the virus is known as the "provirus" and behaves similar to a cellular gene [45]. The site of integration within the cellular DNA is thought to be random although some evidences suggest that integration might happen preferentially within symmetric sequences [62].

1.5.3.4 Transcription and RNA export

The LTR element drives transcription as it harbors binding sites for cellular transcription factors [45]. The basal transcriptional activity from the LTR is very low when cellular factors are solely involved but it is greatly increased in the presence of HIV-1 Tat protein [41]. Tat is synthesized early during basal transcription. Tat functions by first

binding to the transactivation response region (TAR), an RNA stem-loop structure found at the 5' end of all viral RNAs [11]. Cyclin T1 and CDK9 [127], two cellular factors, are recruited by Tat where CDK9 phosphorylates the C-terminal domain of RNA polymerase II [59]. This results in increased processivity of RNA polymerase II and RNA synthesis is increased by at least 100-fold [45].

Transcription from the HIV-1 LTR generates over 40 different RNA species due to alternative splicing of the primary RNA transcript [81]. They are classified into three categories of RNAs: unspliced, partially spliced and multiply spliced. The unspliced RNAs (9 kb) are translated to give rise to $Pr55^{Gag}$ and $Pr55^{GagPol}$ and they function as genomic RNA for progeny virions. The partially spliced RNAs (~4 kb) are translated to give Env, Vif, Vpu and Vpr proteins. The multiply spliced RNAs (~2 kb) are translated to give Rev, Tat and Nef [117]. As intron-containing RNAs are prevented from nuclear export, the virus has developed a mechanism to counteract this in order to export its partially spliced and unspliced RNAs. Indeed, the Rev protein recognizes a highly structured RNA element, termed the Rev responsive element (RRE), found on unspliced and partially spliced RNAs [99]. Rev is expressed early in the cell as it is encoded by the multiply spliced RNA. Rev contains a nuclear localization signal (NLS) that enables it to travel back to the nucleus [77]. When Rev binds to RRE, it recruits CRM1/exportin 1, which is involved in nuclear export, to gain access the cytoplasm [99]. Viral proteins are translated by cellular machineries.

1.5.3.5 Virus assembly, budding and maturation

Structural proteins are derived from the $Pr55^{Gag}$, $Pr160^{GagPol}$ and Env polyproteins. Env is transported through the endoplasmic reticulum and Golgi where it is modified and cleaved by cellular proteases to yield gp120 and gp40/41 before trafficking to the plasma membrane [24]. Following synthesis, $Pr55^{Gag}$ and $Pr160^{GagPol}$ polyproteins are transported by cellular vesicular trafficking machinery to the plasma membrane for assembly [22]. The MA domain of $Pr55^{Gag}$ is modified with addition of myristic acid, which is essential for binding to the plasma membrane [54]. Equally important is a stretch of positively charged basic residues on MA which is attracted to negatively charged acidic phospholipids found on the plasma membrane [92]. It is believed that MA first binds to the plasma membrane via its basic residues before the myristoyl group is inserted because binding causes a conformational change to expose the myristic acid [103]. As $Pr55^{Gag}$ molecules begin to accumulate on the plasma membrane, they start to oligomerize through the C-terminal domain of CA, the SP1 peptide and the NC domain [45]. $Pr55^{Gag}$ drives the assembly process and recruits full-length viral genomic RNA along with viral components and several cellular factors. In fact, $Pr55^{Gag}$ alone is able to form virus-like particles in the absence of other viral proteins [51]. Packaging of the viral genomic RNA is achieved through recognition of the packaging signal (Ψ) only found in full-length viral RNA by the NC domain of $Pr55^{Gag}$ [45].

Virus budding is mediated by cellular machineries recruited by the p6 domain of $Pr55^{Gag}$. An amino acid motif composed of proline-threonine-alanine-proline (PTAP) near the N-terminus of p6 interacts with Tsg101, a component of the endosomal sorting complexes required for transport I (ESCRT-I) involved in endosomal protein sorting [82].

This interaction was shown to facilitate viral budding. As the virion buds out from the plasma membrane, the lipid bilayer forms the viral membrane with a layer of $Pr55^{Gag}$ polyproteins associated with the inner membrane [48]. The virion is immature at this point. PR enzyme cleaves $Pr55^{Gag}$ and $Pr160^{GagPol}$ polyproteins into individual proteins, which would reorganize in the virion to produce a mature particle with a condensed conical core [48]. MA remains associated with the inner viral membrane, NC coats the two copies of viral genomic RNA and CA assembles into the conical core (Figure 5).

1.6 Interferon

1.6.1 What are interferons?

Interferons (IFNs) are cytokines that are produced following a viral infection and contribute to blocking viral spread to uninfected cells. Their antiviral properties were discovered in 1957 as they inhibited the replication of influenza virus [65]. In addition to inhibiting viral replication, IFNs possess antiproliferative and immunomodulatory properties [97]. These molecules consist of the first line of defense against pathogen invasion as they are part of the innate immune system. In clinical practice, IFNs are currently used as therapeutics to treat chronic viral infections such as hepatitis C virus (HCV), multiple sclerosis and numerous cancers [12].

Production of IFNs is induced upon recognition of pathogen-associated molecular patterns (PAMPs), which are molecules or motifs uniquely found in pathogens, by pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs). Following a PAMP-PRR interaction, a signaling cascade involving numerous cellular factors is initiated. Depending on which PRR is triggered, there are slight variations in terms of the

cellular factors involved in the signaling pathway [101]. At the end of this signaling cascade, transcription factors are imported to the nucleus where they would bind to the promoter region of the IFN gene, leading to the recruitment of the transcriptional machinery and ultimately to the expression of the IFN gene [101].

Based on the receptor they signal through, three classes of IFN have been identified and named: type I, II and III IFN. Type I and III IFN are induced following a viral infection but not type II IFN. Type I IFN in human comprises of IFNα (13 subtypes), IFNβ, IFNκ, IFNε and IFNω. IFNδ and IFNτ also belong to type I IFN but these are only found in pigs and cattle respectively [97]. Type I IFN binds to a heterodimeric receptor composed of IFNAR1 and IFNAR2 (Figure 8). Among the type I IFN, IFNα and IFNβ possess well-defined antiviral activities while less is known for the other types. In addition, the 13 subtypes of IFNα are encoded by 13 different genes clustered on chromosome 9 along with IFNβ, IFNε and IFNω genes [56]. Type II IFN consists of only one type, IFNγ, and binds to the heterodimeric receptor composed of IFNGR1/IFNGR2. IFNγ is not directly involved in an antiviral response; instead, it plays a role in the regulation of the adaptive immune response [66]. Type III IFN comprises of IFNλ1, IFNλ2 and IFNλ3 and they bind to a receptor composed of IL-10R2 and IFNLR1. The discovery of type III IFN is relatively recent and although they signal through a different receptor complex than type I IFN, they are structurally and functionally similar to type I IFN [124]. It has been suggested that type III IFN is the ancestor of type I IFN and it gave rise to the complex antiviral system found in vertebrates [71].

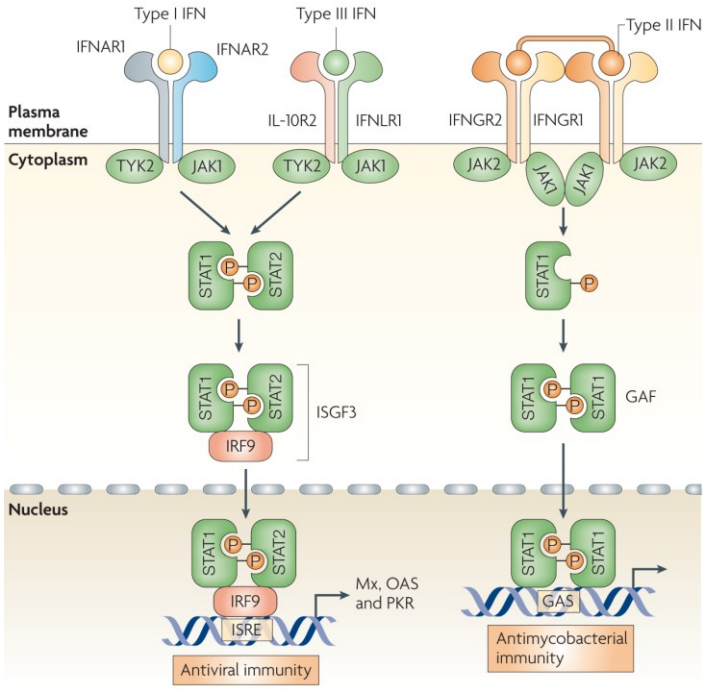


Figure 8: Signaling pathway of interferon [104]. Type I, II and III IFNs bind to their respective receptor complex on the cell surface and initiate a signaling cascade leading to the expression of interferon-stimulated genes, which are the effectors of the antiviral response. Refer to text for details.

1.6.2 Type I IFN elicits its antiviral activity through interferon-stimulated genes

Type I IFN elicits its antiviral activity through a set of genes, known as interferon-stimulated genes (ISGs). Once type I IFN is produced following a viral infection, it binds to its ubiquitously expressed receptor IFNAR1/2. This interaction signals to Janus activated kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) to transphosphorylate the receptor chain leading to the recruitment and phosphorylation of signal transducers and activators of transcription (STATs) (Figure 8). STAT1 and STAT2 form a heterodimer and associate with IFN-regulatory factor 9 (IRF9) to form a complex named IFN-

stimulated gene factor 3 (ISGF3). ISGF3 is translocated to the nucleus where it binds to IFN-stimulated response element (ISRE) found in the promoter region of some ISGs and the transcription of those genes is then initiated. Other pathways to induce ISGs include the formation of STAT homodimers, the use of other STAT proteins, additional transcription factors and kinases [122]. The combination of factors involved appears to be cell-type specific and the biological function resulting from this will differ among different cell types [122]. Thus, a single type of IFN can lead to the induction of different sets of ISGs in different cell types. In addition, the six STAT genes in human (STAT1, STAT2, STAT3, STAT4, STAT5 and STAT6) are IFN-inducible; thus, initial binding of type I IFN to its receptor will induce the expression of factors involved in amplifying the expression of the ISGs [97]. Some STAT dimers bind to another DNA element known as IFN-γ-activated site (GAS) found in the promoter of a number of ISGs [97]. Besides, the promoter region of all ISGs is not identical. Some have the ISRE only, some have the GAS element only, and others have both elements; thus, the regulation in the expression of ISGs is a complex interplay between the signaling molecules and the ISGs themselves. As new information is being discovered, it has become evident that IFN-mediated signaling is more complex than previously thought.

ISGs are the effectors of the IFN response. Binding of type I IFN to its receptor leads to the induction of more than 300 ISGs [32]. Only a few of these ISGs have been studied extensively and shown to exhibit antiviral activities. These include: dsRNA-dependent protein kinase (PKR) [50], 2',5'-oligoadenylate synthetase (OAS) and ribonuclease L (RNase L) pathway [113], IFN-stimulated protein of 15 kDa (ISG15) [70] and myxovirus resistance (Mx) proteins GTPase [80]. In addition to these, other less well

characterized ISGs, such as apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like (APOBEC) [76], ISG20 [30], members of the tripartite-motif-containing (TRIM) proteins [120], adenosine deaminase acting on RNA (ADAR1) [119] and bone marrow stromal cell antigen 2 (BST-2) [118], exhibit equally important antiviral functions.

1.6.3 Viral evasion strategies against IFN

Over time, viruses have evolved many mechanisms to evade the IFN system. There are five main strategies: 1) interfering with host-cell gene expression and/or protein synthesis; 2) minimizing IFN production by either limiting expression of PAMPs and/or directly blocking the signaling pathway to IFN expression; 3) blocking the IFN signaling pathway that leads to the expression of ISGs; 4) interfering with the function of specific ISGs via a viral antagonist; and 5) having a replication strategy that is insensitive to the antiviral activities of IFN [101]. As example, the nonstructural 1 (NS1) protein of influenza A virus interferes with the expression of cellular genes by inhibiting the processing and export of cellular mRNAs [69,107]. The nonstructural 3/4A (NS3/4A) protein of HCV blocks the production of IFN by cleaving Cardif, which is an adaptor protein in the retinoic acid inducible gene I (RIG-I) signaling pathway involved in IFN production [85]. The V protein of simian virus 5 prevents IFN signaling by targeting STAT1 for ubiquitin-mediated proteasome degradation [33]. The Vpu protein of HIV-1counteracts the interferon-inducible BST-2 protein, which is a restriction factor preventing virus release from the cell surface, by an yet undetermined mechanism [89,123]. In addition to these stratagems, certain viruses may possess more than one viral antagonist and/or use more than one strategy in order to evade the IFN system more efficiently.

1.6.4 Effects of interferon on HIV-1 replication

Soon after the discovery of HIV-1, it has been shown that type I IFN severely inhibits HIV-1 replication in cultured cells. This effect is observed in both primary cells, such as peripheral blood mononuclear cells (PBMCs) and macrophages, as well as in monocytes and T cell lines [61,68,74,98]. The inhibitory effect occurs at multiple steps of the viral life cycle; thus, suggesting that several ISGs may be involved. In fact, early studies have shown that type I IFN inhibits virus fusion with host cell membrane [129], viral reverse transcription [110], viral gene expression [111] and virus release from the cell surface [89,123]. Many ISGs have been shown to be implicated in restricting HIV-1 replication. As example, the 2',5'-OAS activates RNase L to degrade HIV-1 RNA as well as cellular RNA to cause cell death [75]. The TAR element found on HIV-1 RNA dimerizes to activate PKR so that viral protein synthesis is inhibited [9,57]. ISG15 prevents cellular cofactors from binding to the HIV-1 budding complex so that viral release is inhibited [91,96]. ISG20 inhibits HIV-1 replication by a yet unknown mechanism [36]. Trim22 may disrupt the proper trafficking of $Pr55^{Gag}$ to the plasma membrane [7]. APOBEC3G and APOBEC3F are cytidine deaminases that are encapsidated by budding particles and they cause hypermutations during viral reverse transcription [20,52]. BST-2/tetherin inhibits viral release by tethering the particles on the cell surface [89,123].

While the IFN system seems to be a powerful defense mechanism in blocking HIV-1 replication, the virus has developed numerous ways to circumvent this. Some of the HIV-1 proteins are able to counteract specific IFN-induced factors. Vpu antagonizes BST-2 by downmodulating its cell surface expression by a yet undetermined mechanism [108]. Vif counteracts APOBEC3G/F by possibly two different mechanisms:

1) Vif recognizes APOBEC3G/F and sends them for degradation by the proteasome; and 2) a degradation-independent mechanism has also been proposed [52]. In addition, HIV-1 infection induces the expression of an RNase L inhibitor to block the 2',5'-OAS/RNase L pathway [83].

1.6.5 Interferon production in vivo

The main producers of type I and type III IFNs during a viral infection, including HIV-1 infection, are plasmacytoid dendritic cells (pDCs) [42,43]. Many other cell types are capable of producing type I IFN during a viral infection; however, pDCs are the most potent ones as they can produce up to 1,000 fold more IFN than the other cell types [112]. pDCs are bone marrow-derived cells that make up a small population of cells found in the peripheral blood and lymphoid organs [115]. They detect viral RNA and DNA via their endosomal TLR7 and TLR9 receptors respectively [42].

It has been observed that the number and the function of circulating pDCs are decreased in HIV-1 infected individuals [39]. The mechanism for this remains unclear. It has been suggested that apoptosis of pDCs is increased and they are replaced by less mature pDCs [43]. Although there is evidence for associating the loss of circulating pDCs with HIV-1 disease progression [43], the benefit for the production of type I IFN in HIV-1 infected individuals remains a controversial topic. Indeed, there is evidence suggesting that the chronic production of type I IFN plays an important role in immune activation, which is detrimental in HIV-1 infection, and increases the expression of death receptors which would lead to increased apoptosis in both infected and uninfected cells [43].

1.7 Interferon-induced transmembrane (IFITM) proteins

Interferon-induced transmembrane (IFITM) proteins, as the name implies, are IFN inducible by both type I and type II [46]. Four members belong to this family: IFITM1 (9-27), IFITM2 (1-8D), IFITM3 (1-8U) and IFITM5 (Bril). IFITM1, 2 and 3 are ubiquitously expressed and they play a role in immune cell signaling [13], cell adhesion [37], oncogenesis [15,38] and germ cell homing [116]. IFITM5 is expressed in osteoblasts and plays a role in mineralization [87]. Due to IFITM5 specific expression pattern, it will not be considered any further in this study. As IFITM proteins comprise of two transmembrane domains, they are localized on membranes such as the plasma membrane, exosomes and the endoplasmic reticulum [14,15]. It is possible that they are also localized on other types of intracellular vesicles such as lysosomes and endosomes.

IFITM proteins belong to the protein superfamily CD225, which is characterized by two transmembrane domains and a highly conserved intracellular loop (pfam04505, CD225). IFITM1, 2, and 3 are small proteins of 13.9, 14.5 and 14.6 kDa respectively [73]. They are highly homologous at the amino acid level such that there is 68% homology between IFITM1 and IFITM2, 70% between IFITM1 and IFITM3, and 91% between IFITM2 and IFITM3 (Figure 9). IFITM1 differs from IFITM2 and 3 at both the N- and C-terminus regions. Compared to IFITM3, IFITM1 lacks the first 21 amino acids and its last 27 residues are different in terms of sequence and it is 13 residues longer in this region.

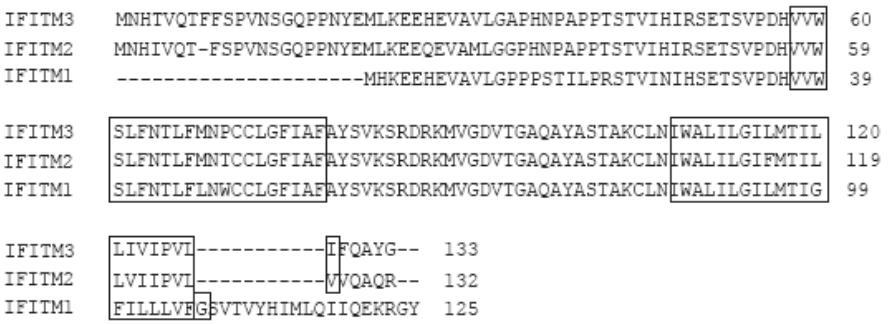


Figure 9: Amino acid sequence alignment of the IFITM proteins. The transmembrane domains are highlighted in boxes.

The promoter regions of the IFITM genes are different. IFITM2 and 3 each have two ISRE elements separated by only three base pairs, whereas IFITM1 has only one ISRE element [73]. Also, IFITM1 lacks a TATA box but has two CCAAT boxes, whereas IFITM2 and 3 lack both TATA and CCAAT boxes [73]. Although they are all IFN-inducible, IFITM2 is induced at a lesser extent due to a point mutation in both of its ISREs [73].

Recently, a group has reported that the IFITM proteins are involved in restricting an early replication step of influenza A virus, West Nile virus and Dengue virus [14]. In addition, by using virus-like particles that are pseudotyped with the envelope proteins of certain viruses, they showed that the IFITM proteins may also restrict Omsk hemorrhagic fever virus and yellow fever virus. However, when they depleted IFITM3 in HeLa-CD4 cells with siRNA oligos, no effect was observed on HIV-1 infection. Which step of the viral life cycle the IFITM proteins interfere with and the underlying mechanism remain unknown.

Project objectives

With the advent of potent ART to treat HIV-1 infection, the field of IFN and its antiviral effects on HIV-1 replication have been cast aside. However, recent discoveries have shown that many cellular restriction factors (APOBEC3G/F, TRIM5α and BST-2) are in fact IFN-inducible. Thus, the main objective of this project is to revisit this theme with the purpose of identifying novel IFN-inducible anti-viral factors.

This project was conducted in multiple steps. First, the cell line to work with had to be selected on the basis of its ability to block HIV-1 replication after IFN treatment. Second, the genes that were upregulated in that cell line following IFN treatment were determined. Third, those genes were individually screened for their effect on HIV-1 replication. Finally, the candidate genes were investigated further to determine at which step they exerted their antiviral activity. Due to time constraint, only one candidate gene was studied in-depth.

Chapter 2-Materials and Methods

2.1 Antibodies

The primary antibodies used for immunoblotting are: mouse monoclonal anti-HIV-1 p24 (Fitzgerald) (1:5000), mouse monoclonal anti-Flag (Sigma-Aldrich) (1:5000), mouse monoclonal anti-β-Tubulin (Santa Cruz Biotechnology) (1:5000), rabbit polyclonal anti-IFITM1 (Proteintech Group) (1:1000), rabbit polyclonal anti-IFITM2 (Proteintech Group) (1:1000), and rabbit polyclonal anti-IFITM3 (Proteintech Group) (1:1000). The secondary antibodies used for immunoblotting are: horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare) (1:5000) and horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare) (1:5000).

Pacific Blue™ (PB)-conjugated mouse monoclonal anti-human CD4 antibody (BD Biosciences) (1:10) was used to stain SupT1 cells in order to set up the parameters of the flow cytometer for the virion fusion assay. R-Phycoerythrin (PE)-conjugated mouse monoclonal anti-human CD4 (BD Biosciences) (1:100) and PE-conjugated mouse monoclonal anti-human CXCR4 (BD Biosciences) (1:200) were used for cell surface immunostaining of SupT1 cells.

2.2 Cell lines and culture conditions

SupT1 cells were maintained in RPMI media 1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 1% Penicillin/Streptomycin (P/S) (Invitrogen), and 1% L-glutamine (Invitrogen). SupT1 cell lines stably expressing shRNAs were maintained in RPMI media 1640 supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 2 μg/mL puromycin (Sigma-Aldrich). Tetracycline/doxycycline

inducible IFITM SupT1 cell lines were maintained in RPMI media 1640 supplemented with 10% Tet system approved FBS (Clontech), 1% P/S, 1% L-glutamine, 2 μg/mL puromycin, and 1 mg/mL G418 sulfate (Invitrogen). Cord blood mononuclear cells (CBMCs) were obtained from Maureen Oliveira (Dr. Mark A. Wainberg's lab). They were maintained in RPMI media 1640 supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 20 U/mL human interleukin-2 (IL-2) (Roche). TZM-bl reporter cells, human embryonic kidney (HEK293T) cells, and GP2-293 packaging cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 10% FBS and 1% P/S. All cells were grown at 37°C in a 5% CO_2 incubator.

2.3 Transfections

Transfections were done with Lipofectamine 2000 (Invitrogen) using Opti-Modified Eagle Medium (opti-MEM) (Invitrogen) according to the manufacturer's instructions. All transfections were done in media without antibiotics and the media was changed 4 to 6 hr following transfection.

2.4 Reverse Transcriptase (RT) assay

To measure the viral RT activity, 10 μl of cultured supernatants were added to 40 μl of reverse transcriptase reaction cocktail [2 M Tris HCl (pH 7.9), 0.2 M $MgCl_2$, 2 M KCl, 20 mM EGTA, 2% Triton X-100, 80% ethylene glycol, 0.2 M DTT, 12 mM GSH, poly(rA)-oligo(dT) (10 U/mL) (Midland Certified Reagent Company), and [^{3}H]dTTP (2.5 μCi/μl) (PerkinElmer)] in 96-well plates. The reaction mix was incubated at 37°C for 3 hr. The reaction was stopped with 150 μl of cold 10% trichloroacetic acid (TCA) and precipitated for 30 minutes at 4°C. MultiScreen glass fiber filter plates (Millipore) were

pre-wet with cold 10% TCA then vaccum-drained using the MultiScreen Manifold (Millipore). The precipitated reaction was spotted on glass fiber filters then washed twice with cold 10% TCA and once with cold 95% ethanol. Filters were transferred into scintillation vials and 3 mL of liquid scintillation cocktail (MP Biomedicals) was added. β-emission was measured using a liquid scintillation counter (Wallac 1410; PerkinElmer) and the results are expressed as counts per minute (CPM).

2.5 Luciferase assay

To assess luciferase activity, TZM-bl cells were lysed in 100 µl 1X passive lysis buffer (Promega) and 10 µl of lysates were added to 50 µl of Luciferase substrate (Promega). Luminescence was measured by a GLOMAX 20/20 luminometer (Promega). The results are expressed as relative light units (RLU).

2.6 Western blot

SupT1 cells and CBMCs were lysed 10 minutes on ice in CytoBuster™ protein extraction reagent (Novagen) and TZM-bl reporter cells were lysed in 1X passive lysis buffer. Cell lysates were ran on SDS-polyacrylamide gels and transferred on PVDF membranes (Roche). Membranes were blocked in a milk-PBST solution (5% milk and 0.05% tween-20 in phosphate buffered saline) then incubated with the primary antibody followed by the secondary antibody. The signals were visualized by applying enhanced chemiluminescence (ECL) substrate (PerkinElmer).

2.7 Preparation of virus stocks

HIV-1 (BH10 isolate) and NLEY1-IRES viruses were generated by transfection of HEK293T cells with plasmids encoding the proviral DNA. The transfection was done in 10 cm plates with 2.0 μg of proviral DNA and 2.0 μg of pcDNA3.1 per plate. After 48 hr, the cultured supernatant was harvested and cleared of cell debris by centrifugation [3,000 rpm, 15 minutes, 4°C]. The supernatant was then divided into aliquots and stored at -80°C.

2.8 Interferon treatment of SupT1 cells and CBMCs

Ten million SupT1 cells or CBMCs were treated with different concentrations of IFNα2b (0, 100, and 1,000 U/mL) (provided by the Jewish General Hospital) in 10 mL of media in 50 mL tissue culture flasks for 16 hr. The cells were then infected with vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped HIV-1 (BH10 isolate). Forty hours after infection, the cells were harvested for Western blot analyses and the cultured supernatants were harvested for reverse transcriptase assay and luciferase assay.

2.9 Microarray analysis

Ten million SupT1 cells were treated with or without IFNα2b (1,000 U/mL) in 5 mL of media for 8 hr or 16 hr in 6-well plates. RNA was extracted using Trizol reagent (Invitrogen) at 0 hr, 8 hr, and 16 hr according to the Invitrogen protocol. RNA was resuspended in RNase-free water (Ambion). The samples were sent to McGill University and Génome Québec Innovation Centre for microarray analysis using Illumina

Expression BeadChips Technology. The raw microarray results were analyzed and put together in an Excel file by René-Pierre Lorgeoux.

2.10 Screening interferon stimulated genes (ISGs) in SupT1 cells

A set of short hairpin RNA (shRNA) clones were purchased from Sigma-Aldrich (MISSION® *shRNA* Transduction Particles) to deplete 63 ISGs with 4 to 5 shRNAs targeting each gene. The control is a non-targeting shRNA. The shRNA screen was conducted in two steps. First, SupT1 cell lines were created to express a single shRNA per cell line. This was done by infecting $1x10^6$ SupT1 cells with 50 µl of lentiviral particles supplemented with polybrene (5 µg/mL) (Sigma-Aldrich) in 0.5 mL media in 24-well plates. After 24 hr, the cells were fed with 1 mL of fresh media. After 24 hr, the cells were transferred to 6-well plates and maintained in 6 mL of media supplemented with 2 µg/mL puromycin to select cells stably transduced with shRNA. The second step of the screening was to test those stable cell lines. One million cells were treated with IFNα2b (500 U/mL) in 1 mL of media in 24-well plates for 16 hr. The cells were then infected with 20 µl of VSV-G pseudotyped HIV-1 (BH10 isolate). After 40 hr, cultured supernatants were collected and 20 µl were used to infect TZM-bl reporter cells to measure the level of infectious HIV-1 produced.

2.11 Creating doxycycline-inducible IFITM SupT1 cell lines

The Flag-tagged IFITM cDNAs were inserted into the pRetroX-Tight-Pur retroviral vector (Clontech) between the *BamHI* and *EcoRI* restriction sites to create DNA clones under the control of a doxycycline/tetracycline-responsive promoter (done by Wei He). Virus stocks were prepared by transfecting 1 µg of each of these plasmids into GP2-

293 packaging cells with 0.1 μg of VSV-G DNA in 6-well plates. After 48 hr, the cultured supernatants containing virus particles were harvested and cleared of cell debris by centrifugation. Inducible cell lines were created by infecting $3x10^6$ SupT1 cells by spinoculation [1,800 rpm, 45 minutes, room temperature] with 0.25 mL of virus expressing the gene of interest together with 0.25 mL of virus expressing the rtTA activator supplemented with polybrene (5 μg/mL) in 24-well plates. After 2 to 3 hr of incubation at 37°C, 1 mL of media was added. After 48 hr, the cells were transferred to 6-well plates and maintained in 6 mL of media supplemented with puromycin (2 μg/mL) and G418 sulfate (1 mg/mL) to select for cells stably transduced by both types of viruses. To verify the expression of IFITM proteins, the cell lines were treated with doxycycline (0.5 μg/mL) for 16 hr and examined by Western blot analyses using an anti-Flag antibody.

2.12 HIV-1 NLEY1-IRES virus one-round infection

One million IFITM SupT1 cells were treated with doxycycline (0.5 μg/mL) in 1 mL of media for 16 hr in 12-well plates. The cells were infected with 0.3 mL of NLEY1-IRES virus by spinoculation [1,800 rpm, 45 minutes, room temperature] supplemented with polybrene (5 μg/mL) in 24-well plates. The NLEY1-IRES virus is a HIV-1 reporter virus which encodes the yellow fluorescence protein (YFP) gene inserted between *Env* and *nef* [72]. YFP is expressed upon viral protein expression. Then the cells were incubated at 37°C for 2 to 3 hr before 1 mL of media was added. After 48 hr, the cells were washed with cold phosphate buffered saline with 2% FBS and fixed in 0.5 mL of 1% paraformaldehyde. Expression of YFP in cells was verified by flow cytometry with a 2-laser FACSCalibur (BD Biosciences) using a laser which generates light at 488 nm

for excitation. Data was acquired and analyzed with CellQuest Pro software (BD Biosciences).

Alternatively, $1x10^6$ IFITM SupT1 cells were first infected with 0.3 mL of NLEY1-IRES virus by spinoculation [same conditions as above] then incubated at 37°C for 2 hr. Unbound viral particles were washed off with media. The cells were then treated with doxycycline (0.5 μg/mL) in 1 mL of media for 48 hr in 24-well plates. The cells were washed and fixed and expression of YFP in cells was verified by flow cytometry with a 2-laser FACSCalibur [same conditions as above].

2.13 Fluorescence Resonance Energy Transfer (FRET)-based virion fusion assay

Viral particles containing β–lactamase-Vpr (BlaM-Vpr) chimera proteins were produced by transfecting HEK293T cells in 10 cm plates with 3 μg of BH10 proviral DNA, 1 μg of pCMV-BlaM-Vpr DNA (obtained from Dr. Warner C. Greene) and 0.5 μg of pcDNA3.1. After 48 hr, the cultured supernatant was harvested and cleared of cell debris. Viruses were concentrated by ultracentrifugation [35,000 rpm, 1 hr, 4°C] in Ultra-Clear centrifuge tubes (Beckman Coulter) placed in a SW41 TI rotor (Beckman Coulter). The viral pellet was resuspended in 1 mL of RPMI 1640 media. Aliquots of the concentrated virus were made and stored at -80°C.

One million IFITM SupT1 cells were treated with doxycycline (0.5 μg/mL) in 1 mL of media for 16 hr. The virion fusion assay was conducted in three steps. First, the cells were infected with 100 μL of concentrated BlaM-Vpr virions by spinoculation [1,800 rpm, 2 hr, 4°C] supplemented with 200 μl of media and polybrene (5 μg/mL) in

24-well plates. The fusion event was allowed to happen by incubation at 37°C for 2 to 3 hr. Then, the cells were washed with CO_2-independent medium (Invitrogen) and loaded with CCF2-AM substrate (Invitrogen) by incubating the cells with 100 µl of loading solution for 1 hr at room temperature in the dark. The latter was prepared by mixing 2 µL of CCF2-AM (1 mM) with 8 µl of 0.1% acetic acid containing 100 mg/mL Pluronic-F127 surfactant (solution B provided by Invitrogen with the CCF2-AM loading kit) and 1 mL of CO_2-independent medium. Finally, the cells were washed with 200 µl of development media and the β–lactamase enzymatic reaction was developed in 200 µl of development media for 16 hr at room temperature in the dark. The latter was prepared by mixing 10 µl of probenecid (250 mM) with 1 mL of CO_2-independent medium and 100 µl of Tet system approved FBS. After 16 hr, the cells were washed once with cold phosphate buffered saline with 2% FBS and fixed in 0.5 mL of 1% paraformaldehyde. Upon viral fusion, BlaM-Vpr would be transferred into target cells and it would enzymatically cleave CCF2-AM, a fluorescent substrate of β-lactamase, leading to a change in emission wavelength (Figure 10). The change from green (520 nm) to blue (447 nm) was analyzed by flow cytometry with a 3-laser LSR II (BD Biosciences) using a laser which generates light at 405 nm for excitation. Data was acquired and analyzed with FACSDiva software (BD Biosciences).

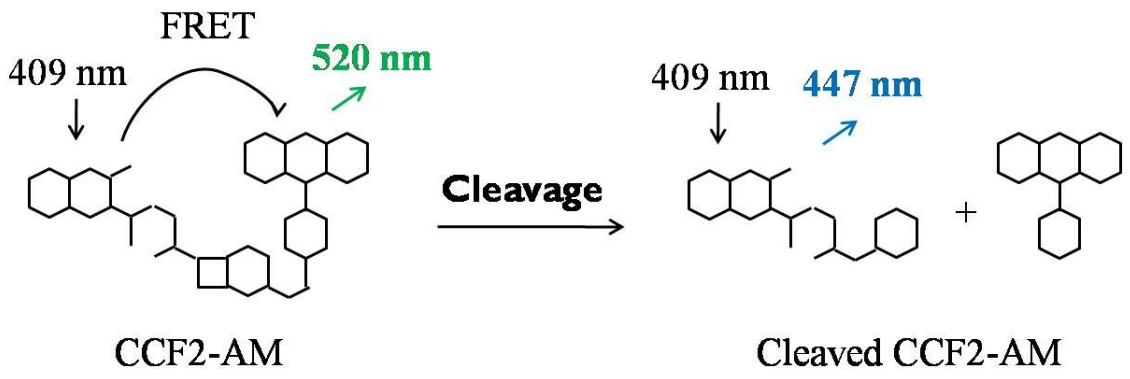


Figure 10: CCF2-AM changes emission wavelength when cleaved. Excitation of the coumarin moiety at 409 nm causes a FRET reaction to the fluorescein moiety, producing a green emission (520 nm). If cleaved, FRET is blocked, and excitation at 409 nm produces a blue emission (447 nm). Adapted from Cavrois M. et al. 2004 [18].

2.14 Depletion of IFITM proteins in TZM-bl

Short interfering RNA (siRNA) purchased from Ambion were used to target IFITM1 (siRNA ID #: s16192, s16193, s228411), IFITM2 (siRNA ID #: s20771, s230492, s230493), and IFITM3 (siRNA ID #: s195033, s195034, s195035) mRNA. TZM-bl cells were seeded in 24-well plates one day before siRNA transfection. When IFITM proteins were depleted individually, 20 pmol of siRNA was transfected. When IFITM proteins were simultaneously depleted, 10 pmol of each siRNA was transfected. Two combinations were used: 1) s16192/ s20771/ s195035 and 2) s16192/ s230492/ s195035. Two siRNA transfections were performed with 24 hr apart. One day after the second transfection, the cells were infected with 20 µl of HIV-1 (BH10 isolate). After 2 hr, unbound viral particles were washed off with media and the cells were incubated for 48 hr. Then, luciferase activity in TZM-bl cells was measured. Knockdown efficiency

was verified by Western blot analyses using anti-IFITM1, anti-IFITM2, and IFITM3 antibodies.

Chapter 3-Results

3.1 Interferon-treated SupT1 cells and cord blood mononuclear cells (CBMCs) are resistant to HIV-1 infection

In order to identify new ISGs responsible for inhibiting HIV-1 infection in cultured cells, the effect of IFNα2b on HIV-1 production was verified in different cell lines and CBMCs. Cells were first treated with different concentrations of IFNα2b (0, 100, 1,000 U/mL) for 16 hr before being infected with VSV-G pseudotyped HIV-1. Forty hours following infection, cells were lysed and verified for $Pr55^{Gag}$ proteins expression by Western blot analyses using an antibody against HIV-1 p24 (Figure 11, top panel). Expression of viral $Pr55^{Gag}$ proteins decreased dramatically in SupT1 cells and CBMCs as the concentration of interferon used to treat cells increased. Also, viral RT activity and level of infectious HIV-1 particles were assessed in the cultured supernatants as measures of viral production (Figure 11, second and third panels respectively). Both assays showed a dramatic decrease in viral production where a 10 to 100-fold decrease in viral RT activity and in levels of infectious particles were observed.

Among the cell lines tested, IFN-treated SupT1 cells showed the strongest inhibition to HIV-1 infection and was comparable to the degree of inhibition observed in CBMCs.

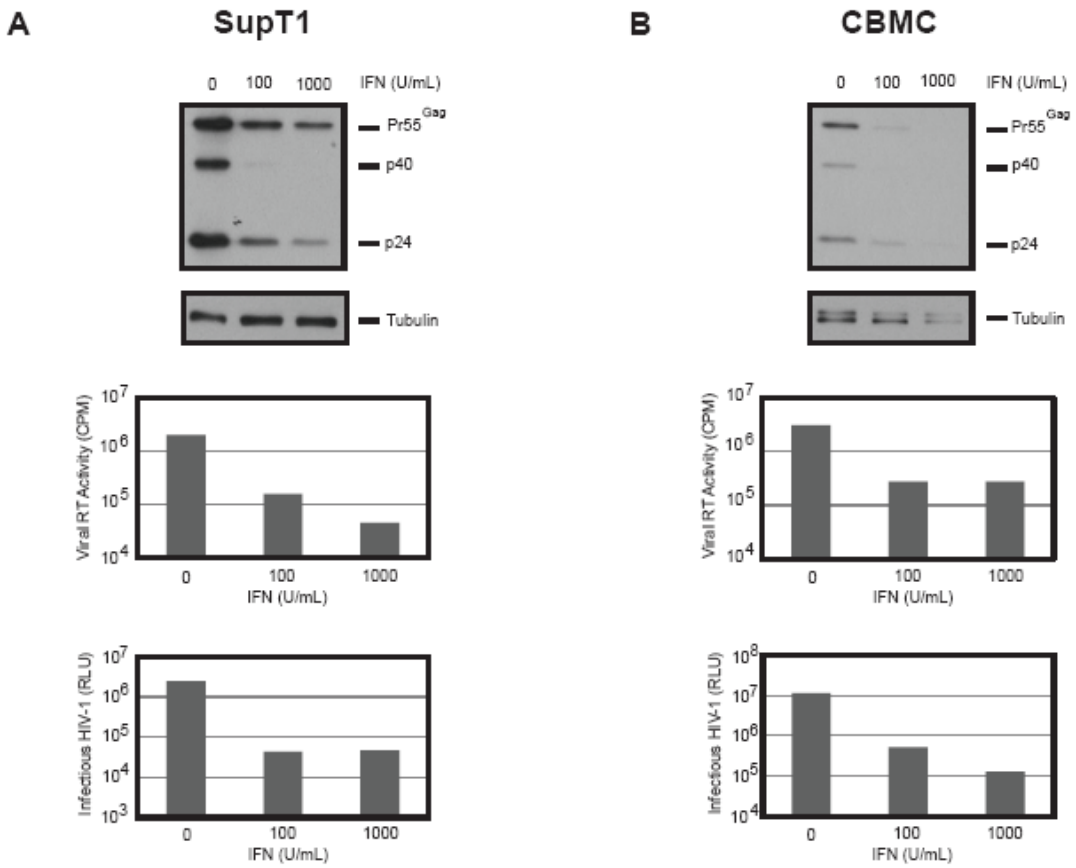


Figure 11: Interferon inhibits HIV-1 infection in SupT1 cells (A) and CBMCs (B). Cells were treated with increasing concentrations of IFNα2b (0, 100, and 1,000 U/mL) for 16 hr before being infected with VSV-G pseudotyped HIV-1. Forty hours following infection, expression of $Pr55^{Gag}$ proteins in whole cell lysates was verified by Western blot analyses using an anti-p24 antibody (top panel). Tubulin was used as the protein loading control. Viral RT activity in the supernatant was measured by RT assay (middle panel). The level of infectious HIV-1 virions released in the supernatant was assessed by infecting TZM-bl reporter cells with 20 µl of supernatant and by measuring the luciferase activity 48 hr later (bottom panel). The results shown are representative of two independent experiments.

3.2 Identification of genes upregulated by IFN-treatment in SupT1 cells using microarray analysis

Since IFN mediates its effect through a set of ISGs, the list of genes upregulated in IFNα2b-treated SupT1 cells was established by microarray analysis. It was determined that the mRNA expression of 94 genes were upregulated by more than 2-fold after 8 hr and 16 hr of treatment (Table 1).

Gene name	Fold of increase (8 hr)	Fold of increase (16 hr)	Tested by MISSION® shRNA
MX1	115.6	141.9	X
IFI44L	71.7	67.4	
EPSTI1	60.8	38.0	X
IFIT1	51.2	26.8	X
IFIT2	49.9	14.3	X
IFIT3	46.0	26.5	X
IFI44	39.4	43.2	X
PRIC285	34.6	9.5	X
MT2A	29.1	10.1	X
IFI6	29.0	25.6	X
HERC5	20.3	8.6	X
MT1A	18.2	6.6	
IFI35	18.1	9.2	X
STAT1	16.0	11.1	X
IFIH1 (MDA5)	15.9	7.4	X
IFITM1	13.5	7.1	X
RSAD2	13.0	5.7	X
ISG15	12.4	10.5	X
HERC6	12.3	9.0	X
OAS2	12.2	12.3	X
GBP1	12.2	3.4	X
IRF7	12.1	6.1	X
TAP1	11.7	4.0	X
IFI27	11.3	14.2	X
GBP2	11.2	3.0	X
SAMD9	10.4	5.2	X
BST2	10.4	10.0	X

OAS1	10.3	6.0	X
PLSCR1	10.2	7.0	X
HS.125087	10.0	10.4	
SP110	9.8	3.9	X
DDX60	9.8	10.2	X
ISG20	9.4	5.2	X
PARP9	9.2	9.7	X
EIF2AK2 (PKR)	9.0	6.8	X
SAMD9L	8.9	4.1	
PARP12	8.1	4.1	X
G1P3	8.0	10.4	
OASL	7.9	2.7	X
MAFA	7.9	7.4	
MX2	7.0	3.2	X
TRIM22	7.0	3.3	X
DDX58	6.9	2.5	X
RASGRP3	6.9	2.3	X
ISGF3G (IRF9)	6.8	3.7	X
XAF1	6.4	4.4	X
CDA	6.0	5.2	X
PARP14	6.0	3.4	X
HLA-B	5.6	4.0	
NT5C3	5.6	3.0	X
STAT2	5.5	3.0	X
RARRES3	5.5	4.0	X
LGP2	5.4	3.2	X
LBA1	5.3	2.4	
GZMB	5.1	2.1	X
DTX3L	5.0	3.0	X

PSMB9	4.9	2.6	X
DNAPTP6	4.9	3.0	X
USP18	4.8	2.6	X
RTP4	4.6	3.0	X
NMI	4.5	3.1	X
LY6E	4.4	2.9	X
TDRD7	4.4	2.4	X
LAP3	4.4	2.4	X
KIAA1618	4.4	3.3	X
PARP10	4.3	2.6	X
SP100	4.2	2.5	X
LYSMD2	4.1	2.5	
FLJ11286	4.0	2.6	
IFIT5	4.0	3.1	X
LOC129607	3.9	2.2	
ZNFX1	3.8	2.1	
TRIM25	3.7	2.2	
LGALS3BP	3.6	2.1	
TAP2	3.6	2.0	
PSMB8	3.2	2.4	
UBE2L6	3.2	2.0	X
MYD88	3.1	2.1	X
HS.489254	3.1	2.5	
ADAR	3.0	2.3	X
PSME2	3.0	3.1	
HDHD1A	2.4	2.0	
NME1	2.3	2.5	
CD38	2.3	2.2	
TFRC	2.3	3.2	

SLA	2.2	2.4
NOLC1	2.2	2.2
HLA-H	2.2	2.3
PTPN6	2.2	2.6
FAM98A	2.1	2.2
B2M	2.1	2.2
PSMD1	2.1	2.0
PSMA3	2.1	2.2
RGPD1	2.0	2.9

Table 1: List of ISGs in SupT1 cells

ISGs identified by microarray analysis using Illumina expression BeadChip Technology (McGill University and Génome Québec Innovation Centre). The genes are organized in decreasing order based on the fold of increase in response to IFNα2b after 8 hr. The fold of increase after 16 hr is also shown. Genes marked with an "X" on the right column were targeted by lentiviral-based shRNA and those unmarked were not covered by Sigma.

3.3 Identification of ISGs responsible for inhibiting HIV-1 infection in SupT1 cells

To determine which of those 94 ISGs is or are responsible for the inhibitory effect observed in SupT1 cells, they were depleted individually using lentivirus-based shRNA (MISSION® Sigma). Among those 94 genes, 63 were covered by Sigma-Aldrich's shRNA library with 4 to 5 shRNA clones per gene. SupT1 cells were transduced with lentiviral particles and cells with the shRNA expressing plasmid stably integrated within their DNA were selected with puromycin (2 μg/mL). Once the selection was completed, the screening experiment was performed as illustrated in Figure 12A. The level of infectious HIV-1 produced was used as the measure of virion production. The results for each ISG are summarized and presented by their fold of change in virus production compared to the control cell line in the presence of interferon (Figure 12B).

Among the 63 ISGs tested, the depletion of five ISGs, interferon-induced transmembrane protein 1 (IFITM1), interferon-alpha-inducible protein 44 (IFI44), receptor (chemosensory) transporter protein 4 (RTP4), epithelial stromal interaction 1 (EPSTI1), and tudor domain containing 7 (TDRD7), was found to rescue HIV-1 production by more than 2-fold (Figure 12B). This effect was observed with two or more shRNA clones per gene. The individual depletion of the 58 other ISGs showed an increase or a decrease of less than 2-fold.

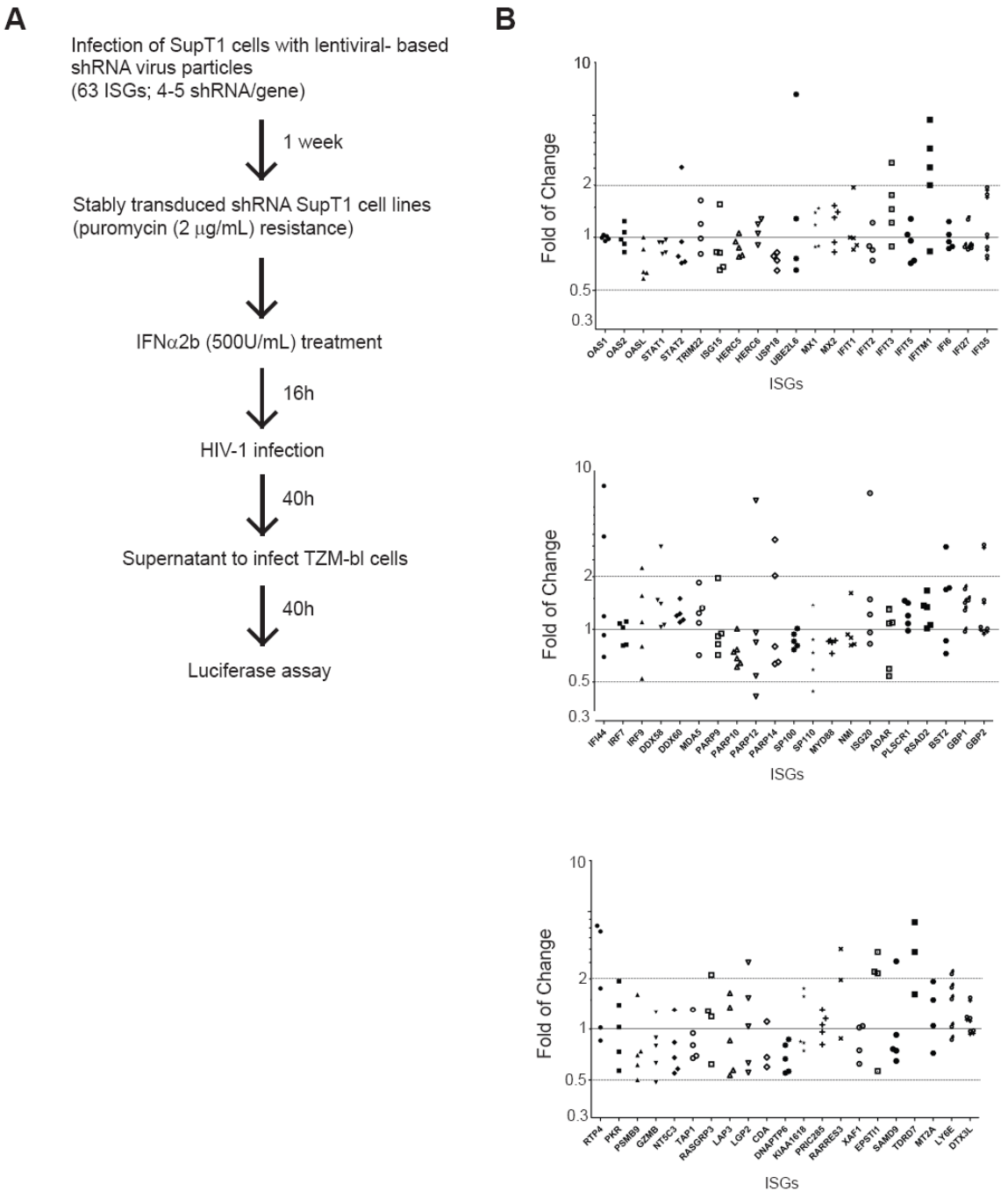


Figure 12: Identification of ISGs responsible for inhibiting HIV-1 infection in SupT1 cells. (A) Flow chart of the screening protocol. (B) Summary of the screening data. The fold of change was calculated by dividing the level of infectious HIV-1 particles produced from the knockdown cell line by the level produced from the control cell line in the presence of IFNα2b. Each data point is the average from a duplicate.

3.4 IFITM proteins inhibit HIV-1 infection

As the depletion of IFITM1 showed a rescue in HIV-1 production by more than 2-fold with 4 out of the 5 shRNA clones, further experiments focused on IFITM1 and its two homologs IFITM2 and IFITM3.

3.4.1 Exogenous expression of IFITM proteins in SupT1 cells inhibits HIV-1 replication

To study a particular ISG outside the context of IFN, further experiments were conducted using tetracycline/doxycycline inducible IFITM SupT1 cell lines.

A time-course infection of IFITM SupT1 cell lines was done. The cells were first treated with doxycycline for 16 hr then infected with wild type HIV-1 (BH10 isolate). Viral production was monitored by sampling the cultured supernatant every other day and by measuring the RT activity present. Cells were passed every four days and kept in media with doxycycline. HIV-1 production was profoundly impaired in SupT1 cells expressing the IFITM proteins exogenously (Figure 13A). At day 16 post-infection, when the viral load peaks in the untreated cells and the doxycycline-treated control cell line, viral production was relatively low or null in the doxycycline-treated IFITM cell lines. This effect was more pronounced in the IFITM1 SupT1 cell line than for the IFITM2 and IFITM3 cell lines. At day 12 post-infection, the expression of $Pr55^{Gag}$ proteins was undetectable in doxycycline-treated IFITM cell lines (Figure 13B).

In addition, it should be noted that the overexpression of IFITM1, 2 and 3 had no effect on cellular proliferation (experiment performed by Qinghua Pan; data not shown).

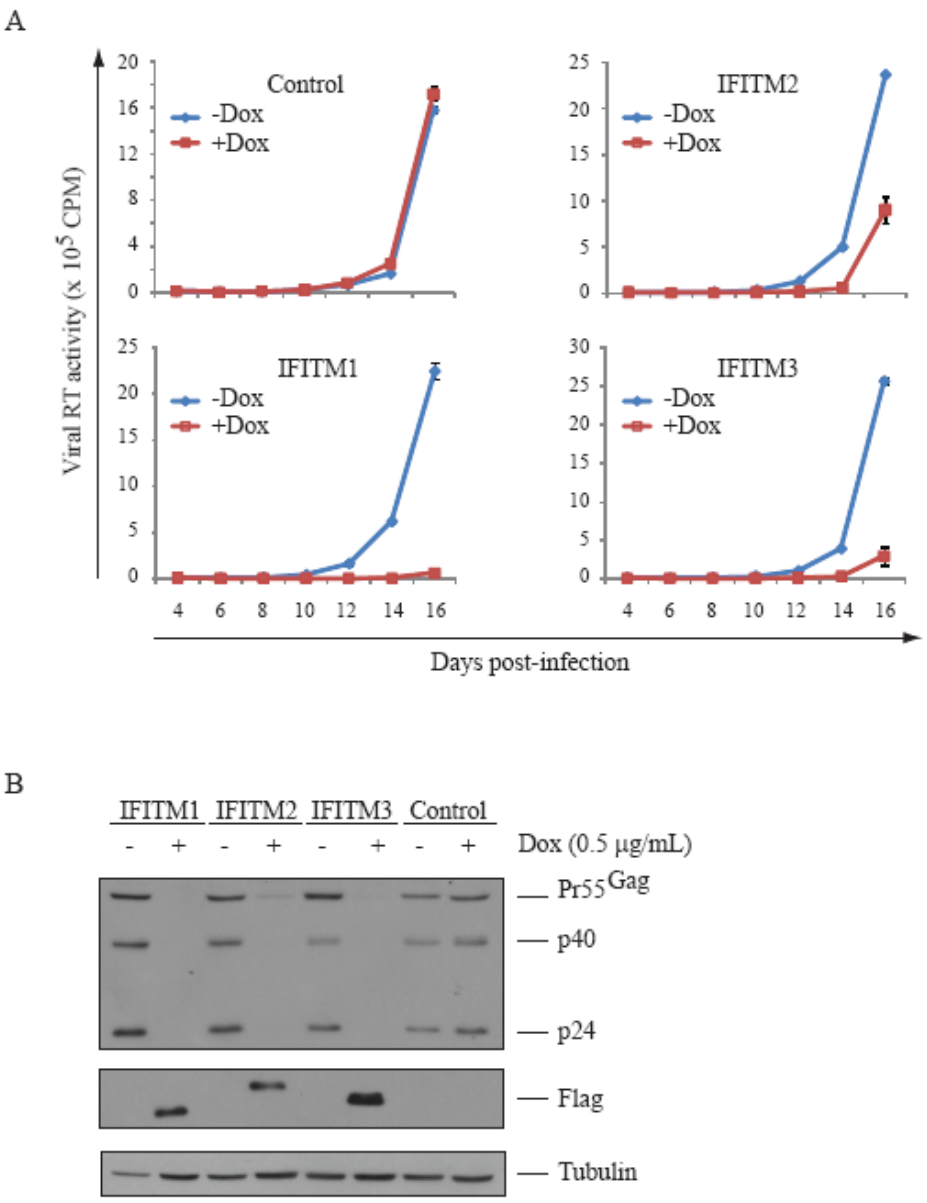


Figure 13: IFITM proteins inhibit HIV-1 infection in a time-course experiment. IFITM SupT1 cell lines were induced with doxycycline (0.5 µg/mL) for 16 hr before HIV-1 infection. (A) The cultured supernatant was sampled every other day until day 16 post-infection and viral RT activity was assessed by RT assay. (B) Cells were harvested on day 12 post-infection and Western blot analyses of cell lysates for $Pr55^{Gag}$ proteins, Flag-IFITM proteins, and tubulin are shown. Tubulin was used as the protein loading control. Dox, doxycycline.

3.4.2 IFITM2 and IFITM3 proteins inhibit an early step in HIV-1 replication cycle

To determine at which step of the viral replication cycle IFITM proteins interfere with, two experiments were conducted by Qinghua Pan to delineate whether they act at an early step or a late step of the life cycle. In the first experiment, IFITM SupT1 cells were treated with doxycycline for 16 hr before being infected. In the second experiment, the cells were infected for 2 hr before doxycycline treatment. In both cases, cells were infected with the NLEY1-IRES reporter virus, a HIV-1 virus that encodes the YFP gene. Expression of YFP in infected cells was assessed by flow cytometry.

When IFITM2 and IFITM3 cell lines were treated with doxycycline before infection, there was a 50% decrease in YFP expressing cells (Figure 14A). However, the percentage of YFP expressing cells remained unchanged when doxycycline was added after infection (Figure 14B). Surprisingly, the percentage of YFP expressing IFITM1 cells showed no decrease regardless whether doxycycline treatment was initiated before or after infection. Thus, IFITM2 and IFITM3 proteins appear to inhibit an early step in HIV-1 replication but where IFITM1 protein inhibits remains unclear.

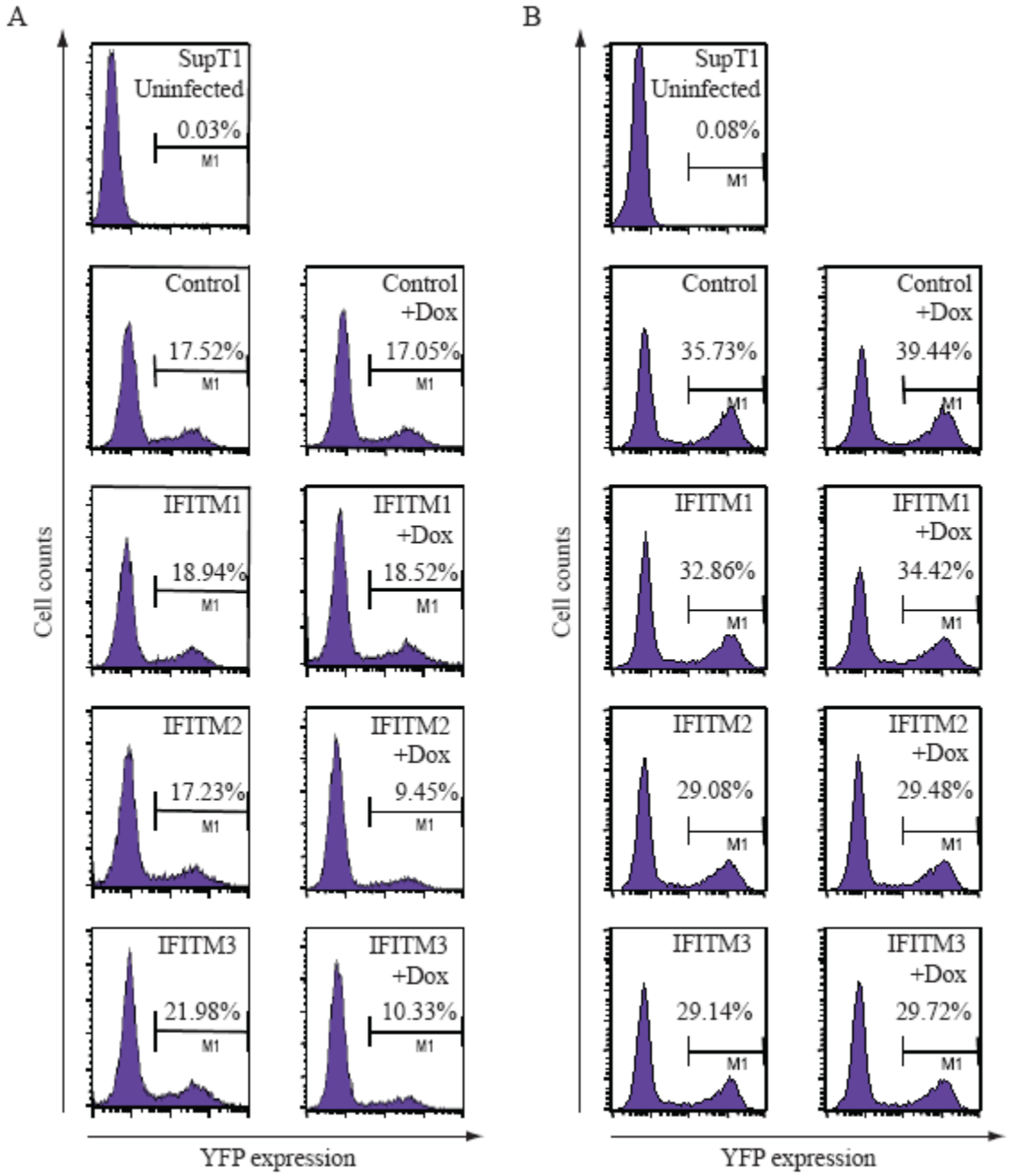


Figure 14: Doxycycline treatment before HIV-1 infection decreases the number of infected IFITM2 and IFITM3-expressing cells. (A) IFITM SupT1 cell lines were treated with doxycycline 16 hr before NLEY1-IRES infection. (B) IFITM SupT1 cell lines were infected with NLEY1-IRES virus 2 hr before doxycycline treatment. The number of YFP expressing cells was scored by flow cytometry. Dox, doxycycline.

3.4.3 IFITM2 and IFITM3 proteins inhibit HIV-1 entry

Since IFITM proteins are transmembrane proteins and they are expressed at the cell surface [29], they may act by interfering with virus entry. To verify this, a FRET-based virion fusion assay was conducted. This assay was done in three steps: 1) production of HIV-1 particles with BlaM-Vpr chimeric proteins incorporated into the virions; 2) infection of target cells where viral fusion resulted in the delivery of BlaM-Vpr proteins into the cytoplasm; and 3) detection by flow cytometry of the cleaved CCF2-AM dye, a fluorescent substrate of β-lactamase enzyme, loaded into the target cells.

It was found that when IFITM2 and IFITM3 cell lines were treated with doxycycline 16 hr before the assay, the percentage of cells with the CCF2-AM substrate cleaved decreased by 3-fold and 2-fold respectively (Figure 15). However, doxycycline-treated IFITM1 cells did not exert a measurable effect on the percentage of cells with the CCF2-AM cleaved. Thus, IFITM2 and IFITM3 proteins but not IFITM1 are able to inhibit HIV-1 entry.

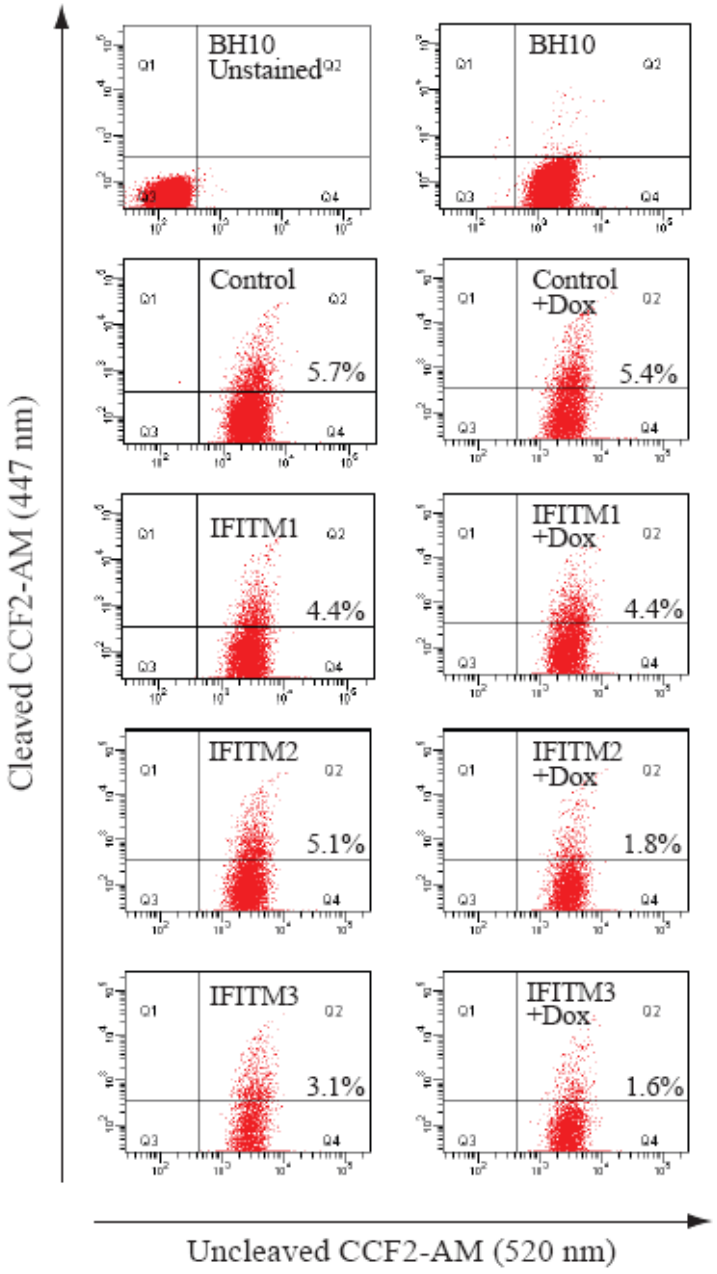


Figure 15: Detection of virion fusion in IFITM SupT1 cell lines. IFITM SupT1 cell lines were treated with doxycycline 16 hr before being infected with HIV-1 containing BlaM-Vpr chimeric proteins. Virion fusion was analyzed by a LSR II multicolor flow cytometer using a purple laser (405 nm) for the excitation of CCF2-AM. Upon excitation, uncleaved CCF2-AM emits green (520 nm) and cleaved CCF2-AM emits blue (447 nm). The number indicates the percentage of blue-expressing cells scored. Dox, doxycycline.

3.4.4 IFITM proteins do not affect the level of CD4 and CXCR4 on the cell surface

Since IFITM2 and IFITM3 expression in SupT1 cells has an effect on HIV-1 entry, it was necessary to determine whether this effect was due to a decrease in receptor or coreceptor expression in those cell lines. To this end, fluorescent-labeled antibodies specific to these two molecules were used for immunostaining. The cells were doxycycline-treated for 16 hr then incubated with 100 µl of diluted antibody for 30 minutes on ice. Cell surface expression was assessed by flow cytometry. It was found that expression of CD4 and CXCR4 on the surface of doxycycline-treated IFITM SupT1 cells was similar to that found on untreated cells (Figure 16).

Oddly, immunostaining of SupT1 cells for CXCR4 showed expression in only 27-46% of the cells when it should be nearly 100% (Figure 16, right panel). On the graphs, we do observe a single population of cells that has shifted to the right when compared to the unstained control; thus, this population of cells does express CXCR4 but the signal detected may be weak. It might be possible that the fluorescent molecules used to label the antibody had lost intensity with time.

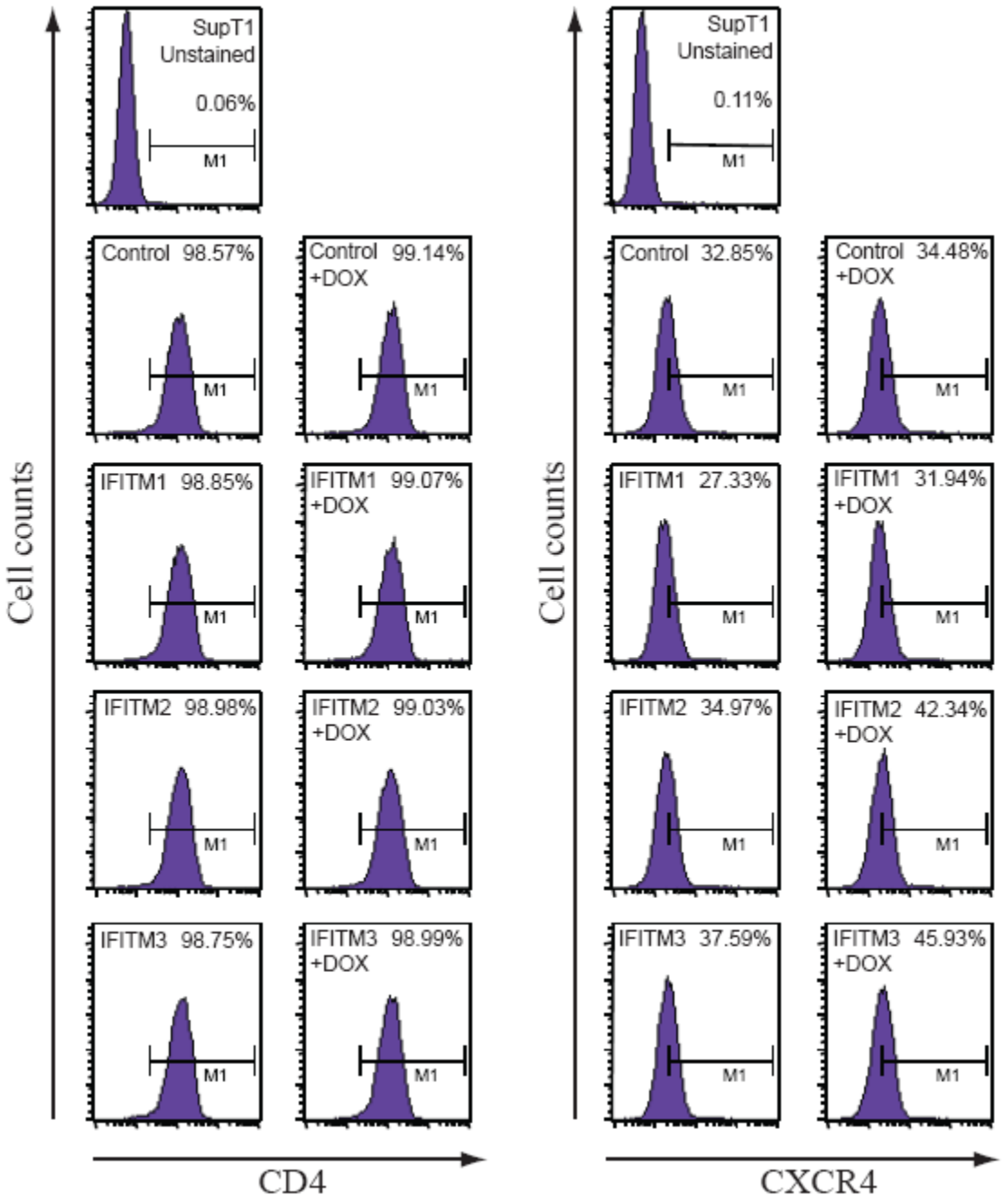
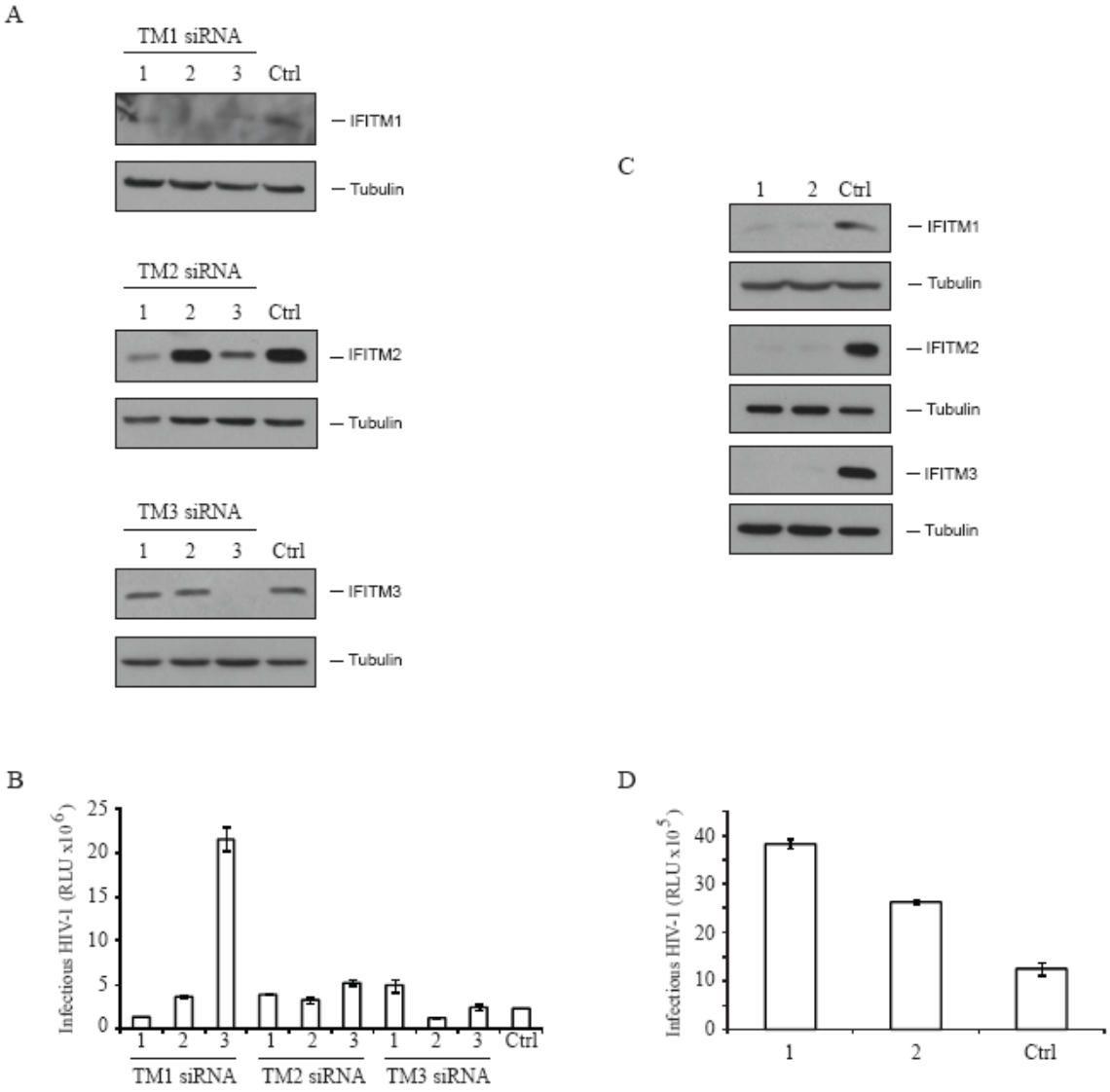


Figure 16: Cell surface expression of CD4 and CXCR4 on IFITM SupT1 cell lines. The cell lines were treated with doxycycline for 16 hr before immunostaining with PE-labeled antibodies specific for CD4 (left) or CXCR4 (right). Fluorescent signals were assessed by flow cytometry with FACSCalibur using a blue laser (488 nm) for excitation. The number indicates the percentage of PE-labeled cells scored. Dox, doxycycline.

3.4.5 The depletion of all three IFITM proteins is necessary for increased HIV-1 infection

As IFITM proteins are highly homologous, the depletion of one of them may not necessarily block their full inhibitory activity because the expression of the two others may compensate for the loss. To verify this, IFITM1, IFITM2, and IFITM3 were simultaneously knocked down in TZM-bl reporter cells before HIV-1 infection. There were three pre-designed siRNAs per gene and they were first tested individually to verify their knockdown efficiency in TZM-bl cells. Among those oligos, siRNA-2 targeting IFITM1, siRNA-1 and siRNA-3 targeting IFITM2 and siRNA-3 targeting IFITM3 resulted in lower protein levels of their targets (Figure 17A). The luciferase activity from those with a positive knockdown was not significantly higher than in the control (Figure 17B). There is however an outlier. siRNA-3 targeting IFITM1 did not completely abolish IFITM1 protein expression but led to a significant increase in luciferase activity. It is possible that this oligo has some off-target activities leading to an enhanced expression of the luciferase gene. Further experiments excluded this oligo and siRNA-2, which led to a complete loss of IFITM1 protein expression, was used instead.

Two combinations of siRNAs were used to simultaneously deplete IFITM1, IFITM2 and IFITM3. Western blot analyses showed that the levels of all three proteins were lowered in both combinations (Figure 17C). The luciferase activity in TZM-bl cells following HIV-1 infection increased by two to three fold when the three IFITM proteins were depleted (Figure 17D).


A
TM1 siRNA
1 2 3 Ctrl
IFITM1
Tubulin
TM2 siRNA
1 2 3 Ctrl
IFITM2
Tubulin
TM3 siRNA
1 2 3 Ctrl
IFITM3
Tubulin
C
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IFITM1
Tubulin
IFITM2
Tubulin
IFITM3
Tubulin
B
Infectious HIV-1 (RLU x10^6)
25
20
15
10
5
0
1 2 3 1 2 3 1 2 3 Ctrl
TM1 siRNA
TM2 siRNA
TM3 siRNA
D
Infectious HIV-1 (RLU x10^5)
40
30
20
10
0
1
2
Ctrl

Figure 17: Depletion of all three IFITM proteins increases TZM-bl reporter cells susceptibility to HIV-1 infection. (A-B) IFITM proteins were depleted individually before HIV-1 infection. There were three siRNA oligos for each. (C-D) IFITM proteins were depleted simultaneously before HIV-1 infection. Two siRNA combinations with oligos targeting all three proteins were used. Western blot analyses show the knockdown efficiency of each oligo. Luciferase activity was measured 40 hr following infection. Ctrl, nontargeting siRNA negative control; TM1, IFITM1; TM2, IFITM2; TM3, IFITM3. Tubulin was used as the protein loading control.

Chapter 4-Discussion

With the main objective of revisiting the field of interferon in regards to HIV-1 replication, a shRNA screen of the genes upregulated by type I IFN in SupT1 cells was performed. As the SupT1 cell line is derived from CD4+ T lymphocytes, results from this study would be more representative of the events in a typical infection setting where CD4 T-cells are the main targets of HIV-1. The screen identified five ISGs whose depletion rescued HIV-1 production at a significant level following IFN treatment and those were: IFITM1, IFI44, RTP4, EPSTI1, and TDRD7. Not much is known about each of these genes and interestingly, with the exception of IFITM1, none of them have been formally associated to an antiviral function yet. IFI44 was initially discovered as a factor associated to microtubules in hepatitis C infected cells and it shows a certain level of antiproliferative activity but its exact function remains unknown [55,63]. RTP4 belongs to the family of receptor transport protein (RTP) known to be involved in the transport of odorant and taste receptors to the cell surface [105]. EPSTI1 is expressed in cancerous breast cells and exerts a regulatory function in those cells [28]. TDRD7 forms protein-protein interactions with other members of its family and is involved in germ cell development [64]. Because of time constraint, only the IFITM family was investigated further in this study. Future directions would consist of investigating the four other candidates and the additional members of the family they belong to. This may reveal yet undiscovered restriction factors the cell possesses to block viral infection. In addition, this would give a better understanding of how IFN can exert such a strong inhibitory effect on viral replication in SupT1 cells.

4.1 Multiple ISGs are involved in inhibiting HIV-1 replication

The depletion of a single ISG per cell line did not show a rescue in viral production equivalent to what was observed when cells were not IFN-treated prior to viral infection. This is consistent with previous findings indicating that more than a single ISG is involved in inhibiting HIV-1 replication. As mentioned earlier, the ISGs found to display antiviral activities against HIV-1 are OAS/RNase L, PKR, ISG15, ISG20, Trim22, APOBEC3G/F and BST-2/tetherin [7,9,20,36,52,57,75,89,91,96,123]. As the wild type HIV-1 was used in this screen, it was expected that the depletion of some of the ISGs with well-characterized anti-viral activities would exhibit no effect on viral production. This is largely due to HIV-1's accessory proteins that can counteract these restriction factors. In our hand, this screen identified five ISGs whose depletion can rescue wild type HIV-1 replication at a significant level in the presence of IFN. It may be possible that they function in synergy to cause the severe inhibitory effect observed in SupT1 cells when cells were treated with IFN prior to infection. To verify this, it would be interesting to deplete all five ISGs simultaneously and to verify if viral production could be rescued to an even greater level. As not all ISGs were covered by Sigma's shRNA library, it is possible that among those left out were some that played a role in restricting HIV-1 replication in SupT1 cells.

In addition, the inability to obtain a greater rescue in viral production may stem from the presence of viral factors that exert a certain level of antagonistic effect, albeit not absolute, on these ISGs. It would be interesting to verify this possibility by using viruses that are deficient in accessory proteins. This method may help identify additional cellular

factors that can block HIV-1 replication but are antagonized by the viral accessory proteins.

Information gathered from previous studies and this screen clearly demonstrate that it is the cumulative effect of the ISGs that is responsible for blocking HIV-1 replication. It is logical to conceive that the simultaneous presence of several inhibitory factors within the cell that can act at different steps of the viral life cycle is more efficient in blocking the virus than if only one were to be present. Throughout evolution, primates have frequently encountered a variety of viruses. Thus, it is not surprising to see that the cell has developed numerous countermeasures to fight them off. It is an advantage for the host to possess more than one countermeasure among its innate immune defense so that viral spread can be prevented or controlled earlier. Resulting from this is that the adaptive immune system may not need to be activated if the virus is eliminated early and the virus would be easier to eradicate if the infection is already contained.

4.2 The IFITM proteins target virus entry

Results from this study demonstrated that all three IFITM proteins strongly inhibited HIV-1 replication in cultured SupT1 cells, where IFITM1 showed the greatest degree of inhibition. However, only IFITM2 and 3 were found to impede with viral entry in host cell. It was not surprising to observe that IFITM2 and 3 exhibited similar phenotypes as they are highly homologous in amino acid sequence. The reason for IFITM1 inability to block entry but yet can strongly inhibit viral replication in cultured cells remains to be investigated. Its shorter N-terminus region and longer C-terminus region may play a role in determining IFITM1 differential function.

While results from this study demonstrated that IFITM1, 2 and 3 proteins possess the potential to inhibit HIV-1 replication, the study from Brass et al., which focused on IFITM3 specifically, showed that IFITM3 inhibited the replication of influenza A virus, West Nile virus and Dengue virus but not HIV-1 [14]. The reason for this discrepancy may be due to the cell line used by Brass et al. to test the effect of IFITM3 on HIV-1. Indeed, this group depleted IFITM3, but not IFITM1 and 2, in HeLa-CD4 cells. Since IFITM1 and 2 are also constitutively expressed in HeLa-CD4 cells, their presence may compensate for the loss of IFITM3; thus, explaining why Brass et al. did not observed a difference with HIV-1.

The detailed mechanism by which IFITM2 and 3 block viral entry remains to be investigated. As the IFITM proteins can also block influenza A virus, West Nile virus and Dengue virus, it suggests that the IFITM proteins recognize a common step or pattern shared by these viruses. One feature that these viruses have in common is the route of entry in the cell which is by endocytosis. This process is mediated by clathrin in the case of influenza A virus, WNV and Dengue virus [88]; whereas dynamin mediates the endocytosis of HIV-1 [86]. Future work is needed to determine whether these proteins act along the endocytosis pathway.

There was a discrepancy between what was observed with the time-course BH10 virus infection and the one-round infection with the NLEY1-IRES virus. Indeed, viral RT activity was undetectable in cultured supernatant until day 14 for IFITM2 and 3 expressing SupT1 cells and was barely detectable on day 16 for IFITM1 cells; whereas, there was a 50% decrease when using the NLEY1-IRES reporter virus to infect IFITM2 and 3 expressing SupT1 cells but there was no change in IFITM1 cells. The reason why

the decrease with NLEY1-IRES virus infection was not absolute such as for the BH10 time-course infection is not known yet. The explanation may lie within the two viruses used. Future directions would require a repeat of these two experiments but using the NLEY1-IRES virus to do the time-course experiment and a BH10 reporter virus to do the one-round infection. Alternatively, the infection protocol itself may have affected the outcome. The BH10 infection consisted of incubating viruses with cells in media and to allow the infection to happen without additional force; whereas spinoculation, where viruses were forced to attach to cells by centrifugal force, was used for the NLEY1-IRES infection.

The IFITM SupT1 cell lines are heterogeneous in that 40%-50% of the cells show high levels of IFITM expression with induction by doxycycline as being measured by flow cytometry analysis (data not shown). It is highly possible that the results from this study under-estimate the degree of inhibition by these proteins. To remediate to this, purer cell lines can be generated by selecting single cell clones expressing both the transactivator gene and the IFITM gene. This may explain why the one-round infection did not show a greater decrease than the 50% observed.

In addition, it would be important to determine whether IFN treatment of SupT1 cells can also block viral entry in order to validate the results observed with the IFITM SupT1 cell lines. This would provide an insight into the physiological relevance of IFN on viral entry.

To sum up, this study has identified the IFITM proteins as restriction factors that confer cellular resistance to HIV-1. All three IFITM proteins were able to severely inhibit

viral replication in cultured cells. IFITM2 and 3 but not IFITM1 blocked the viral entry step. As the very first step of the viral life cycle is blocked by these proteins, they are especially interesting in terms of drug development. There is a clear advantage to blocking the early steps rather than the late steps. Indeed, preventing the virus from entering the cell will prevent it from initiating further damage to the cell and ultimately to the host. The reason why HIV-1 cannot be completely eradicated from an infected individual is due to its ability to integrate its viral DNA into the host DNA. If the virus is prevented from reaching this step, then viral reservoir is prevented. Thus, future studies are warranted to determine the exact inhibitory mechanism of these proteins as they possess the potential for the design of novel antiviral therapies.

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