The Anti-HIV-1 Host Protein MxB Promotes Cell Apoptosis

Master's Thesis

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Abstract (English)

MxB is an interferon-inducible myxovirus resistance (Mx) protein that has recently been reported to restrict HIV-1 infection. Before the discovery of its anti-HIV-1 activity, MxB was believed to be non-antiviral but to serve normal cellular functions. However, the role of MxB in regulating cell apoptosis has not been reported.

In our study, overexpression of MxB resulted in a dramatically increased apoptotic cell number in U87-$CD4^+$/$CXCR4^+$ cells. Annexin V staining revealed that MxB induces apoptosis in a dose-dependent manner. An additive effect on apoptosis was observed when these MxB-expressing U87 cells were exposed to treatment of sodium arsenite. Moreover, the knockout of endogenous MxB expression reduced oxidative stress-induced apoptosis in U87MG cells upon IFNα induction. In addition, SupT1 cells expressing MxB was also shown to be more sensitive to stress-induced apoptosis as MxB accelerated the apoptosis inducement under the condition of sodium arsenite treatment. Deleting the N-terminal region, disrupting the GTPase activity, and blocking the higher-ordered oligomerization of MxB resulted in a reduced apoptosis promotion activity. Completely blocking the dimerization of MxB, however, significantly enhanced its activity in apoptosis promotion. These data helps identify the functional regions of MxB that are involved in its pro-apoptotic activity. Taken together, results of our study suggest that MxB promotes cell apoptosis and enhances the sensitivity of different cell types to stress-induced apoptosis.

Future studies would aim to illustrate the mechanism of MxB-induced cell apoptosis. We will also investigate the possible link between the pro-apoptotic activity and the antiviral function of MxB.

Résumé (Français)

Il a récemment été démontré que la protéine de résistance aux *Mixovirus* (Mx) inductible par interferon MxB restreint l'infection au VIH-1. Avant la découverte de cette activité anti-VIH-1, MxB semblait servir pour les fonctions cellulaires normales et ne pas avoir de rôle antiviral. Cependant, le rôle de MxB en tant que régulateur de l'apoptose n'a pas été reporté.

Dans notre étude, la surexpression de MxB a entraîné une augmentation radical du nombre de cellules apoptotiques dans les cellules U87-$CD4^{+}$/$CXCR4^{+}$. Un marquage à l'Annexine V a révélé que MxB induit l'apoptose de manière dose-dépendante. Un effet pharmacologique additif sur l'apoptose a été observé lorsque des cellules U87 exprimant MxB ont été soumises à un traitement par de l'arséniate de sodium. En outre, le knock-out de l'expression endogène de MxB a réduit l'apoptose oxydative induite par le stress dans les cellules U87MG après induction par IFNα. De plus, nous avons montré que des cellules SupT1 exprimant MxB étaient plus sensibles à l'apoptose induite par le stress du fait que MxB accélère l'apoptose résultant du traitement par l'arséniate de sodium. En supprimant la région N-terminale, perturbant l'activité GTPase, et bloquant le haut degré d'oligomérisation de MxB, la propriété pro-apoptotique de MxB a été réduite.

Cependant, le blocage total de la dimérisation de MxB a significativement augmenté son activité pro-apoptotique. Ces données aident à identifier les régions fonctionnelles de MxB impliquées dans son activité pro-apoptotique. Dans leur ensemble, les résultats de notre étude suggèrent que MxB entraine l'apoptose cellulaire et augmente la sensibilité de différents types cellulaires à l'apoptose induite par le stress.

De futures études viseraient à élucider le mécanisme de l'apoptose induite par MxB. Nous explorerons aussi le lien putatif entre l'activité pro-apoptotique et la fonction antivirale de MxB.

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List of Abbreviations

Ab	Antibody
AIDS	Acquired Immunodeficiency Syndrome
AIM	Apoptosis inhibitor of macrophage
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ART	Antiretroviral Therapy
BAK	BCL-2 antagonist/killer
BAX	BCL-2-associated X protein
BH3	BCL-2 homology 3
CA	Capsid
Cas9	CRISPR associated protein 9
CCR5	C-C chemokine receptor type 5
CDS	coding DNA sequence
CD4	Cluster of differentiation 4
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCR4	CXC chemokine receptor 4
DCAF1	DDB1-Cul4A-associated-factor-1
DISC	death-induced signaling complex
DNA	Deoxyribonucleic acid
Dox	Doxycycline
Env	Envelope
ESCRT	cellular endosomal sorting complex required for transport
FASL	FAS ligand
FBS	Fetal bovine serum
gp	Glycoprotein
GRID	gay-related immune deficiency

gRNA	CRISPR guide RNA
HAART	Highly Active Antiretroviral Therapy HCV Hepatitis C virus
HIV-1	Human immunodeficiency virus type 1
HIV-2	Human immunodeficiency virus type 2
IFITM	Interferon induced transmembrane IFN Interferon
IN	Integrase
ISG	Interferon stimulated gene
kD	Kilodaltons
KO	Knockout
LTR	Long terminal repeat
µg	Microgram
µl	Microliter
µM	Micromolar
M	Molar
MA	Matrix
MDM	monocyte-derived macrophages
mg	Milligram
ml	Milliliter
MLV	Murine leukemia virus
mM	Millimolar
mRNA	Messenger RNA
Mx	Myxovirus resistance
NC	Nucleocapsid
Nef	Negative-regulation factor
ng	Nanogram
NLS	nuclear localization signal
nm	Nanometer

NS	Nonstructural
ns	non-significant
PBS	Phosphate-buffered saline
PCP	Pneumocystis pneumonia
PE	Phycoerythrin
PFA	Paraformaldehyde
PIs	Protease Inhibitors
PIC	Pre-integration complex
PKR	dsRNA-dependent protein kinase
Pol	Polymerase
PR	Protease
PRR	pattern recognition receptor
$Pr55^{Gag}$	Group-specific antigen
P/S	Penicillin/Streptomycin
RIG-I	Retinoic acid inducible gene I
RING	really interesting new gene
RME	Receptor-mediated endocytosis
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRE	Rev responsive element
RT	Reverse transcriptase
RTC	Reverse transcription complex
SAMHD1	Sterile Alpha Motif (SAM)-containing HD-domain containing protein 1
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SIV	Simian Immunodeficiency Virus
STAT	Signal transducers and activators of transcription
TAR	transactivation response

Tat	Trans-activator of transcription
TRIM	Tripartite-motif-containing TYK2 Tyrosine kinase 2
U	Unit
UTR	Untranslated Region
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
Vpx	Viral protein X
VSV-G	Vesicular stomatitis virus glycoprotein
WT	Wild Type
7-AAD	7-Aminoactinomycin D

Chapter 1-Introduction

1.1 Human Immunodeficiency Virus

1.1.1 History and Classification

Human immunodeficiency virus (HIV) is the viral pathogen that causes Acquired Immunodeficiency Syndrome (AIDS). The first cases of AIDS were reported in 1981 when 5 young homosexual men with no clinically apparent immunodeficiency developed Kaposi's Sarcoma, pneumocystis pneumonia (PCP) or/and cytomegalovirus (CMV) infections[1-3]. The disease was firstly called "gay compromise syndrome" or "gay-related immune deficiency (GRID)" until the next year when people realized that the disease was also spreading among hemophiliacs and heroin users[4,5]. In the same year, the disease was renamed Acquired Immunodeficiency Syndrome[6]. In 1983, Dr. Luc Montagnier's group firstly isolated a new retrovirus from the lymph nodes of a patient that developed the symptoms of AIDS[7]. In the following years, with a number of more-detailed reports published, the retrovirus was finally confirmed to be the etiological agent of AIDS and was officially named as human immunodeficiency virus[8-10].

HIV belongs to *Lentivirus*, a genus of viruses of the *Retroviridae* family. It is further categorized into two distinct species: HIV-1 and HIV-2, with HIV-1 being the predominant species worldwide. HIV-1 originates from the cross-species transmission of SIV from chimpanzee (SIVcpz)[11]. HIV-2, which arises from the transmission of SIV from sooty mangabeys (SIVsm), is less prevalent (mainly prevalent in West Africa) and less pathogenic compared to HIV-1[12-14] (Figure 1).

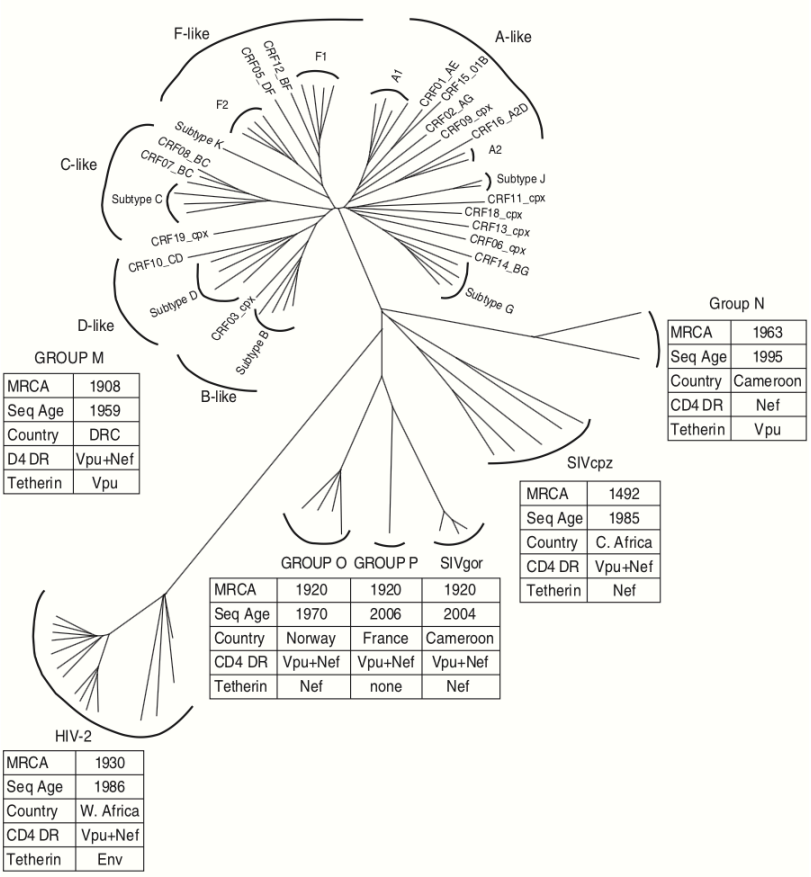


Figure 1. HIV strains cluster with chimpanzee and gorilla SIV in phylogenetic analysis[15]. The genetic similarities between different HIV and SIV strains are compared by full genome sequence aligning. The phylogenetic trees are shown based on nucleotide distance. In the inserted tables, MRCA refers to the time to the most recent common ancestor. Seq Age: oldest available sequence. Country: the country or location of the oldest sequence. CD4 DR: CD4 down-regulation by Nef and/or Vpu. Tetherin: tetherin antagonism by Nef, Vpu or Env. HIV-2 derives from SIVsm and it shares 50–60% sequence identity with HIV-1. HIV-1 Groups M and N are more closely related to SIVcpz while groups O and P are more closely related to SIVgor.

HIV-1 is divided into four distinct lineages based on viral sequence homology, namely, Groups M (main), N (new), O (outlier) and P (putative) (Figure 1). Each of these phylogenetic lineages results from an independent cross-species transmission event of SIVs that infect African apes[16]. Group M is the largest HIV-1 group and is responsible for the global HIV-1 epidemic. This group has further evolved into nine distinct subtypes (or clades): A, B, C, D, F, G, H, J, and K[17]. It is believed that the M and N groups of HIV-1 originate from SIV strains (SIVcpz*Ptt*) carried by chimpanzees in southern Cameroon[18]. The O and P groups of HIV-1, however, are more likely to be of gorilla origin[16].

1.1.2 Disease and transmission

1.1.2.1 Transmission of HIV

HIV is transmitted through bodily fluids, including blood, semen, pre-seminal fluid, rectal fluids, vaginal fluids, and breast milk. Currently, sexual transmission is the major cause of new infections. Other ways of HIV transmission include sharing needles with HIV-infected patients during intravenous injections, transfusion with infected blood, and transmitting from mother to child during pregnancy, birth, or breastfeeding.

It is commonly believed that blocking the viral transmission is the simplest and the most effective way to control HIV infection. Sexual transmission can be substantially reduced by barrier protections (i.e. use of latex condoms)[19]. Vertical transmission can also be effectively controlled by anti-retroviral therapy (ART) during pregnancy, delivery and the breastfeeding period[20].

1.1.2.2 Acute HIV infection

In the absence of treatment, a typical HIV infection advances in three distinct stages: acute (or primary) HIV infection, chronic HIV infection (also known as clinical latency or asymptomatic phase), and AIDS (Figure 2).

The acute HIV infection occurs within the first few weeks following the primary infection. During this phase, the founder virus is widely disseminated, predominantly to lymphoid tissues, which is reflected by a sharp increase in HIV RNA in plasma and a dramatic loss of $CD4^+$ T-cells. The host innate immune system is also activated, releasing a number of associated cytokines that subsequently activate the downstream effectors[21]. In most cases, patients develop flu-like symptoms including fever, sore throat, headache, and muscle and joint pain, which last approximately two weeks[22-24]. Painless swelling of lymph nodes may also occur during the second week of infection[23,24]. After a certain period of rapid viral replication, which varies from 21 to 119 days, the viral load stabilizes at a certain level known as the viral set point. This is a consequence of the Cytotoxic T cell production by the host immune system that fights against the constantly produced viral particles[25].

1.1.2.3 Chronic HIV infection

The acute HIV infection usually lasts for approximately 6-12 weeks and is followed by the chronic infection, which may last 2-15 years[21]. During the chronic infection, the virus is still present while symptoms are minimal. With the activation of the robust host adaptive immune response, the plasma viral RNA load drops to a low level whereas the patient's $CD4^+$ T-cell count is partially restored[26]. As viral replication is still proceeding at a relatively low level, immune cells continually secrete proinflammatory cytokines and

chemokines, including IL-1 and TNFα, which further deplete the $CD4^+$ T-cells and weaken the host immune system[26].

1.1.2.4 AIDS

As the infection progresses, the patient's immune system is continually damaged due to the persistent immune activation[27,28]. When the $CD4^+$ T-cell count falls below 200 cells/mm^3, the patient is considered to have progressed to AIDS. In this phase, as the host immune control of pathogenic organisms can no longer be maintained, the level of viremia rises and the viral variation starts to explode[29]. Consequently, patients are vulnerable to opportunistic infections, which lead to the development of more severe symptoms such as rapid weight loss, recurring fever, prolonged swelling of the lymph glands, memory loss, depression, and other neurologic disorders[30].

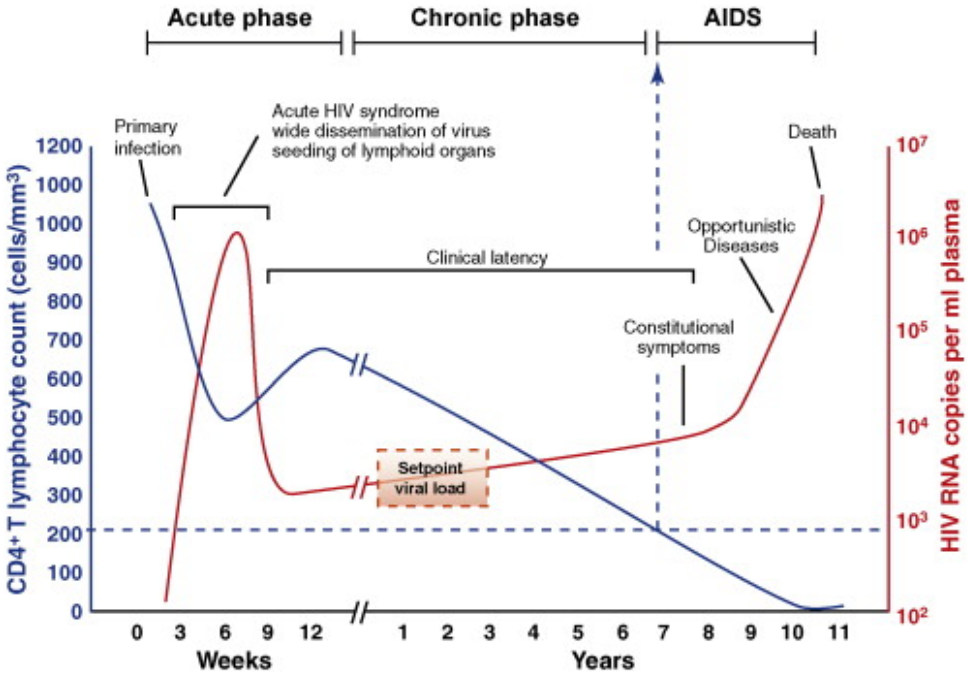


Figure 2. A typical natural time course of HIV infection[31]. The pattern of the $CD4^+$ T-cell decline is shown in blue. The curve of viremia proceeding is highlighted in red.

1.1.3 Virology

1.1.3.1 HIV-1 genome and viral proteins

Similar to other retroviruses, the full HIV genome is encoded on a 9.7kb-long RNA strand that consists of nine open reading frames flanked by two long terminal repeats (LTR) (Figure 3). Nine viral proteins are encoded by overlapping genes on the HIV-1 genome, including three polyproteins: Group-specific antigen (Gag), polymerase (Pol), envelope (Env), four accessory proteins: viral infectivity factor (Vif), viral protein R (Vpr), viral protein U (Vpu), negative-regulation factor (Nef), and two regulatory proteins: transactivator (Tat) and regulator of viral expression (Rev).

Gag is a polyprotein that is further cleaved by the viral protease into three structural proteins: p17 (matrix or MA), p24 (capsid or CA), p7 (nucleocapsid or NC), and one additional protein p6[32]. The matrix protein p17 participates in the early stages of HIV-1 replication and is important for RNA targeting to the plasma membrane, incorporation of envelope into the viral particle and virion assembly[33]. P24 is important for incorporation of the Gag-Pol precursor into viral particles[34]. It also helps recruit Cyclophilin A (CypA), which enhances the viral infectivity[35]. The nucleocapsid protein P7 is important for HIV-1 RNA packaging[36]. Other than encapsulating and protecting the viral RNA, P7 also participates in reverse transcription and viral assembly[36,37]. P6 facilitates Vpr packaging and virus releasing[38].

The HIV-1 *pol* gene lacks an initiation codon. Thus, the viral Pol protein is primarily translated as $Pr160^{GagPol}$, a Gag-Pol fusion product, resulting from a ribosomal frameshift during the translation of $Pr55^{Gag}$[39]. The Gag-Pol precursor is subsequently cleaved by viral protease, during which Pol itself forms three viral enzymes: protease (PR), reverse

transcriptase (RT), and integrase (IN)[39]. The HIV-1 protease PR cleaves the Gag-Pol precursor as described above. RT catalyzes reverse transcription during which the double-stranded proviral DNA is formed. IN is responsible for the integration of the proviral DNA into the host cell chromosomes.

The Env protein precursor (gp160) is cleaved by cellular proteases into two viral proteins: the surface membrane protein (SU) gp120 and the trans-membrane protein (TM) gp41, both of which are involved in HIV-1 entry, facilitating the target cells attachment and the cell membrane fusion, respectively[40,41].

The two viral regulatory proteins, Tat and Rev, are also required for HIV-1 replication. Tat targets the transactivation response (TAR) element at the 5' end of viral RNA and it regulates transcription of the long terminal repeat (LTR)[42]. Rev localizes to nucleus of the host cell and it plays important roles in exporting HIV-1 RNAs from the nucleus, regulating viral protein expression, and maintaining proper balance between early and late viral genes[43-46].

Unlike HIV-1 regulatory proteins, Vif, Vpr, Vpu and Nef are referred to as accessory proteins because they have been shown to be dispensable for viral replication in many cell culture systems[47]. However, they have essential roles in viral replication and pathogenesis in vivo, such as counteracting anti-HIV-1 host restriction factors[48,49].

1.1.3.2 HIV-1 particle structure

A mature HIV-1 particle is spherical in shape and has a diameter of about 120 nm (Figure 4)[50]. The HIV-1 viron is enveloped in a lipid bilayer that is formed when the newly produced virus particle buds from the host cell, surrounded with part of the host cell membrane. Surface protein Env complex, a gp120-gp41 heterodimer located on the

viral envelope, is composed of a gp120 cap that reaches out of the surface and a gp41 stem that is protruding through the membrane. Three Env complexes assemble non-covalently via their gp41 portion into a trimeric glycoprotein spike, which is crucial for the virus binding to its cellular receptor CD4. A matrix composed of an association of the viral protein MA is formed underneath the viral envelope, which ensures the integrity of the virion particle. HIV-1 CA proteins polymerize to form a conical-shaped viral core. Within the HIV-1 core, two copies of single-strand viral genomic RNA are tightly bound to NC proteins, which protect them from nuclease digestion. Also enclosed within the HIV-1 particle are viral enzymes, regulatory proteins and accessory proteins.

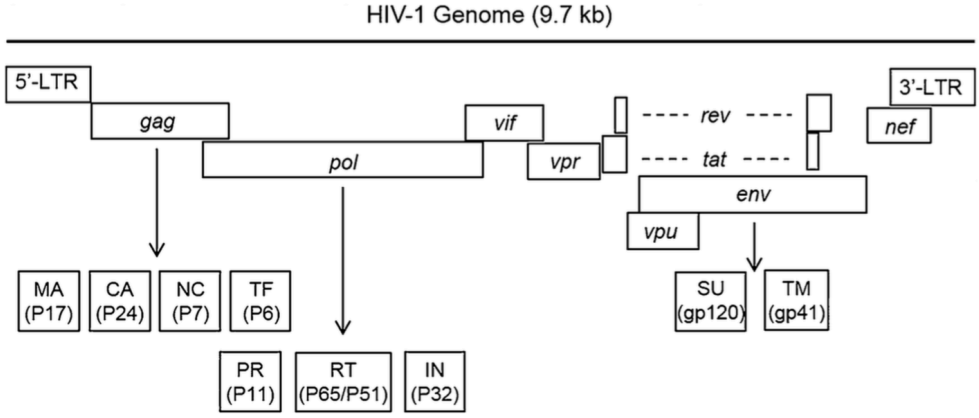


Figure 3. Schematic diagram of the HIV-1 genome[51]. The full HIV-1 genome is about 9.7kb. Nine viral proteins are encoded, including three polyproteins: Gag, Pol, Env, four accessory proteins: Vif, Vpr, Vpu, Nef, and two regulatory proteins: Tat and Rev.

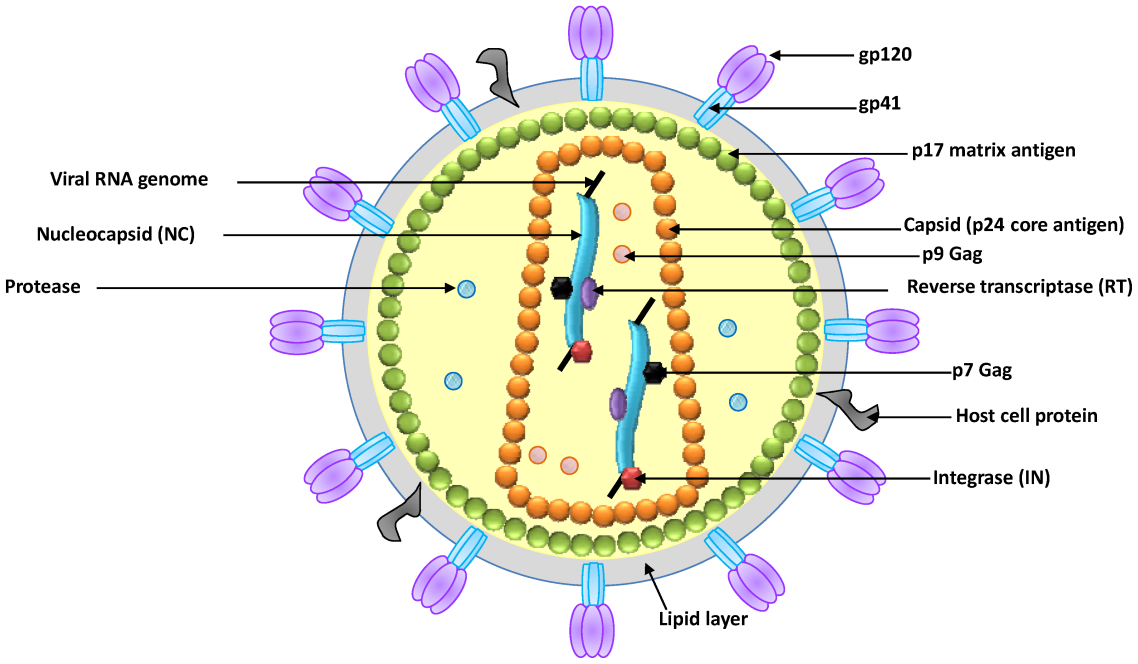


Figure 4. Schematic structure of a mature HIV-1 particle[52]. Gp120/gp41 complex on exterior, p17 matrix protein, p24 capsid protein, and RNA bound with necessary regulatory factors are shown.

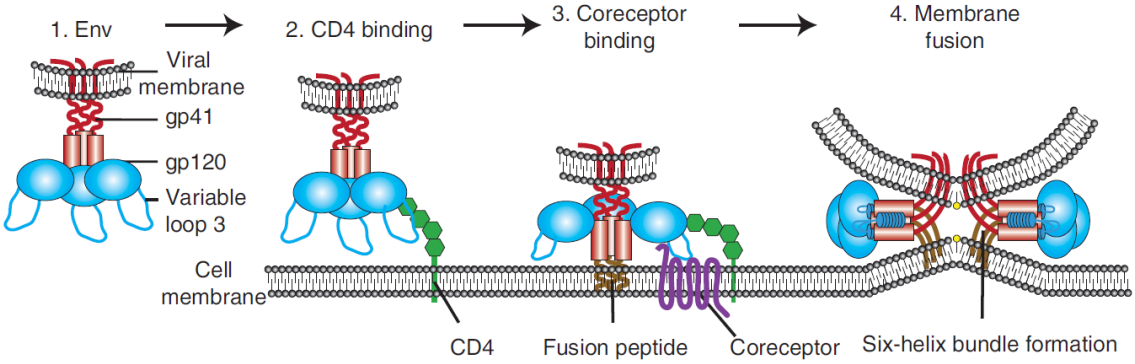


Figure 5. HIV-1 Entry[53]. The virus entry process is shown, including the cell surface receptor/coreceptor binding, and fusion between the viral and host cell membranes.

1.1.3.3 HIV-1 replication cycle

HIV-1 replication cycle can be briefly divided into seven stages: binding, fusion, reverse transcription, integration, replication, assembly, and budding.

The viral particles recognize and bind to their primary cell-surface receptor CD4 that is expressed on the surface of $CD4^+$ T-cells, macrophages and dendritic cells. Then the gp120-gp41 trimer undergoes a conformational change, leading to the exposure of the variable loop 3 (V3)[54]. The envelope V3 loop is a high-affinity binding site that interacts with the CXCR4 or CCR5 coreceptors after this conformational change[54]. After coreceptor binding, the gp120-gp41 complex undergoes another conformational change, which exposes the fusion peptide in gp41 to trigger the fusion step[53].

The fusion between viral envelope and plasma membrane of the target cell starts when the fusion peptide inserts into the cellular membrane. Subsequently, a six-helix bundle is formed as a consequence of the interaction between HR1 and HR2 domains of the Env trimer[55]. This six-helix bundle brings viral and host cell membranes in close contact, promoting the formation of fusion pore through which the viral capsid can pass to enter the target cell (See in Figure 5)[55].

Shortly after entry, HIV-1 core undergoes uncoating, a multistep process that is linked to reverse transcription and nuclear import. During this process, the viral matrix is disassembled and the core is rearranged to form a reverse transcription complex (RTC) and is then released into the cytoplasm. The RTC is composed of HIV-1 RNA, NC and RT, which reverse transcripts the viral RNA into double-stranded DNA. Reverse transcription is initiated when a cellular lysyl-$tRNA^{Lys,\ 3}$ binds to primer binding site (PBS) on the viral genome, leading to an RNA-primed RT elongation[56]. HIV-1 RT has

both DNA polymerase and RNase H activities, which allow it to degrade the remaining RNA strand after the first-strand synthesis and subsequently synthesize the second DNA strand using the preformed single-stranded DNA as template (Figure 6)[57].

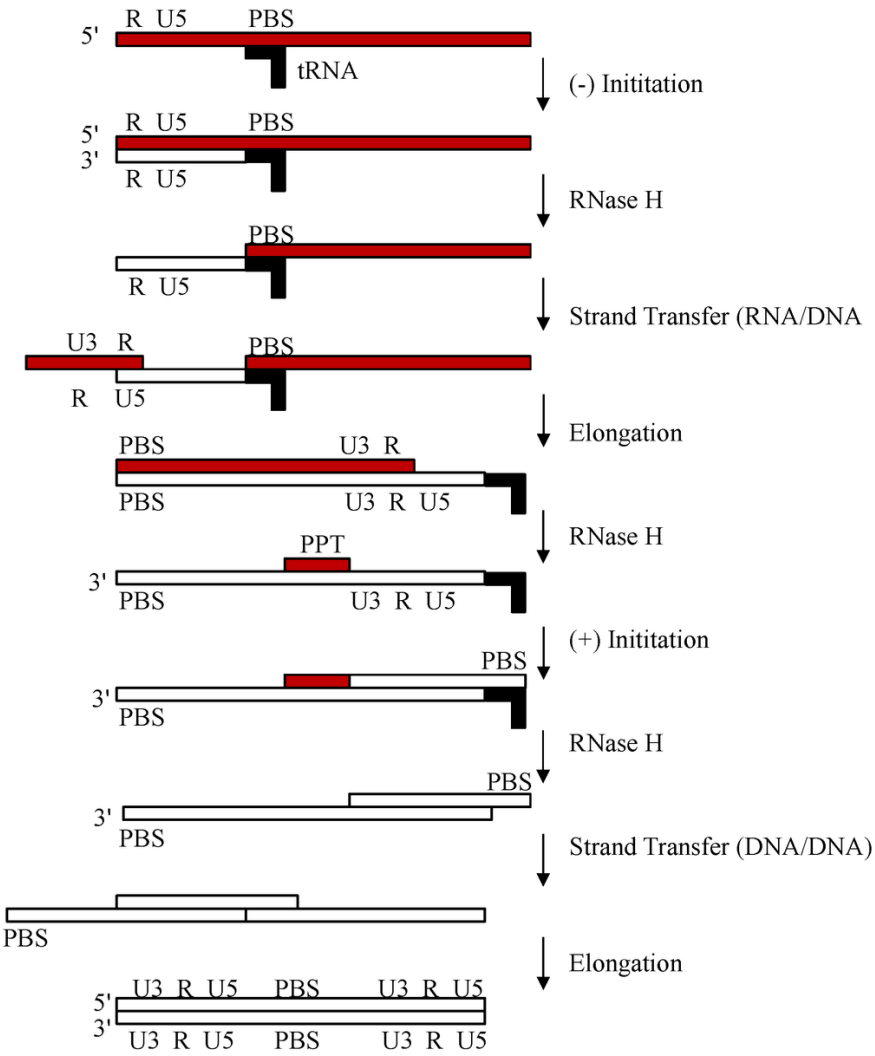


Figure 6. Schematic of HIV-1 Reverse transcription[56]. The viral RNA template is shown in red. Primer binding site is labeled as PBS. The integrase-competent dsDNA (bottom) is formed after reverse transcription, and will be inserted into the host genome.

Once reverse transcription has been completed, the pre-called RTC with the viral genomic RNA replaced by viral dsDNA is then referred to as pre-integration complex (PIC). The PIC is composed of viral DNA, which contains a nuclear import signal[58], viral proteins including IN, Vpr and MA, which possess nuclear localization signals (NLSs)[59], and some host proteins. The nuclear localization signals may contribute to PIC migrating to the nuclear pores and entering the nucleus. Following the nuclear import, the viral DNA is stably integrated into host cell chromosome by the HIV-1 integrase (Summarized in Figure 7). Briefly, IN binds to the LTR region at each end of the viral DNA. It then cleaves off the two nucleotides GT from the 3' end of viral DNA. This process occurs in the cytoplasm and is referred to as 3'-processing reaction[60]. The HIV-1 IN also removes four to six nucleotides from the cellular DNA, forming a gap where the viral DNA can insert[60]. Next, strand transfer occurs when sticky ends on the viral DNA and the cleaved ends of cellular DNA are joined together. The integration process is completed when host cell DNA repair enzymes fill the gaps that are formed between the integrated viral DNA and the cellular DNA after strand transfer[61].

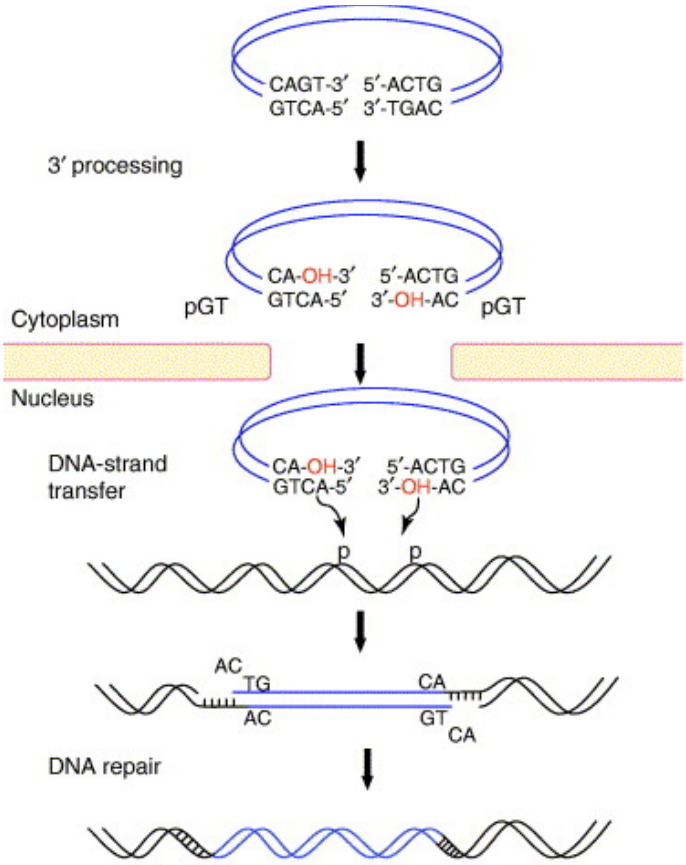


Figure 7. Schematic of HIV-1 integration. The 3'-processing reaction occurs in the cytoplasm, whereas DNA-strand transfer takes place in the nucleus. The PIC is imported into the nucleus. The strand-transfer reaction, repair of the remaining gaps are shown.

Once integrated into the host genome, the HIV-1 proviral DNA is incorporated into nucleosomes like normal cellular genes[62]. At this point, the provirus may either lie dormant as in the latent stage of HIV infection or be actively transcribed to produce new viral particles, mainly depending on nucleosomal organization and epigenetic control of the provirus[63]. The transcription process is initiated by the viral LTR element, which harbors binding sites for cellular transcription factors[64]. Low-level of viral transcription is common, however, in order to achieve a high viral production, both cellular transcription factors and two viral proteins, Tat and Rev, need to be present[65,66]. Tat is synthesized during an early step of basal transcription. It then binds to the transactivation response (TAR) region on the nascent RNA to relieve a cellular block. Two cellular factors, Cyclin T1 and CDK9, are subsequently recruited, which increase the processivity of RNA polymerase II and thus result in high-levels of viral RNA production[67,68]. During the primary transcription process, a polycistronic pre-mRNA is synthesized. It contains multiple splicing sites and can be alternatively spliced to generate more than 40 different mRNAs, which can be divided into three classes: ~9kb full-length RNA, ~4kb singly spliced RNA and ~2kb multiply spliced RNA[68]. In the host cells, RNAs that contain introns are prevented from nuclear export. Thus, only multiply spliced RNAs can be exported into the cytoplasm where they are translated to form Tat, Rev and Nef[69]. However, when the viral protein Rev is synthesized, it enters the nucleus and binds to the Rev responsive element (RRE) on singly spliced and unspliced RNAs, which allows the export of these transcripts into the cytoplasm[70]. In the cytoplasm, singly spliced RNAs are translated to form Env, Vif, Vpu and Vpr proteins, while unspliced RNAs serve as the viral genetic material and are also translated to form Gag and Gag-Pol polyproteins

(Figure 8)[69]. Notably, a balance between spliced and unspliced viral RNAs must be achieved in order to ensure a spreading HIV infection[68].

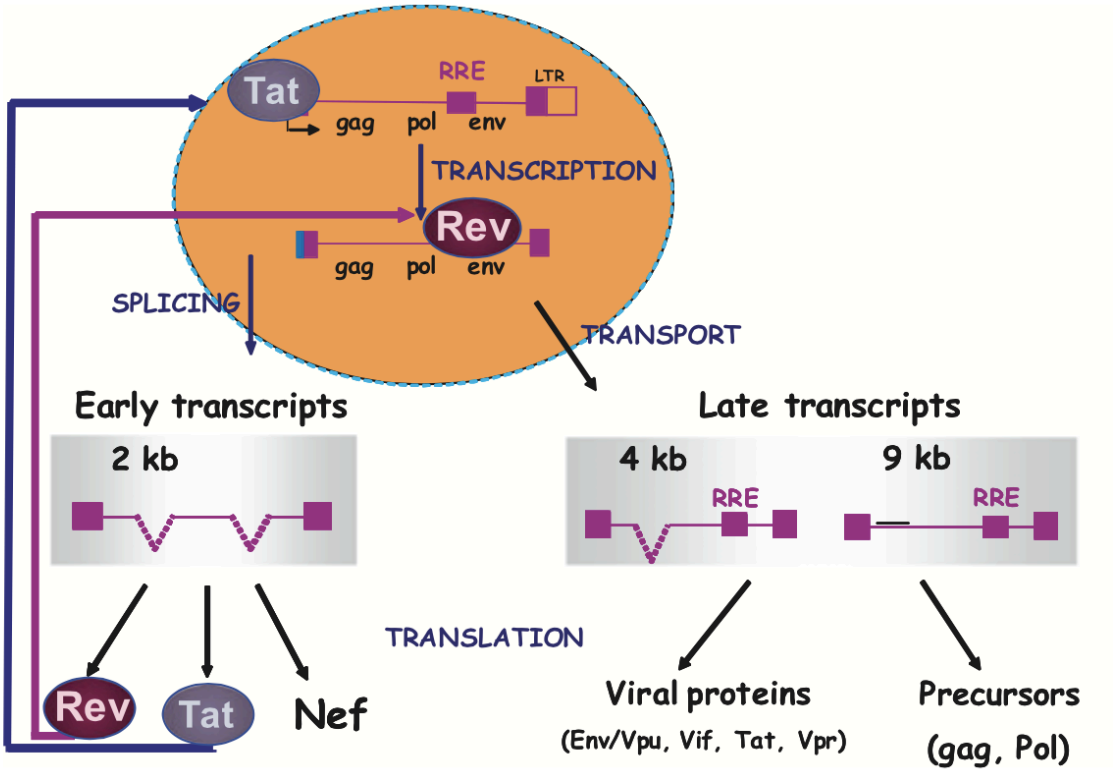


Figure 8. Early and late transcripts of HIV-1[69]. The integrated viral DNA is used as template to firstly produce viral early transcripts Rev, Tat and Nef. Tat enters the cell nucleus and mediates transcription transactivation. Rev binds to the 4kb singly spliced and the 9kb unspliced late transcripts in the cell nucleus and facilitates the nuclear export of these late mRNAs.

HIV-1 structural proteins are derived from Gag, Gag-Pol and Env polyproteins. The Env glycoproteins traffic from the rough endoplasmic reticulum (RER) to the Golgi apparatus, during which they are further cleaved by cellular proteases to form gp120 and gp41[71]. They then traffic in vesicles and finally reach the plasma membrane[71]. The Gag precursor, which contains MA, CA, NC and p6 domains as well as two spacer peptides, is synthesized from full-length viral RNAs as described. The MA domain guides the translocation of Gag proteins to the plasma membrane where they accumulate and oligomerize through its C-terminal CA domain, SP1 peptide and NC domain[64,72]. Besides facilitating the assembly process, NC additionally recruits the viral RNA genome into the virions. During assembly, MA also promotes incorporation of the viral Env glycoproteins into the host cell membrane where the new virus will take as part of its envelope. This is achieved by basic residues that are found at the top of MA. They interact with negatively charged lipids in the inner leaflet of the host cell membrane[72].

After the immature Gag lattice is assembled at the plasma membrane, the viral particle is then released from the cell surface via a membrane fission process. This step of viron budding is catalyzed by cellular endosomal sorting complex required for transport (ESCRT) apparatus that is recruited by the p6 domain of Gag[73]. Briefly, the p6 domain bears a highly conserved PTAP motif near its N-terminus. This motif directly interacts with tumour susceptibility gene 101 (TSG101), a cellular protein found in ESCRT-I[74-76]. This ESCRT-recruiting process may be additionally promoted by Gag ubiquitylation, which is found in many retroviruses[77]. ESCRT machinery then drives membrane scission, during which the viral membrane is formed by taking part of the plasma membrane with a layer of $Pr55^{Gag}$ polyproteins associating with the inner leaflet[50]. Shortly after budding,

maturation is initiated when the viral protease cleaves Pr^{Gag} and $Pr160^{GagPol}$ polyprotein precursors, leading to the formation of a conical capsid core (Figure).

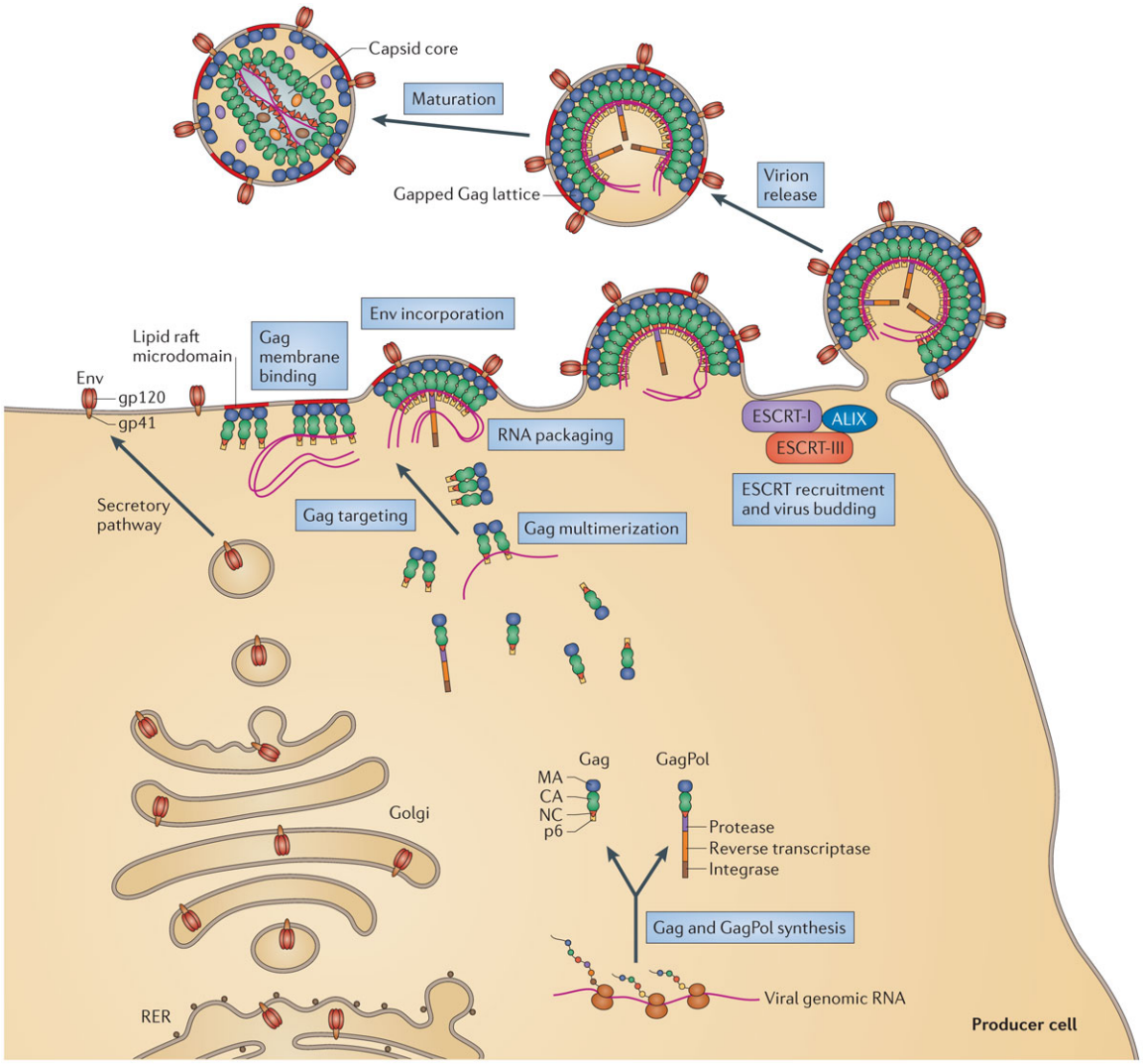


Figure 9. Late stages of the HIV-1 replication cycle[72]. Viral mRNA translation, Env trafficking to host cell membrane, Gag multimerization and binding to host cell membrane, RNA packaging, nascent viral particle releasing, and maturation are shown.

1.2 Host restriction factors against HIV-1

1.2.1 What are restriction factors?

Restriction factors are a group of cellular proteins that inhibit viral pathogens at different stages of their replication cycle[78]. They serve as key effectors during the host innate immune response and also provide effective barriers to cross-species transmissions of viral pathogens[79,80]. In addition, restriction factors also play an important role in limiting viral spread in new host species[81].

Although diverse in structures and functions, host restriction factors share four defining characteristics[82,83]. Most importantly, restriction factors must dominantly cause a dramatic drop in the viral pathogen's infectivity. Secondly, they are cell-intrinsic proteins that are often interferon-inducible and are thus tied to the host's innate immune response[82]. Thirdly, due to the non-stop arms race during the long-standing virus-host co-evolution, restriction factors often exhibit evolutionary signatures, with mutations maintained in a population only when they confer selective advantages[84]. Finally, the viruses have evolved equally potent mechanisms to counteract restriction factors that are true threats to their replications[85].

A number of anti-HIV-1 host restriction factors have been identified over the past two decades (Summarized in Figure 10). Studying these HIV-1 restriction factors and the viral counteracting mechanisms help develop deeper knowledge and strategies for new therapeutic interventions[82].

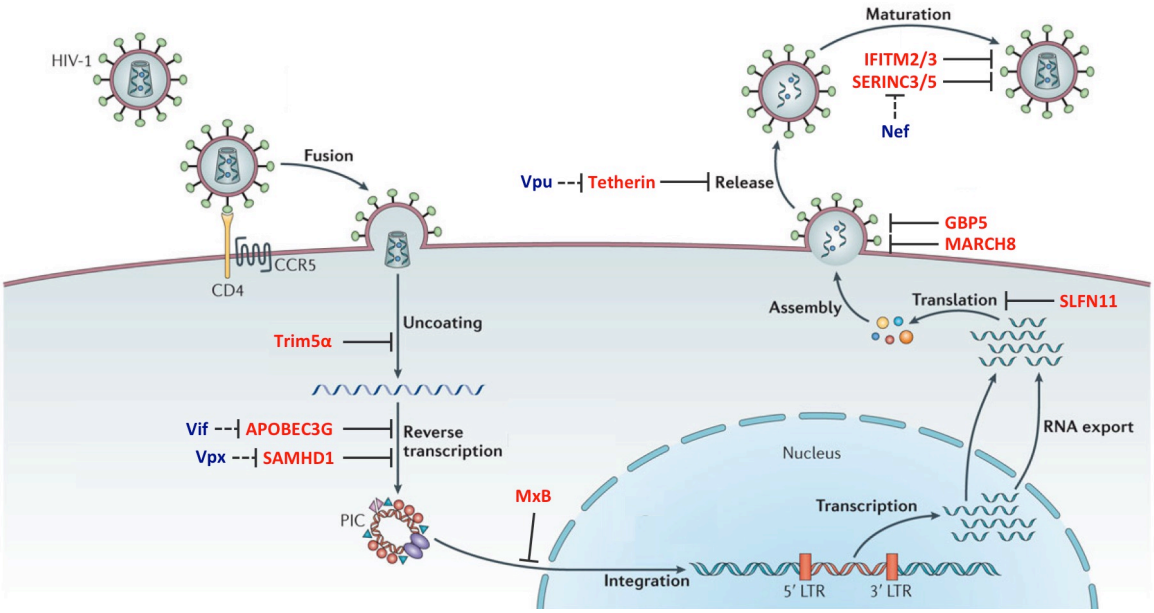


Figure 10. Host restriction factors targeting different stages of HIV-1 replication cycle and viral counteracting mechanisms[86]. Restriction factors are highlighted in red and the viral antagonists are highlighted in blue. Briefly, soon after HIV-1 entry, Trim5α binds to the viral capsids and accelerates its uncoating. The reverse transcription step is targeted by APOPBEC3G and SAMHD1, which are counteracted by the viral protein Vif and Vpx, respectively. APOPBEC3G causes viral DNA hypermutation, leading to degradation of the mutated viral DNA by host DNA repair enzymes. SAMHD1 reduces cellular nucleotide pool to a level that is too low for reverse transcription to proceed. MxB binds to HIV-1 capsids and prevent the nuclear import of the viral DNA. SLFN11 blocks viral mRNA translation by reducing tRNAs bearing the viral codon bias. GBP5 and MARCH8 impair the Env incorporation into the nascent HIV-1 particles by reducing cell surface Env proteins. Tetherin, which is counteracted by viral protein Vpu, captures the newly produced virions onto the producer cell surface. Infectivity of the progeny virus is strongly inhibited by incorporation of IFITM2/3 and SERINC3/5, and the latter proteins are antagonized by HIV-1 protein Nef.

1.2.2 APOBEC3G

The anti-HIV-1 activity of APOBEC3G was firstly identified through studies to understand the function of HIV-1 Vif protein, which was found to be dispensable for viral replication in some cell lines but not in $CD4^+$ T-cells or other cell lines[87,88]. In the cell fusion experiment, hybrid cells inherited the nonpermissive phenotype, attributing causation to a dominant-acting restriction factor[89]. Finally, a cDNA subtraction-based screen revealed that APOBEC3G is the anti-HIV-1 restriction factor that is counteracted by viral protein Vif[90].

APOBEC3G is a member of Apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3) subfamily that contains seven APOBEC3 proteins[91]. APOBEC3 subfamily belongs to the cytidine deaminase family, which includes another four members: activation-induced cytidine deaminase (AID), APOBEC1, APOBEC2, and APOBEC4[92]. Typically, APOBEC proteins have one or two copies of zinc-coordinating deaminase domain (the Z domain) that confers them RNA/DNA cytidine deaminase activities[93]. Due to the structural and functional similarities, other APOBEC3 proteins, including APOBEC3A, 3B, 3C, 3F, and 3D/E, have also been shown to restrict *vif*-deficient HIV-1 to varying degrees[94-98].

APOBEC3G has two copies of Z domains; the C-terminal Z domain catalyzes DNA C-to-U deamination, whereas the N-terminal pseudocatalytic Z domain possesses no enzymatic activity but has high affinity for RNA-binding and mediates the protein incorporation into HIV-1 particles (Figure 11)[99,100]. During viral particle assembly in the producer cell, APOBEC3G is packaged into the virion as a result of an RNA-dependent interaction between its N-terminal Z domain and the HIV-1 Gag protein[101,102]. Thus,

when the target cell is infected, APOBEC3G is also delivered into the cell, where its C-terminal Z domain deaminates cytosines during reverse transcription, causing C-to-U hypermutations on newly synthesized minus-strand viral cDNAs[103-105]. Host DNA repair enzymes recognize these uridine-containing DNAs for degradation. In addition, these minus-strand DNAs can further cause the DNA G-to-A hypermutations on the reverse complement plus-strand, which compromises viral genome integrity[92].

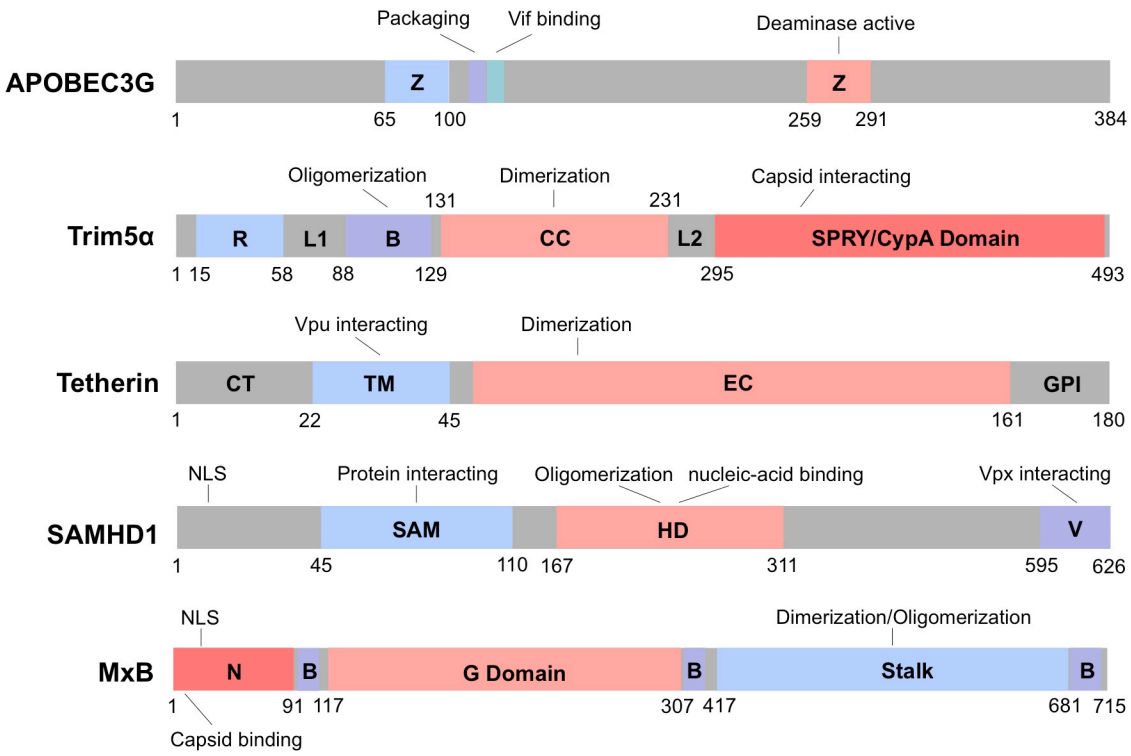


Figure 11. Schematic domain organizations of human APOBEC3G, Trim5α, Tetherin, SAMHD1 and MxB. Major domains/motifs are highlighted in color. Functions and attributes of different domains are indicated. The numbers of amino acids are also indicated.

APOBEC3G has also been shown to directly block HIV-1 reverse transcription and integration through a deamination-independent mechanism[106-110]. Reverse transcription is suppressed by APOBEC3G-mediated inhibition of $tRNA^{lys3}$-viral RNA annealing[110], transcripts elongation[108], and DNA strand transfer[106]. Notably, although this anti-HIV-1 mechanism is known as deamination-independent, the deaminase activity of APOBEC3G is still required[92,111-113].

The antiviral activity of APOBEC3G is counteracted by the viral protein Vif[114]. Briefly, Vif interacts with APOBEC3G and Cul5 E3 ubiquitin ligase complex via different motifs, leading to the assembly of an APOBEC3G-Vif-ligase complex, which subsequently induces polyubiquitylation and proteasomal degradation of APOBEC3G and, likely, Vif itself[114-118].

1.2.3 TRIM5α

TRIM5α was identified as a host restriction factor against HIV-1 in 2004[119]. It was firstly observed that HIV-1 replication was blocked before reverse transcription in Old World Monkey cells[120-122]. Similar to the case of APOBEC3G, the restriction is dominant in heterokaryons in the cell fusion experiment[120,123]. In addition, the virus was capable to avoid this restriction when the capsid sequence was manipulated[124]. Evidence above leads to the prediction that a cellular antiretroviral factor must exist and target viral capsids[123]. Thus, a cDNA-based screen of rhesus macaque genes was performed, leading to the identification of TRIM5α[119].

TRIM5α is cytoplasmic protein that belongs to the tripartite motif (TRIM)-containing protein family[125]. Human TRIM5α protein has an N-terminal TRIM domain and a C-terminal SPRY domain that are connected by linker 2 (L2) region. The TRIM domain

is further divided into a RING finger (R) motif that possesses E3 ubiquitin ligase activity, a B-box (B) motif that is linked to the R motif via linker 1 (L1) region, and a central coiled-coil (CC) motif that drives the protein dimerization (Figure 11)[126,127]. Some monkey species have another highly related antiretroviral protein known as TRIM-CypA, in which the SPRY domain of TRIM5α is functionally replaced by another protein cyclophilin A (CypA) [128].

It is not fully understood how TRIM5α directly acts to block retroviral infections. One possible mechanism is that TRIM5α directly interacts with viral capsids and accelerates the uncoating process, which leads to premature uncoating of the virus and proteasomal degradation of the viral RTC[129]. Both the RING finger motif and B-box motif are essential for the full antiviral activity of TRIM5α. The B-box motif contributes to higher-ordered oligomerazaiton of TRIM5α, which increases the interaction between TRIM5α and the viral capsid lattice[130]. The RING finger motif possesses E3 ubiquitin ligase activity[126], and TRIM5α-HIV-1 complex has been found in association with proteasomal subunits in cell[131]. Moreover, inhibition of the proteasomal pathway disrupts TRIM5α-promoted capsid disassembly, resulting in restoration of the viral reverse transcription[132]. In addition, TRIM5α may also block nuclear translocation of the viral PIC, which is the other possible inhibitory mechanism that has been proposed[132].

Besides directly inhibiting viral replication, TRIM5α also functions as a pattern recognition receptor (PRR), an immune sensor that activates the NF-κB pathway[133]. Briefly, TRIM5α is activated upon interaction with the retroviral capsid lattice[133]. The activated TRIM5α then interacts with E2 ubiquitin-conjugating protein UBC13 and Ubiquitin-conjugating enzyme complex UEV1A. The interaction triggers the production

of unanchored K63-linked ubiquitin chain, which subsequently results in the activation of TAK1. Consequently, the NF-κB signaling pathway is activated, leading to the innate PRR-mediated immune response[92].

TRIM5α functions as a restriction factor in a highly species-specific manner. It shows no antiviral activities against retroviruses that are naturally found in the same host species. On the contrary, it effectively inhibits retroviruses that are found in other host species. For example, HIV-1 is inhibited by TRIM5α proteins that are found in Old World Monkey species[119], but not by human TRIM5α, which in turn inhibits MLV and EIAV[134,135]. Hence, TRIM5α provides a formidable barrier to cross-species transmission of primate lentiviruses[80,136].

1.2.4 SAMHD1

Vpx, which is duplicated from Vpr, is a viral accessory protein encoded by HIV-2 and most SIV strains, but not HIV-1[137]. Although both Vpx and Vpr have the activities in enhancing viral replication in MDMs, such activity of Vpx has been found to be significantly higher than that of Vpr[138]. THP-1 cells, which are non-permissive to HIV-1 infection, became permissive when transduced to overexpress Vpx[139]. Heterokaryons formed by the fusion of permissive and non-permissive cell lines showed resistance to HIV-1 infection, which can be eliminated by an additional Vpx expression[140]. Evidence above supports the hypothesis that Vpx counteracts a dominant inhibitor that targets the viral reverse transcription step[138-140]. This restriction factor, SAMHD1, was subsequently identified by a combined procedure of tandem affinity chromatography and mass spectrometry[141,142].

SAMHD1 consists of an N-terminal nuclear localization domain, a sterile alpha motif (SAM) domain, a histidine-aspartic (HD) domain, and a C-terminal variable domain (Figure 11). The N-terminal domain of SAMHD1 has an NLS, which guides SAMHD1 localization in the cell nucleus[143]. The SAM domain is likely to be responsible for its protein-protein interaction[144], whereas the HD domain plays an important role in its oligomerization and nucleic-acid binding[145,146].

SAMHD1 is a dimeric dGTP-regulated deoxynucleoside triphosphohydrolase that hydrolyses cellular dNTPs in resting or non-dividing cells[147-149]. During the early step of HIV-1 infection, SAMHD1 blocks reverse transcription by reducing the intracellular dNTP pool to the level where reverse transcription cannot proceed[149]. There are also studies supporting another model of SAMHD1-mediated HIV-1 restriction. SAMHD1 additionally displays an RNase activity[150] and selectively targets RNA in the form of DNA-RNA duplexes[151]. Thus, it blocks the viral replication by catalyzing the degradation of HIV genomic RNA on the cDNA-RNA hybrid that is formed during the first step of reverse transcription[151,152].

SAMHD1-mediated restriction is counteracted by Vpx. Briefly, Vpx interacts with DDB1-Cul4A-associated-factor-1 (DCAF1) protein, which is a substrate of the Cul4A E3 ubiquitin ligase[153]. Vpx also binds to SAMHD1, leading to the recruitment of the protein to Cul4A-DCAF1 complex, followed by proteasomal degradation of SAMHD1[153].

1.2.5 Tetherin

The HIV-1 accessory protein Vpu has been found to be essential for efficient viral particle release from some cell lines, namely, HeLa cells, but not from others, such as COS cells[154,155]. Cell fusion experiments suggested the existence of a dominant inhibitor

that can be counteracted by Vpu[156]. Further experiments implied that this inhibitor is a membrane-associated cell-surface protein, as the trapped virions were released following treatment with subtilisin protease[157]. It was further shown to be IFN-inducible[158]. This restriction factor was finally identified by a cDNA microarray system-based comparison between cells with or without IFN-α treatment and was named tetherin[159].

Tetherin is a type II single-pass transmembrane protein. It comprises a transmembrane (TM) domain that is closely connected to a short N-terminal cytoplasmic tail (CT), a C-terminal glycophosphatidylinositol (GPI) lipid anchor, and a coiled-coil extracellular (EC) domain that connects the TM domain and the GPI lipid anchor[160] (Figure 11). At the cell surface, the GPI anchor mediates the attachment of tetherin to specific regions on membrane that are known as lipid rafts[160]. Similarly, the TM domain is also anchored to the lipid bilayer membrane, with the CT reaching inside the cytoplasm where it interacts with the actin cytoskeleton[160,161]. The EC domain is responsible for the protein dimerization[162]. In a dimer, two tetherin monomers are connected via intermolecular disulfide bonds formed among three cysteine residues in the EC domain (Figure 12)[163].

In the absence of Vpu, tetherin blocks the release of newly produced HIV-1 particles by directly tethering them on the surface of the producer cell[158]. Although the mechanism of this tethrin-mediated viron capture is not fully understood at the structural level, it is clear that the membrane anchoring activities of both TM and GPI, as well as the dimerization activity of the EC domain, are strictly required[164]. Moreover, studies using different approaches support the extended parallel/anti-parallel homodimer model of tetherin, in which two parallelly/anti-parallelly and covalently linked EC domains are oriented perpendicular to the cellular and viral membranes (Figure 12)[165-169]. The

captured viral particles accumulate in endosomes following internalization via receptor-mediated endocytosis (RME), and are subsequently degraded in lysosomes[157]. Similar as TRIM5α, tetherin also inhibits HIV-1 by serving as an innate immune sensor for HIV-1 assembly, which subsequently induces proinflammatory responses through NFκB signaling pathway[170].

The HIV accessory protein Vpu counteracts the antiviral function of tetherin. Vpu binds directly to tetherin through interaction between the Vpu TM domain and the tetherin TM domain[171,172]. This TM-TM interaction leads to coimmunoprecipitation of tetherin and Vpu[173,174], as well as internalization of tetherin from the cell surface[171]. In addition, Vpu-binding may also recruits tetherin to proteasomes for degradation[175,176]. It is also noteworthy that most of SIV strains lack Vpu expression, instead, they counteract tetherin-mediated restriction using another viral accessory protein, Nef[177]. In some other cases, the Env proteins of some non-human primate lentivirus species also possess the activities in counteracting tetherin[178,179].

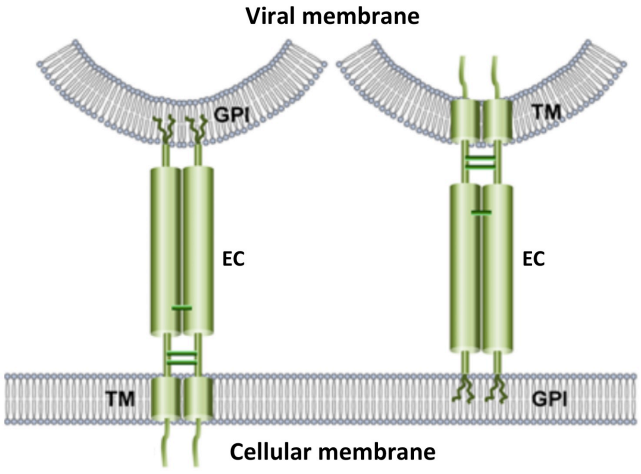


Figure 12. Extended homodimer model of tetherin[92]. Two tetherin monomers are perpendicularly anchored in both membranes with the same (parallel) or opposite (anti-parallel) orientations.

1.2.6 IFITM

The Interferon induced transmembrane proteins (IFITM) were firstly identified as anti-HIV-1 restriction factors through an shRNA-based ISG screening in SupT1 cells[180]. IFITM proteins belong to the dispanins protein family, which is characterized by a common structure with two membrane-associated domains[181]. Humans have five IFITM proteins: IFITM1, IFITM2, IFITM3, IFITM5 and IFITM10, with IFITM1, IFITM2 and IFITM3 possessing anti-viral activities[180,182].

Human IFITM proteins are composed of an N-terminal domain (NTD), an intramembrane domain (M1), a conserved intracellular loop (CIL); a transmembrane domain (M2); and a highly variable C-terminal domain (CTD) (Figure 13)[183]. It is not fully understood how this structure of IFITM proteins contributes to their anti-HIV-1 activities. However, it has been reported that the intracellular region and the C-terminal domain of IFITM1 is important for its anti-HIV-1 function[180,184]. Moreover, the N-terminal domain of IFITM3 has also been found to modulate its activity in inhibiting influenza A virus, but not HIV-1, by altering its subcellular localization[185].

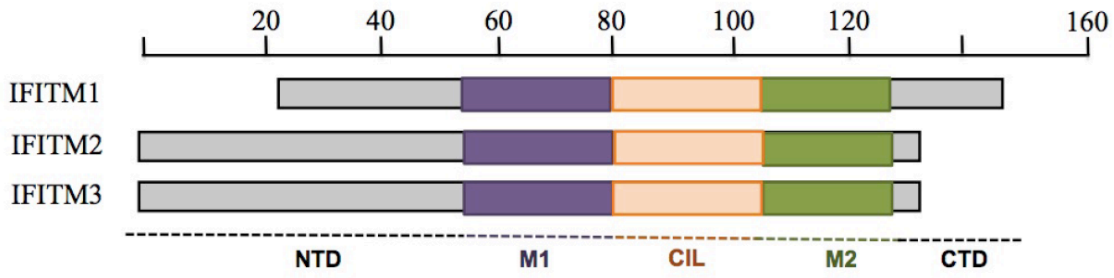


Figure 13. Domain organizations of human IFITM1, IFITM2 and IFITM3[186]. The N- and C-terminal domains are shown in grey. The intramembrane domain (purple), conserved intracellular loop (light orange) and transbambrane domain (green) are shown.

IFITM proteins inhibit HIV-1 in both virus-producing cells and target cells[187], with IFITM1 selectively inhibiting HIV-1 replication at an early step after integration but before Gag protein expression, while IFITM2 and IFITM3 inhibiting viral entry[180,183]. In producer cells, IFITM2 and IFITM3 are incorporated into nascent HIV-1 particles during assembly by interacting with Env, which decreases the viral infectivity by negatively affecting the newly produced virion entry into the target cell[187-189]. In target cells, IFITMs may also interfere with viral protein expression that is specifically mediated by double-stranded viral RNAs[190]. The IFITM restriction has been found to be overcome by viral Env mutants[189].

1.2.7 SLFN11

The anti-HIV-1 activity of Schlafen (SLFN)-11, a protein member of the SLFN family, was revealed in 2012[191]. Similar to other ISGs, the expression of SLFN11 is strongly induced by type I IFN. Induction of SLFN-11 subsequently leads to a selectively reduced expression level of viral proteins but not host proteins[191]. Although the mechanism of SLFN11-mediated HIV-1 restriction is not fully understood at the structural level, it has been shown that SLFN11 inhibits the viral replication at a late stage of the viral mRNA translation in a codon-usage-dependent manner[191]. More specifically, SLFN11 binds directly to cellular tRNAs, leading to a selective reduction of the tRNAs that are bearing rare viral codon bias by an unknown mechanism[191]. Consequently, HIV-introduced change in the tRNA pool, which is crucial for the viral replication, is prevented by SLFN-11 on the basis of codon usage discrimination[191,192].

1.2.8 MxB

In 2013, three groups independently and simultaneously reported the anti-HIV-1 activity of human MxB, one of the two closely related IFN-inducible myxovirus resistance (MX) proteins in humans[193-195]. The two MX proteins, MxA and MxB, are large GTPases that belong to the dynamin-like family of proteins and both have anti-viral activities against different virus species[196,197].

MxB is composed of an N-terminal region, a GTPase (G) domain, and a C-terminal stalk domain, which are connected by three bundle signaling element (BSE/B) domains (Figure 11)[198]. The N-terminal region of MxB contains both an HIV-1 capsid-binding motif that is crucial for its anti-HIV-1 activity[199,200] and a nuclear localization signal (NLS) that helps MxB localizes to the nuclear rim[196]. The C-terminal stalk domain of MxB is responsible for the protein oligomerization, which is also required for the restriction of HIV-1[201,202].

MxB directly binds to HIV-1 capsids via a triple-Arginine motif in its N-terminal region[199] and blocks the virus replication after reverse transcription but before integration, through a mechanism that is not fully understood[193-195]. Although the NLS of MxB has been shown to be dispensable for its anti-HIV-1 activity[199,200], many studies support the idea that MxB restricts HIV-1 by deterring the nuclear import and integration of the viral DNA[195,202-205]. It has also been reported that MxB may prevent the uncoating process of HIV-1[197]. Notably, MxB only binds to the intact HIV-1 capsid assemblies, but not to the CA protein monomer or hexamers, which indicates a possible role of MxB as an immune sensor for HIV-1 capsids[206]. In addition, the anti-HIV-1 activity of MxB also requires dimerization, but not higher-ordered oligomerization, of the protein[201,206]. As viral

capsids are the targets of MxB, HIV-1 has been found to escape MxB restriction by introducing mutations in its capsid sequence[193-195].

1.2.9 Newly identified anti-HIV-1 host factors

In recent two years, several host proteins have been identified as anti-HIV-1 factors, including GBP5, MARCH8, and SERINC3/5.

Guanylate Binding Protein (GBP) 5 is an IFN-inducible GTPase that belongs to the GBP subfamily. It was identified as a potential HIV-1 restriction factor in 2015 by a genome-wide screen[207]. In virus-producing cells, GBP5 interferes with HIV-1 Env processing and incorporation, which reduces the infectivity of newly produced viral particles[208]. Moreover, the Golgi localization of GBP5 is crucial for its antiviral activity, whereas the GTPase activity is dispensable[208]. GBP5 restriction is counteracted by naturally occurring start codon mutations in the viral *vpu* gene, which leads to an increase of Env expression and a tolerable loss of Vpu function[208].

Membrane-associated RING-CH 8 (MARCH8) belongs to the MARCH family of RING-finger E3 ubiquitin ligases[209,210]. The anti-lentiviral function of MARCH8 was firstly observed when a MARCH8-expressing lentiviral vector showed extremely low infectivity when used for transduction[211]. Similar as GBP5, MARCH8 targets HIV-1 Env in the virus-producing cells and reduces the infectivity of newly produced virions[208,210]. Briefly, MARCH8 downregulates the viral Env from surface of the infected cell, leading to reduced incorporation of Env into the nascent viral particles[211]. The viral infectivity is thus decreased at the step of entry into target cells[211].

The anti-HIV-1 activities of serine incorporator 3 (SERINC3) and SERINC5 were reported in 2015 by two groups using different approaches[212,213]. Although it still remains

debatable whether these two proteins can be classified as host restriction factors due to the fact that they are not IFN-inducible and no positive-selection signature has been observed[212], it is clear that they are potent inhibitors of HIV-1 infectivity and are counteracted by the HIV-1 Nef protein[212,213]. SERINC3 and SERINC5 are multipass transmembrane proteins that have around 9-10 transmembrane domains. When expressed in the virus-producing cells, they localize to the plasma membrane and incorporate into the nascent HIV-1 particles during virions budding[212,213]. Similar to the cases of IFITM2/3, GBP5 and MARCH8, the incorporation of SERINC3/5 potently impairs the infectivity of the newly produced HIV-1 particles by negatively affecting their entry into target cells[212,213]. The HIV-1 accessory protein Nef antagonizes SERINC3/5 restriction by preventing the incorporation of SERINC3/5 into HIV-1 virions[212,213]. More specifically, Nef downregulates SERINC5 from the cell surface by redirecting the protein to a Rab7-positive endosomal compartment[212].

1.3 Cell death: apoptosis, autophagy, necrosis and cornification

Cell death occurs through different mechanisms. Based on the underlying mechanisms, the Nomenclature Committee on Cell Death (NCCD) proposed that cell death could be classified into four major types: apoptosis, autophagy, necrosis and cornification (Table 1), as well as several atypical modalities including mitotic catastrophe, excitotoxicity, anoikis, and Wallerian degeneration[214,215]. The four typical cell death modalities will be briefly introduced below.

Table 1. Four typical modalities of cell death[215]

Cell death mode	Morphological features
Apoptosis	Rounding-up of the cell Retraction of pseudopodes Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, *in vivo*
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, *in vivo*
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation (loss of corneocytes) by protease activation
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation

1.3.1 Apoptosis

Apoptosis is the major mode of programmed cell death (PCD) in multicellular organisms that is accompanied by characteristic cell changes including cell shrinkage, plasma membrane blebbing, global mRNA decay, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation[214]. Two major activation mechanisms have been found to initiate cell apoptosis: the intrinsic pathway (or the mitochondrial pathway) and the extrinsic pathway (or the death-receptor pathway) (Figure 12)[216].

In the mitochondrial pathway, the initiation of apoptosis is determined by a tripartite apoptotic switch, which consists of three protein subgroups of the BCL-2 family: BH3-only proteins, pro-survival cell guardians, and pro-apoptotic effector proteins BAX and BAK[217]. Once activated by cytotoxic stimuli, such as oncogenic stress, BH3-only proteins initiate apoptosis by inhibiting the pro-survival cell guardians, leading to the activation of the pro-apoptotic effectors BAX and BAK. The activated BAX and BAK form protein oligomers that disrupt the mitochondrial outer membrane, enabling the release of cytochrome c, an apoptogenic factor, to the cytosol[218]. Cytochrome c promotes the activation of caspase-9 on APAF1, which subsequently activates the effector caspases, such as caspase-3[219].

The death-receptor pathway is activated by death receptors of the tumor necrosis factor receptor (TNFR) family, among which the cell surface-bound receptor FAS (first apoptosis signal) is best characterized. FAS is activated when ligating to its physiological ligand FASL on the cell plasma membrane, leading to the assembly of a death-inducing signaling complex (DISC) which contains caspase-8, caspase-10, FADD and c-FLIP[220].

FAS interacts with FADD and promotes the FADD-mediated recruitment of caspase-8, which in turn activates effector caspases and triggers the execution of the apoptotic cell[221].

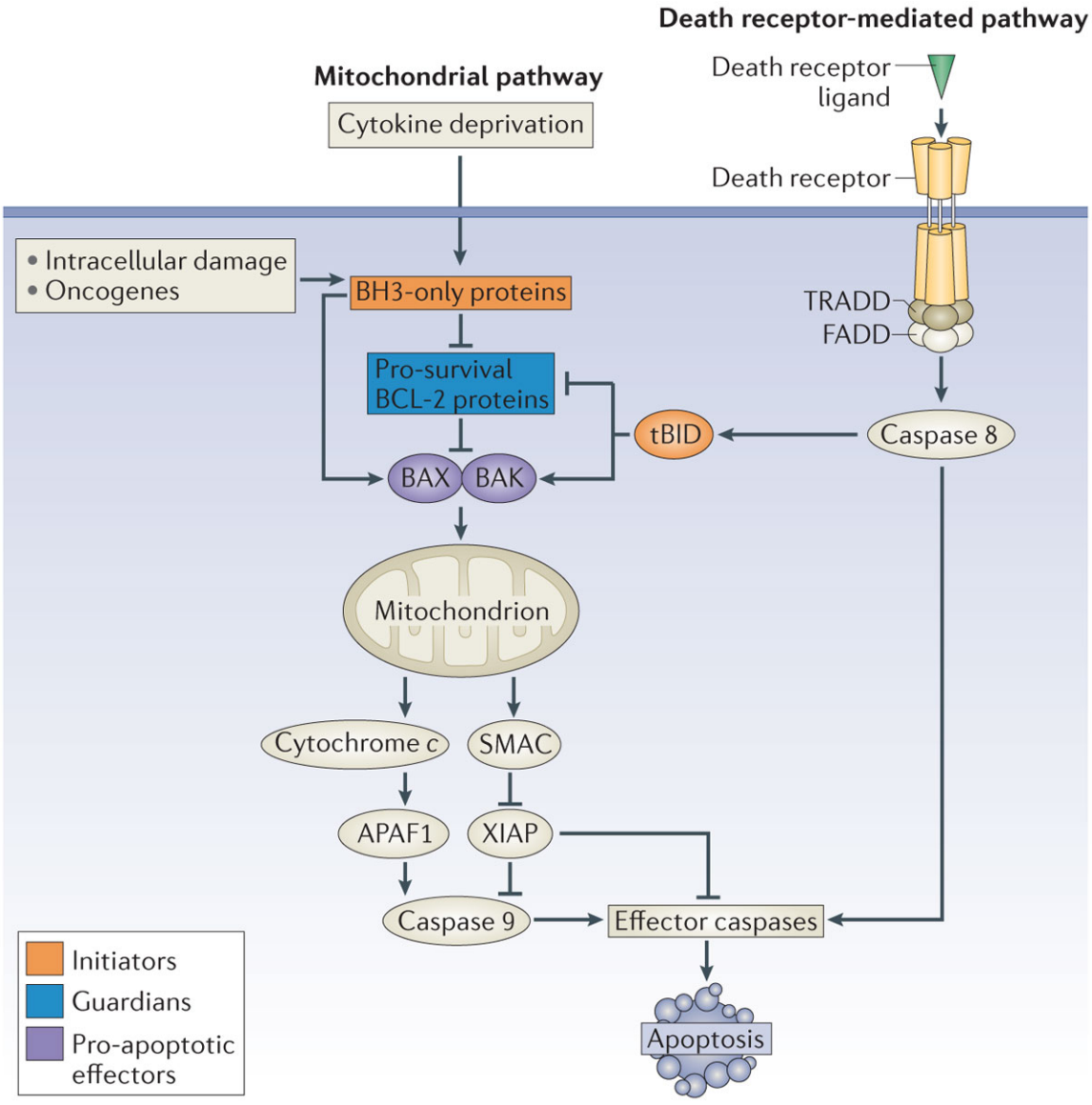


Figure 14. Two major apoptotic pathways in mammalian cells[217]. The mitochondrial pathway (left) and death receptor pathway (right) are shown

1.3.2 Autophagy

Autophagy, also known as type 2 PCD, is a self-degradative process that leads to a highly organized non-apoptotic form of cell death[222]. This process is mainly characterized by degradation of cytoplasmic components at lysosomes, thus to eliminate misfolded or aggregated proteins, damaged cellular organelles, as well as intracellular pathogens[223]. Autophagy is further classified into three types: macro-autophagy, micro-autophagy, and chaperone-mediated autophagy, among which macro-autophagy is best characterized. Unless specified, the term "autophagy" usually refers to macro-autophagy. The process of macro-autophagy is summarized below (figure 15)[222]. Briefly, the target cytoplasmic component is firstly enclosed by a unique membrane called the phagophore or isolation membrane to form a double-membraned vesicle known as the autophagosome. Next, the outer membrane of autophagosome is fused with an endosome and then a lysosome, leading to the formation of the autophagosome where the internal material is degraded by lysosomal acid.

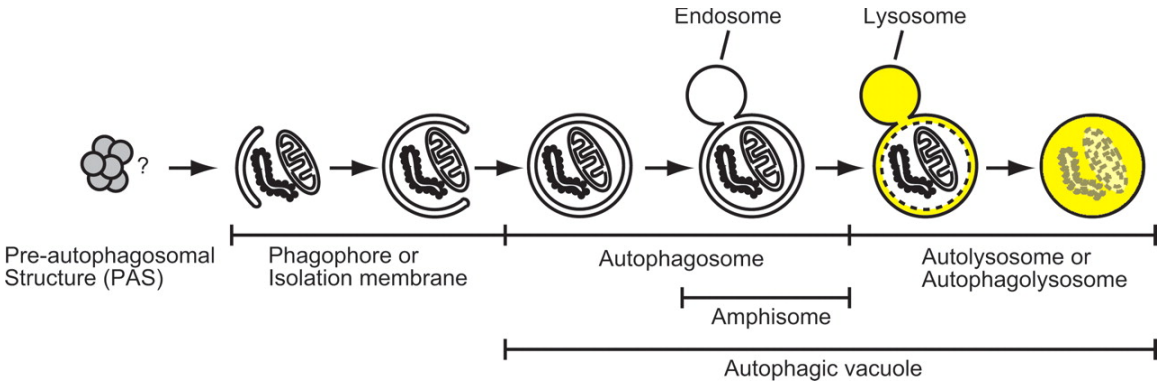


Figure 15. Summary of macro-autophagy in mammalian cells[222]. The process from the PAS (yeast) initiation to autophagosomal contents degradation is shown.

1.3.3 Necrosis

Necrosis, also known as necrotic cell death, is usually considered as a premature cell death caused by autolysis of injured cells[224]. Morphological characteristics of necrosis include an initial gain in cell volume, organelle swelling, early plasma membrane rupture and subsequent loss of intracellular contents[225]. On the contrary to the previous opinion that necrosis is an accidental and unregulated cellular event, studies have shown the existence of programmed necrosis, also known as necroptosis, which is executed by regulated mechanisms (Figure 16)[226,227]. Under normal conditions, a balance between apoptosis and necrosis is reached due to an inhibitory function of the death-receptor apoptotic pathway on necrosis. Briefly, the execution of necrosis requires the enzymatic activity of protein RIP kinase 1 (RIPK1)[228], which functions together with another highly related protein RIPK3[229]. The two proteins form a RIPK1-RIPK3 pro-necrotic complex necrosome that regulates programmed necrosis[230]. When death-receptor apoptotic pathway is intact, caspase-8 cleaves both RIPK1 and RIPK3, thus inhibiting the necrotic cell death[231]. Other components of DISC, such as FADD and FLIP, have also been shown to negatively regulate the necrosome function[226].

1.3.4 Cornification

Cornification is a specific form of PCD that occurs in the epidermis of multicellular organisms, leading to the formation of a dead keratinocyte specifically named corneocyte. Corneocyte contains an amalgam of specific proteins and lipids, which enable it to serve varies functions, such as mechanical resistance, elasticity, water repellence and structural stability. Cornification is a specific process of terminal epithelial differentiation during which cells express a plethora of enzymes including proteases, as well as their

substrates[232]. Specific lipids are also synthesized and released into the extracellular space where they interact with cornified envelope proteins to build up an epidermal barrier[215].

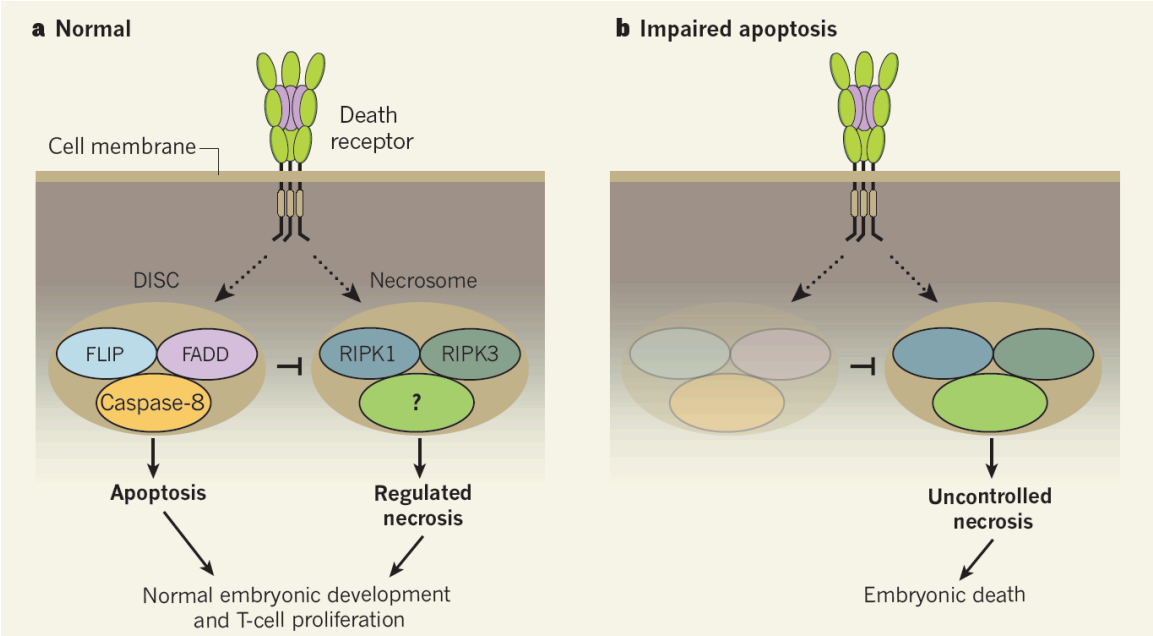


Figure 16. Crosstalk between DISC and the necrosome in embryonic development[227]. **a.** Under normal condition, a balance between apoptosis and necrosis is reached, leading to normal embryonic development and T-cell proliferation. **b.** When the DISC is impaired, the death-receptor pathway is thus disrupted, leading to uncontrolled necrosis and, subsequently, the death of the embryo.

1.4 Rationale, hypothesis and objectives

It has long been known that type I and type II IFNs play important roles in promoting apoptosis in multiple cell types with both the mitochondrial and the death-receptor pathways involved[233-236]. Although the mechanisms behind their pro-apoptotic effects are not fully understood, type I IFNs themselves are not likely to be the direct effectors; rather, they function by up-regulating certain ISGs that either serve as or subsequently induce downstream intermediate effectors of cell apoptosis[236,237]. Many ISGs that are involved in cell apoptosis pathways have been identified, including pro-apoptotic innate sensors such as TLRs[238], as well as some caspases such as caspase-4 and caspase-8[237]. Some other ISGs have also been shown to possess pro-apoptotic activities, implying that they may play roles in IFN-induced apoptosis. Among these ISGs, human MxA, which is a potent antiviral host protein, promotes cell apoptosis triggered by endoplasmic reticulum stress under the conditions of the influenza virus infection as well as apoptotic stimuli induction[239-241]. Enhanced apoptosis in virus-infected cells leads to a reduced virus production, therefore, this pro-apoptotic activity of MxA may further contribute to its antiviral function[239]. Similarly, MxB is also an IFN-inducible antiviral host protein that selectively inhibits lentiviruses including HIV-1[193-195]. In our study, significantly increased cell death has been observed when U87-$CD4^+$/$CXCR4^+$ cells were transduced to overexpress MxB, implying a possible pro-apoptotic activity of MxB that is similar to MxA. We therefore hypothesize that MxB plays a role in promoting cell apoptosis.

In this project, we aimed to investigate a potential new function of the IFN-inducible anti-HIV-1 host protein, MxB, in apoptosis promotion. In order to verify if MxB induces cell apoptosis, the apoptotic cell ratio was monitored in U87-$CD4^+$/$CXCR4^+$ cells that

were transduced to overexpress MxB by Annexin V staining. The cell samples were collected at different time points in order to understand the dynamic process of the MxB-induced apoptosis. As MxA has been reported to enhance cell death induced by the influenza virus infection as well as other apoptotic stimuli, we tested if MxB also promotes apoptosis under different conditions including HIV-1 infection, oxidative stress, as well as serum starvation. Activated caspase-3 level was also detected to further verify the MxB-promoted apoptosis. Next, we wanted to see if the IFN-induced endogenous expression of MxB is sufficient in apoptosis promotion. To test this, the MxB knockout experiment was performed in U87MG cells with the CRISPR/Cas9 system. Finally, we tested the pro-apoptotic activities of different MxB loss-of-function mutants. By doing so, we hoped to identify the functional regions that are essential for the activity of MxB in promoting cell apoptosis.

Chapter 2-Materials and Methods

2.1 Antibodies

The primary antibodies used for immunoblotting are: mouse monoclonal anti-Flag (Sigma-Aldrich) (1:5000), mouse monoclonal anti-β-Tubulin (Santa Cruz Biotechnology) (1:5000), rabbit polyclonal anti-MxB (1:500), cleaved caspase-3 (Asp175) antibody (Cell Signalling) (1:1000). The secondary antibodies used for immunoblotting are: horseradish peroxidase-linked donkey anti-rabbit IgG (GE Healthcare) (1:10000) and horseradish peroxidase-linked sheep anti-mouse IgG (GE Healthcare) (1:10000).

The antibodies used to stain cells for flow cytometry are: antibody for the detection of FLAG™ conjugated proteins (MOUSE) Monoclonal Antibody (Rockland) (1:1000), Fluorescein Isothiocyanate (FITC)-conjugated mouse monoclonal KC57 antibody (1:100), Phycoerythrin (RD1)-conjugated mouse monoclonal KC57 antibody (1:100).

2.2 Cell lines and culture conditions

U87MG cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Wisent Bio Products) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher), 1% Penicillin/Streptomycin (P/S) (Thermo Fisher), and 1% L-glutamine (Thermo Fisher).

U87-$CD4^+$/$CXCR4^+$ cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher) supplemented with 15% FBS, 1% P/S, 1% L-glutamine, 2 μg/mL puromycin (Sigma-Aldrich), and 1 mg/mL G418 sulfate (Invitrogen).

U87MG-MxB-Knockout cell lines were maintained in EMEM supplemented with 10% FBS, 1% P/S, 1% L-glutamine, and 1 μg/ml puromycin.

SupT1 cells were maintained in RPMI media 1640 (Thermo Fisher) supplemented with

10% FBS, 1% P/S, and 1% L-glutamine.

Tetracycline/doxycycline inducible Control-/MxA-/MxB-/IFITM-expressing SupT1 cell lines were maintained in RPMI media 1640 supplemented with 10% FBS, 1% P/S, 1% L-glutamine, 2 μg/mL puromycin, and 1 mg/mL G418 sulfate.

Human embryonic kidney (HEK293T) cells were maintained in DMEM supplemented with 10% FBS and 1% P/S.

Cells were grown in CO_2 incubators at 37°C with 5% CO_2 concentration.

2.3 Transfections

4×10^6 HEC 293T cells were seeded in 10cm cell culture dishes 20 hours before transfections. 8.5μg plasmids, 25.5μl PEI (1μg/μl) and 1ml Opti-Modified Eagle Medium (opti-MEM) (Invitrogen) were mixed by vortexing and incubated in room temperature for 20 minutes. The mixtures were then added into the dishes to transfect cells. The media was changed 4 to 6 hours following transfection.

2.4 Preparation of retroviral vectors

MLV-based retroviral vectors were produced by cotransfection of HEK293T cells with plasmids encoding the MLV-gag/pol, VSV-G and the target proteins. The transfection was done in 10cm plates with 4.0μg of MLV-gag/pol, 4.0μg of plasmids encoding the target proteins and 0.5μg of VSV-G. 48 hours after transfection, the cultured supernatant was harvested and centrifuged at 3000rpm, 4°C for 20 minutes to remove the cell debris. The supernatant was then divided into aliquots and stored at - 80°C.

2.5 Transduction

0.3×10^6 U87-$CD4^+$/$CXCR4^+$ cells were seeded 6-well tissue culture plates with 1.5ml of drug-free media 16 hours before transduction. Indicated amount of retroviral vectors

were added to each well with 3µl of polybrene (5mg/µl). The cells were spinoculated at 1800rpm, room temperature (25°C) for 45 minutes. The media was changed 24 hours following transduction.

2.6 PE Annexin V/7-AAD staining

PE Annexin V/7-AAD staining was performed with the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen™). U87-CD4^{+}/CXCR4^{+} cells were harvested by trypsinization and were centrifuged together with the cultured supernatant at 1200rpm, 4°C for 5mins. The cells were then moved to 1.5ml centrifuge tubes and were washed twice with cold Phosphate-buffered saline (PBS) (Thermo Fisher). The cells were resuspended in 50µl of the 1× Binding Buffer with 2µl PE Annexin V and 2µl 7-AAD. The cells were incubated for 15 min at RT in the dark and were then washed once with 1ml of the 1× Binding Buffer. The cells were finally fixed in 1ml of 2% Paraformaldehyde (PFA) and stored at 4°C in the dark or subjected to further intercellular staining and flow cytometry.

2.7 Intercellular staining

Cell samples were fixed in 2% PFA at 4°C for 30 minutes and were washed once with 1ml of PBS containing 2% of FBS. Cells were washed twice with the 1× Permeabilization buffer. Antibodies were diluted in the 1× Permeabilization buffer according to the manufacturer's instructions. The cells were resuspended with 50µl of the diluted antibodies and were incubated on ice for 1 hour in the dark. After the incubation, the cells were washed three times with the 1× Permeabilization buffer and were then resuspended in 500µl of PBS containing 2% of FBS. The samples were analyzed by flow cytometry immediately.

2.8 Preparation of virus stocks

The VSV-G-pseudotyped HIV-1 is a chimeric virus composed of the HIV-1 core and the envelope glycoprotein of vesicular stomatitis virus (VSV-G). In our study, the VSV-G-pseudotyped virus (NL4-3/VSV-G) was produced by cotransfection of HEK293T cells with plasmids encoding the proviral DNA of the HIV-1 stain NL4-3 and VSV-G. The virus was collected and stored as described in *Chapter 2.4*.

2.9 Western blot

U87-$CD4^+$/$CXCR4^+$ cells, SupT1 cells and U87MG cells were harvested and washed once in cold PBS. The cells were then lysed for 30 minutes on ice in the lysis buffer. Cell lysates were purified by centrifuging the lysed cells at 32000rpm, 4°C for 15 minutes. 60µl of the purified cell lysates were mixed with 20µl 4×loading buffer and 10µl of the mixed sample was loaded and run on SDS-polyacrylamide gels (SDS-PAGE). Proteins were transferred on PVDF membranes (Roche) and the membranes were blocked in 5% non-fat milk dissolved in PBST (0.05% Tween-20 in PBS) at RT for 1 hour. The membranes were then incubated with the primary antibody at RT for 2 hours or at 4°C overnight, washed 5 times with PBST solution and then incubated with the secondary antibody for 1 hour. The signals were visualized by applying enhanced chemiluminescence (ECL) substrate (PerkinElmer).

2.10 Constructing MxB-Knockout U87MG cell lines

The MxB-Knockout U87 cell lines were constructed using the CRISPR/Cas9 system. The LentiCRISPR v2 plasmid was purchased from Addgene. The target guide sequences were designed with the optimized CHRISPER design tool: tools.genome-engineering.org. (Figure 17) and were cloned into the lentiCRISPRv2 backbone according to the

manufacturer's instructions. Lentivirus vectors were produced by cotransfection of HEK293T cells with plasimds encoding LentiCRISPRv2 backbone, VSV-G and psPAX2. U87MG cells were then transduced with the lentivirus vectors to express the Cas9 nucleases that target different regions of MxB gene. Two days after transduction, the cells were selected with 2 μg/ml of puromycin for 7 days. The selected cell lines were kept in media containing 1 μg/ml of puromycin.

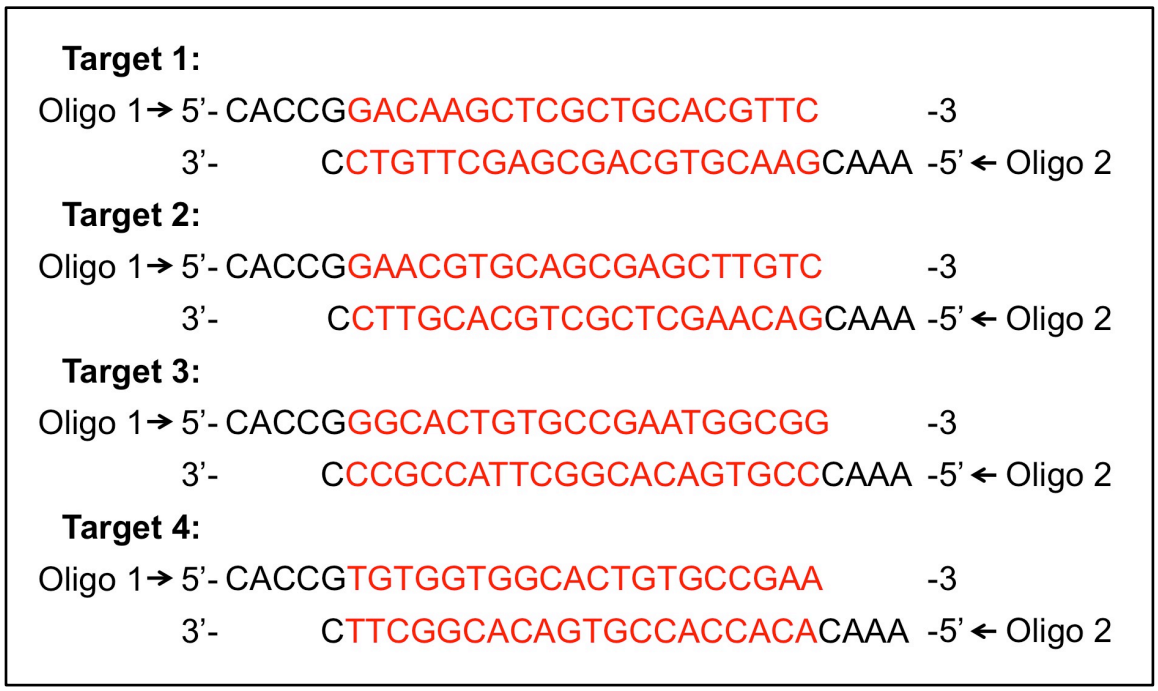


Figure 17 Oligo sequences designed for MxB Knockout using CRISPR/Cas9 system. Four pairs of oligos indicating different target sequences were designed with the optimized CHRISPER design tools (tools.genome-engineering.org) and are shown in red. Target 1 and Target 2 were selected on the 5'UTR of MxB, Target 3 and Target 4 were selected on the CDS of MxB.

2.11 Constructing doxycycline-inducible SupT1 cell lines

Doxycycline-inducible MxA, MxB and IFITM3 SupT1 cells were constructed previously as described[193]. Retroviral vectors were constructed by transfecting the vector DNAs (Retro-X Tet-On, Retro-X-Pur, Retro-X-MxA, Retro-X-MxB or Retro-X-IFITM3) into the GP2-293 packaging cells (Clontech). Retrovirus particles were collected as described in *Chapter 2.4*. 3×10^6 SupT1 cells were spinoculated with the Retro-X TetOn virus particles at 1800rpm, room temperature for 45 minutes. The stably transduced cells were selected with 1mg/ml G418 and were then infected with retroviral vectors encoding Retro-X-MxA, Retro-X-MxB, and Retro-X-IFITM3. The cells were kept in media supplemented with both 2μg/ml puromycin and 1mg/ml G418 for further selection. The expression of the target proteins was verified by western blot and flow cytometry following doxycycline induction.

2.12 Sodium arsenite, arsenic trioxide and hydrogen peroxide treatment

U87-$CD4^+$/$CXCR4^+$ cells were transduced to overexpress the target proteins. The cells were kept for another 3 days before the treatment. 2×10^6 of Doxycycline-inducible SupT1 cells were seeded in 12-well tissue culture plates and were treated with 500ng/ml doxycycline for 16 hours to induce the expressions of the target proteins. 0.4×10^6 of the MxB-Knockout U87MG cells were seeded in 6-well tissue culture plates for 24 hours and were then treated with 500Units/ml of IFNα2b (provided by the Jewish General Hospital) for 16 hours. Indicated concentrations of sodium arsenite ($NaAsO_2$), Arsenic Trioxide (As_2O_3) or Hydrogen Peroxide (H_2O_2) were then added into the media and were kept for 24 hours before the cells were harvested for further experiments.

2.13 Serum starvation

U87-CD4^{+}/CXCR4^{+} cells were transduced to overexpress the target proteins. 24 hours after transduction, the cells were kept in serum-free DMEM supplemented with 1% P/S, 1% L-glutamine, 2 μg/mL puromycin, and 1 mg/mL G418 sulfate for 72 hours before the cells were harvested for further experiments.

Chapter 3-Results

3.1 MxB induces apoptosis in U87-CD4$^+$/CXCR4$^+$ cells in a dose-dependent manner

In order to verify if MxB induces cell apoptosis, U87-CD4$^+$/CXCR4$^+$ cells were transduced to overexpress flag-tagged MxA or MxB proteins. Cells were kept in DMEM containing puromycin and G418 in order to maintain the expression of CD4 and CXCR4. Five days after transduction, cells overexpressing MxB exhibited a significantly increased apoptosis ratio (~4-fold) compared to the control group. However, the apoptosis ratio of cells that were overexpressing MxA was decreased (Figure 18A). This result suggested that MxB, but not MxA, induces cell apoptosis in U87-CD4$^+$/CXCR4$^+$ cells.

Next, we tested if the ratio of this MxB-induced apoptosis is correlated to the expression level of MxB. U87-CD4$^+$/CXCR4$^+$ cells were transduced with escalated-dose retroviral vectors. The result showed that the apoptosis ratio of MxB-expressing cells was increased as we increased the level of MxB expression (Figure 18B). On the contrary, the apoptosis ratio was not significantly changed in either the MxA or the control groups that were transduced by retroviral vectors at doses of up to 200µl. At the dose of 500µl, however, both the MxA and the control group showed an increase (~1.5-1.8 fold) in apoptotic cell ratio, indicating that 500µl dose is too high for this experiment. Therefore, in the following experiments, 200µl of retroviral vectors were used for transduction of U87-CD4$^+$/CXCR4$^+$ cells.

Taken together, the results above suggested that human MxB protein induces apoptosis in U87-CD4$^+$/CXCR4$^+$ cells in a dose-dependent manner.

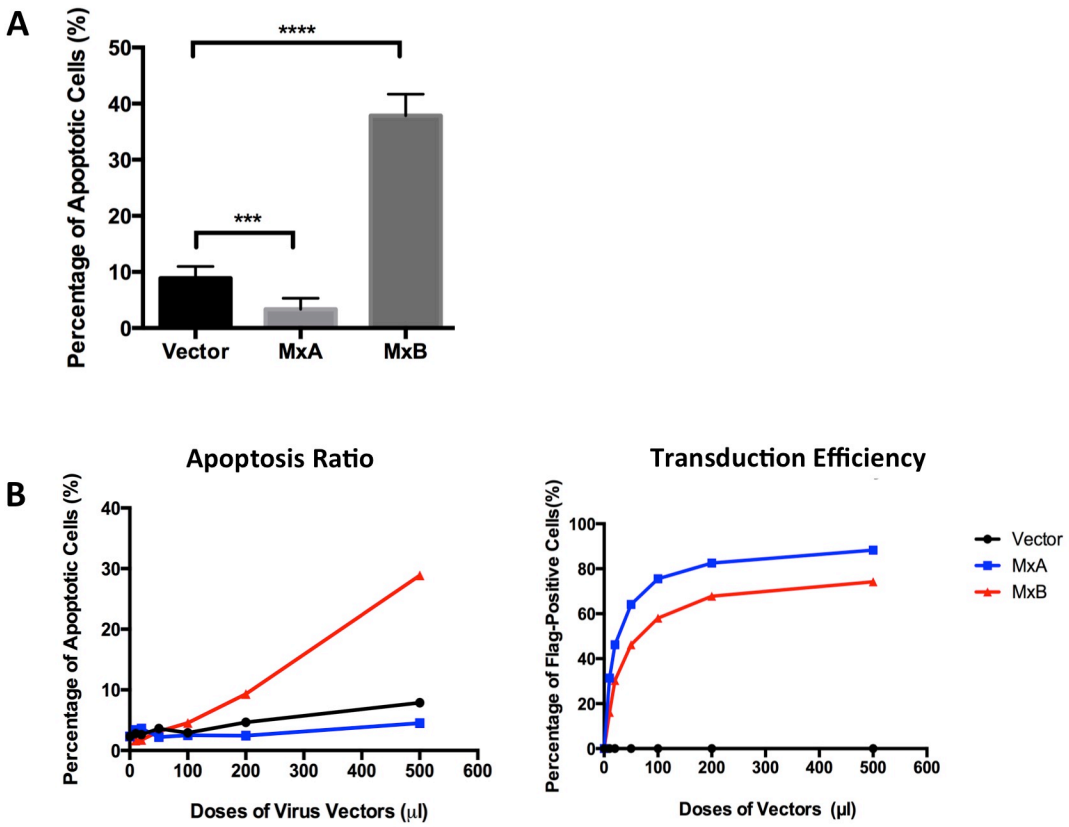


Figure 18 MxB-induced apoptosis in U87-CD4$^+$/CXCR4$^+$ cells (A) MxB induces cell apoptosis. U87-CD4$^+$/CXCR4$^+$ cells were transduced with 500μl retroviral vectors that packaged RNAs encoding MxA or MxB. 500μl blank vector of pQCXIP2.0 was transduced in the control group. Cells were kept in DMEM containing puromycin (2μg/ml) and G418 (1mg/ml) for another 5 days. Cell apoptosis was then detected by PE Annexin V staining. The data represents 5 independent experiments. (B) Dose-dependent promotion of apoptosis by MxB. U87-CD4$^+$/CXCR4$^+$ cells were transduced with 50μl, 100μl, 200μl, or 500μl MxA or MxB retroviral vectors. Same doses of pQCXIP2.0 blank vector were transduced in the control group. Cells were kept in DMEM containing puromycin and G418. Five days after transduction, cells were harvested and stained with PE Annexin V or anti-flag antibody to examine the apoptosis ratio and the transduction efficiency, respectively.

3.2 MxA inhibits apoptosis while MxB induces apoptosis

To understand the dynamic process of MxB-induced apoptosis in U87-$CD4^+$/$CXCR4^+$ cells, a time-course experiment has been performed to monitor the apoptosis ratio from day 2 to day 5 after transduction. In the MxB group, apoptotic cell number began to rise significantly on day 3 after transduction and peaked on Day 4 (Figure 19A). On day 5, however, the percentage of apoptotic cell population decreased and shifted to Annexin V negative region, likely because cells expressing high level of MxB underwent apoptosis and died earlier, shrinking to a size too small to be gated. In contrast, the number of apoptotic cells in the MxA group, which was consistently lower than that of the control group, remains unchanged until day 4, suggesting a delayed apoptosis in MxA-expressing cells (Figure 19A). More strikingly, in the MxA group, apoptosis ratio in the flag-positive population was significantly lower compared to that in the flag-negative population (Figure 19B). These results are strong evidence suggesting that apoptosis is inhibited in U87-$CD4^+$/$CXCR4^+$ cells by expression of MxA.

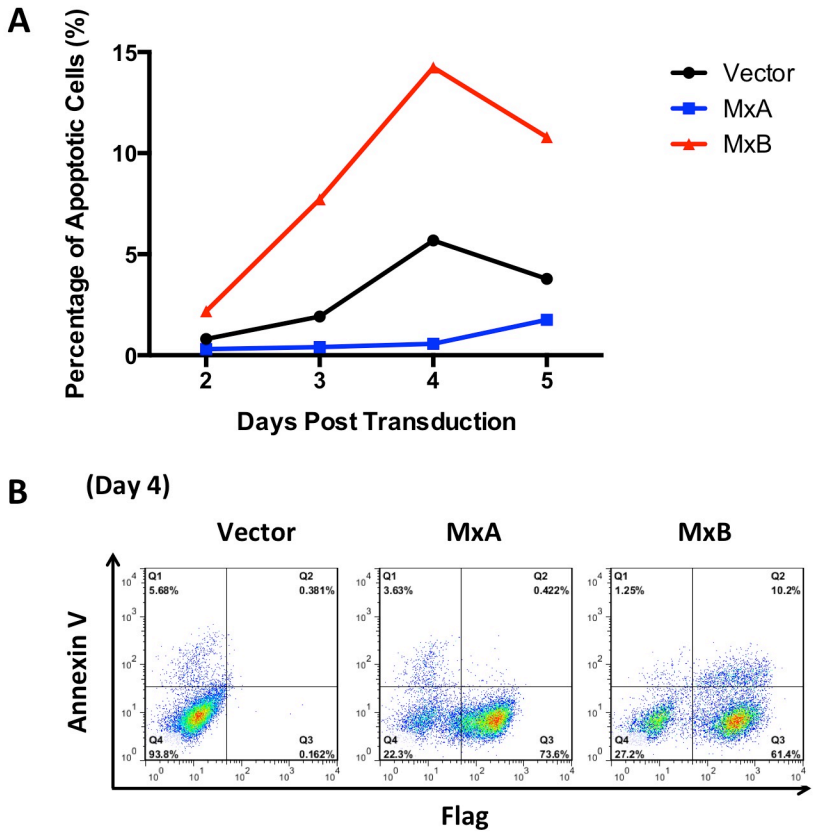


Figure 19. Time-course experiment of MxB-induced apoptosis. U87-CD4^{+}/ CXCR4^{+} cells were transduced with 200µl MxA or MxB retroviral vectors. 200µl blank vector was transduced in the control group. Cells were kept in DMEM containing puromycin and G418. Apoptotic cell percentages and the percentages of the target protein-expressing cells were measured on day 2, 3, 4 and 5 by flow cytometry following the double staining with PE Annexin V and anti-flag antibody. Figure B shows the original flow cytometry data of the control, MxA, and MxB group on Day 4.

3.3 HIV-1 infection doesn't promote MxB-induced apoptosis

MxA, a restriction factor against influenza virus, has been shown to promote apoptosis under the condition of influenza virus infection[239]. In order to test if HIV-1 infection also plays a role in MxB-induced apoptosis, VSV-G-pseudotyped HIV-1 (NL4-3/VSV-G) was used to infect U87-$CD4^+$/ $CXCR4^+$ cells that were transduced to overexpress MxA or MxB. Puromycin and G418 were removed from the media during the experiment to prevent possible interference with viral infection. Apoptosis ratio and infection efficiency were measured 48 hours after viral infection. The apoptosis ratio was not significantly changed in all the groups that were tested, indicating that HIV-1 infection is not likely to be a condition that promotes MxB-induced apoptosis. Also, notably, as MxB inhibits HIV-1 infection, the p24-positive cell number in MxB-expressing cells was much lower compared to that in the MxA-expressing cells and the control cells. The absence of significant change in the cell apoptosis ratio in the MxB group could also be caused by the low infection efficiency (<0.5%). Nevertheless, this experiments suggested that the existence of infectious HIV-1 viral particles does not promote MxB-induced apoptosis.

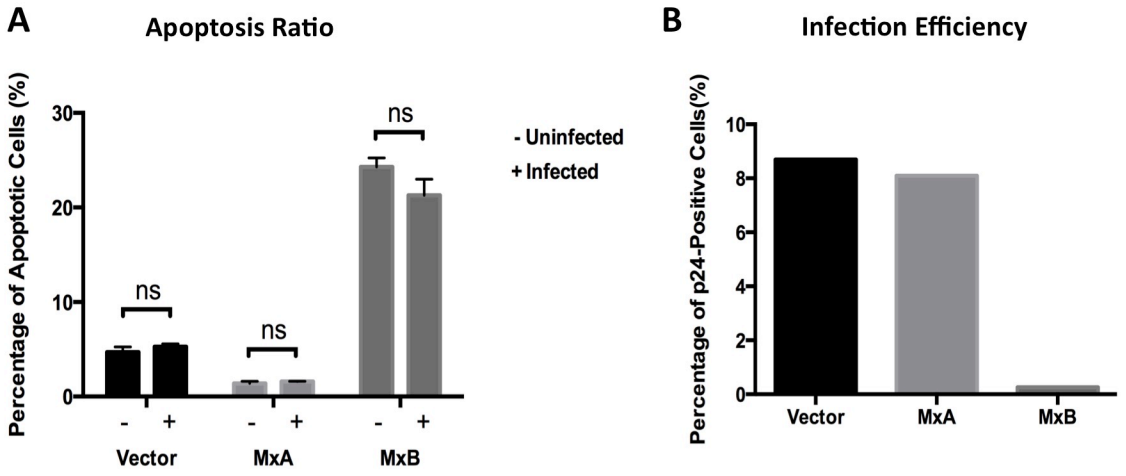


Figure 20. HIV-1 infection in MxB transduced cells. U87-CD4^{+}/ CXCR4^{+} cells were transduced with 200µl MxA or MxB retroviral vectors. 200µl blank vector was transduced in the control group. Cells were kept in DMEM without puromycin and G418. 24 hours after transduction, the cells were infected with 30µl VSV-G-pseudotyped HIV-1 (NL4-3/VSV-G). Cells were stained with PE Annexin V and anti-flag antibody to test the apoptosis ratio 4 days after transduction. The infection efficiency was tested with anti-flag antibody and KC57 antibody that detects HIV-1 capsid protein p24.

3.4 MxB promotes oxidative stress-induced apoptosis in different cell lines

Oxidative stress, a condition in which the generation of reactive oxygen species (ROS) overwhelms the cell's natural antioxidant defences, plays a central role in regulating cell apoptosis[242]. It has been shown that oxidative stress leads to apoptosis in multiple cell types via both the mitochondria-dependent and mitochondria-independent pathways[243]. In order to test if MxB plays a role in promoting oxidative stress-induced apoptosis, we compared the apoptosis ratio in both U87-CD4$^+$/ CXCR4$^+$ cells and SupT1 cells that were overexpressing MxB under normal condition or under the condition of sodium arsenite treatment.

3.4.1 MxB promotes oxidative stress-induced apoptosis in U87-CD4$^+$/ CXCR4$^+$ cells

U87-CD4$^+$/ CXCR4$^+$ cells were shown not to be sensitive to the oxidative stress, as in the control group, the apoptotic cell ratio remained unchanged with up to 5μM sodium arsenite treatment for 24 hours (Figure 21A and B) and the expression of activated caspase-3 was only moderately increased (Figure 21C). The apoptosis ratio of MxB-expressing cells, however, was significantly increased after treated with 5μM sodium arsenite for 24 hours (Figure 21A and B). Activated caspase-3 expression was also shown to be significantly up-regulated in the MxB-expressing cells with the sodium arsenite treatment (Figure 21C). As puromycin and G418 were removed from the media during this experiment, we again compared the apoptotic cell ratio between the cells that were transduced to overexpress MxB and the control cells without oxidative stress. In consistence with the previous result, MxB-expressing cells showed a significantly increased apoptosis ratio (Figure 21A and B). Moreover, the MxB-expressing cells also expressed a higher level of activated caspase-3 (Figure 21C), indicating that MxB itself

truly induces apoptosis. It is also worth mentioning that, in consistence with the previous results, the apoptosis ratio and the activated caspase-3 expression in MxA-expressing cells were significantly decreased when compared to the control cells under all conditions (Figure 21A-C). These results further support the finding that MxA inhibits apoptosis.

Additionally, we tested if MxB also promotes apoptosis under other stress conditions. In our experiment, MxB promoted all apoptosis induced by oxidative stress, such as in the cases of sodium arsenite, arsenic trioxide as well hydrogen peroxide (Figure 21D). All the three conditions aforementioned significantly increased the apoptosis ratio in MxB-expressing cells to different degrees (Figure 21D). However, serum starvation did not enhance the MxB-induced apoptosis (Figure 21D). These results suggested that MxB promotes oxidative stress-induced apoptosis in U87-CD4$^+$/CXCR4$^+$ cells.

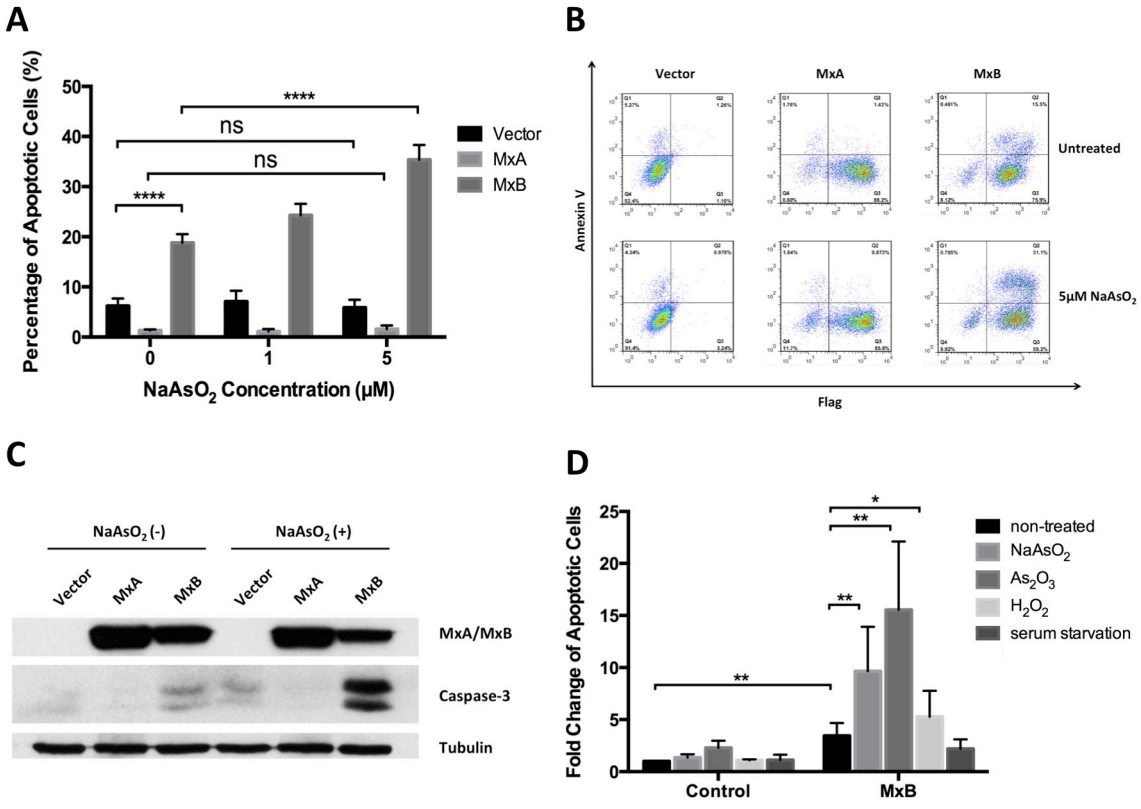


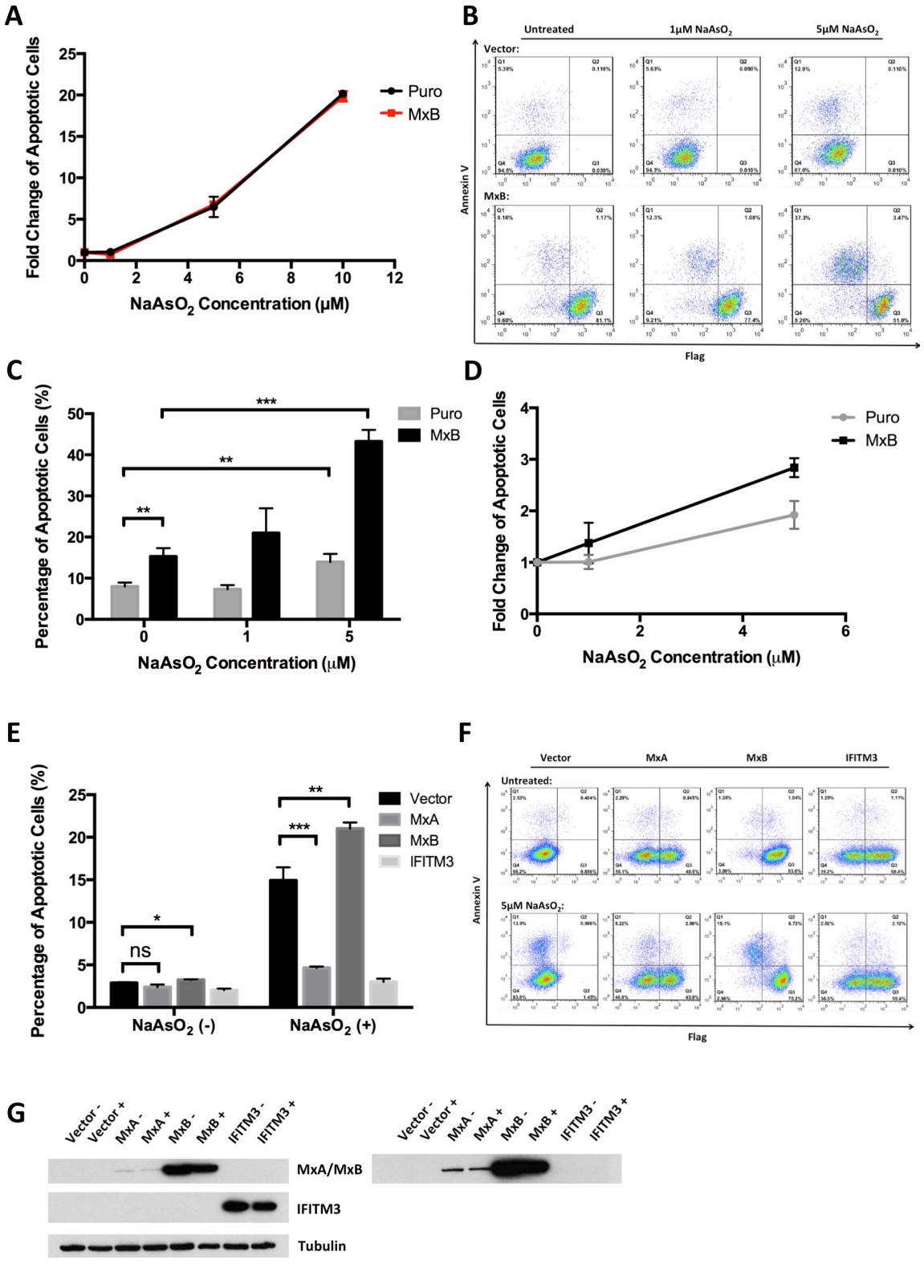
Figure 21. MxB promotes oxidative stress-induced apoptosis in U87-CD4$^+$/CXCR4$^+$ cells. (A-C) U87-CD4$^+$/CXCR4$^+$ cells were transduced with 200µl MxA or MxB retroviral vectors. 200µl blank vector was transduced in the control group. Cells were kept in DMEM without puromycin and G418. 72 hours after transduction, the cells were treated with 1µM or 5µM sodium arsenite ($NaAsO_2$) for another 24 hours before harvested. The Annexin V positive cell ratio and cleaved caspase-3 expression were measured. (D) U87-CD4$^+$/CXCR4$^+$ cells were transduced as described above. 72 hours after transduction, the cells were treated with 5µM sodium arsenite ($NaAsO_2$), 5µM arsenic trioxide (As_2O_3), and 500µM hydrogen peroxide (H_2O_2) as described in *2.12*. Serum starvation was applied as described in *2.13*. The cells were harvested and stained with PE Annexin V and anti-flag antibody.

3.4.2 MxB promotes oxidative stress-induced apoptosis in SupT1 cell lines

Unlike U87-$CD4^+$/ $CXCR4^+$ cells, SupT1 cells showed more sensitivity to oxidative stress-induced apoptosis (Figure 22A and D). Doxycycline-inducible MxB SupT1 cell line was firstly shown to have the same sensitivity to oxidative stress as the control cell line when not induced with doxycycline to express MxB (Figure 22A). The cell lines were then treated with doxycycline for 16 hours to induce MxB expression, followed by the exposure to different concentrations of sodium arsenite for another 24 hours. Under the normal condition without oxidative stress, MxB-expressing SupT1 cells showed a higher apoptosis ratio (~1.8-fold) compared to the control group (Figure 22B and C), indicating that MxB also induces cell apoptosis in SupT1 cells. As we expected, sodium arsenite treatment significantly increased the apoptosis ratio in both groups (Figure 22B and C). In addition, SupT1 cells expressing MxB was also shown to be more sensitive to stress-induced apoptosis as MxB accelerated the apoptosis inducement under the condition of sodium arsenite treatment compared to the control group (Figure 22B and D). It should also be noted that, different from U87 cells, the apoptotic SupT1 cells in the MxB group were not MxB-positive (Figure 22B). Moreover, while the apoptotic cell number was increased with higher concentrations of sodium arsenite treatment, the number of MxB-expressing cells was decreased (Figure 22B). Two possible reasons could lead to this result. Firstly, the MxB-expressing cells may lose MxB expression after undergoing apoptosis, which causes the initial $Flag^+$/Annexin V^- cell population shift to the $Flag^-$/Annexin V^+ quadrant. Secondly, the sodium arsenite may interfere with the doxycycline-inducible protein expression system, leading to a reduced flag-positive cell population. In the second case, the data wouldn't be valid to support our conclusion.

In order to test this, two additional doxycycline-inducible SupT1 cell lines, MxA and IFITM3, were included. Although the cell population in the $Flag^-$/Annexin V^+ quadrant increased in both MxA and IFITM SupT1 cell lines upon 5μM sodium arsenite treatment, the cell population in the $Flag^+$/Annexin V^- quadrant showed no decrease (Figure 22F). Western blot revealed a reduced flag-tagged protein expression in both MxB and IFITM group, but not in MxA group, after the cells were treated with sodium arsenite (Figure 22G). This result suggested that, although sodium arsenite treatment may cause a reduced protein expression level, the protein-expressing cell numbers were not interfered. In this case, we can conclude that the reduced MxB-positive cell population we observed was caused by apoptosis, and the $Flag^-$/Annexin V^+ cells in the MxB group are the formerly MxB-expressing cells that underwent apoptosis. Moreover, although expressed in a significantly reduced level, MxA was shown to inhibit apoptosis in SupT1 cells under the oxidative stress condition (Figure 22E). IFITM3-expressing SupT1 cells also showed a significantly reduced sensitivity to oxidative stressed-induced apoptosis (Figure 22E). This work is mainly focused on MxB and MxA.

Taken together, these results suggested that MxB induces apoptosis and promotes oxidative stress-induced cell apoptosis in both U87-$CD4^+$/ $CXCR4^+$ cells and SupT1 cells.


A
Fold Change of Apoptotic Cells
NaAsO2 Concentration (μM)
Puro
MxB
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Untreated
1μM NaAsO2
5μM NaAsO2
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MxB:
Annexin V
Flag
C
Percentage of Apoptotic Cells (%)
NaAsO2 Concentration (μM)
Puro
MxB
D
Fold Change of Apoptotic Cells
NaAsO2 Concentration (μM)
Puro
MxB
E
Percentage of Apoptotic Cells (%)
Vector
MxA
MxB
IFITM3
ns
NaAsO2 (-)
NaAsO2 (+)
F
Vector
MxA
MxB
IFITM3
Untreated:
5μM NaAsO2:
Annexin V
Flag
G
Vector -
Vector +
MxA -
MxA +
MxB -
MxB +
IFITM3 -
IFITM3 +
MxA/MxB
IFITM3
Tubulin

Figure 22. MxB induces apoptosis and promotes oxidative stress-induced cell apoptosis in SupT1 cells. (A) Doxycycline-inducible MxB SupT1 cell line and the control cell line (Puro) were treated with escalated concentrations (1μM, 5μM and 10μM) of sodium arsenite for 24 hours. Cells were stained with PE Annexin V to detect the apoptosis ratio. The relative fold change of the apoptosis ratio was normalized according the cell apoptosis ratio without sodium arsenite treatment. (B-D) Doxycycline-inducible MxB SupT1 cell line and the control cell line were treated with 500ng/ml doxycycline for 16 hours before 1μM or 5μM sodium arsenite were added to the cells. Doxycycline and sodium arsenite were kept in cells for another 24 hours. Cells were stained with PE Annexin V and anti-flag antibody. (B) The flow cytometry data is representative of 3 independent experiments that are (C) summarized and (D) normalized according the cell apoptosis ratio without sodium arsenite treatment. (E-G) Doxycycline-inducible MxA, MxB and IFITM3 SupT1 cell lines and the control cell line (Vector) were treated with 500ng/ml doxycycline for 16 hours before 5μM sodium arsenite were added to the cells. Doxycycline and sodium arsenite were kept in cells for another 24 hours. Cells were stained with PE Annexin V and anti-flag antibody. (E) Summary of 3 independent experiments and (F) one group of representative original flow cytometry data were shown. (G) One group of the cells were harvested for western blot with anti-flag antibody to detect the target protein expression level.

3.5 MxA reduces MxB-induced apoptosis

During the innate immune responses against viral infection, type I interferon induces the transcription of a large number of ISGs, including both MxA and MxB. In macrophages as well as other types of cells, these two proteins can be detected simultaneously upon type-1 interferon induction. As we have shown that MxA inhibits apoptosis while MxB induces apoptosis, we next wanted to see what would happen to the cells when these two proteins are simultaneously expressed. In order to test this, one additional group of U87-$CD4^+$/ $CXCR4^+$ cells was cotransduced with both the MxA-retroviral vector and the MxB-retroviral vector to overexpress the two proteins simultaneously. The apoptosis ratio was measured after the cells were treated with or without 5μM sodium arsenite for 24 hours.

Under both conditions, the apoptosis ratio of cells that were overexpressing both MxA and MxB was significantly decreased when compared to the MxB group (Figure 23A and B), indicating that MxA inhibits cell apoptosis in MxB-expressing cells. Specifically, without oxidative stress, MxA reduced MxB-induced apoptosis to the level that is comparable to the control group (Figure 23A and B). Under the stress condition, however, the apoptosis ratio of the cells that were expressing both MxA and MxB was still significantly higher compared to the control group, which was again shown not to be sensitive to oxidative stress-induced apoptosis (Figure 23A and B). The result of this experiment showed that, when expressed together, MxA reduced MxB-induced apoptosis, revealing a possible role of MxA in balancing the apoptosis-inducing activity of MxB under normal conditions. Moreover, as cells expressing both MxA and MxB were still sensitive to oxidative stress, we believe that, MxA does not eliminate the activity of MxB

in promoting oxidative stress-induced apoptosis, although the apoptosis ratio was reduced to a more moderate level.

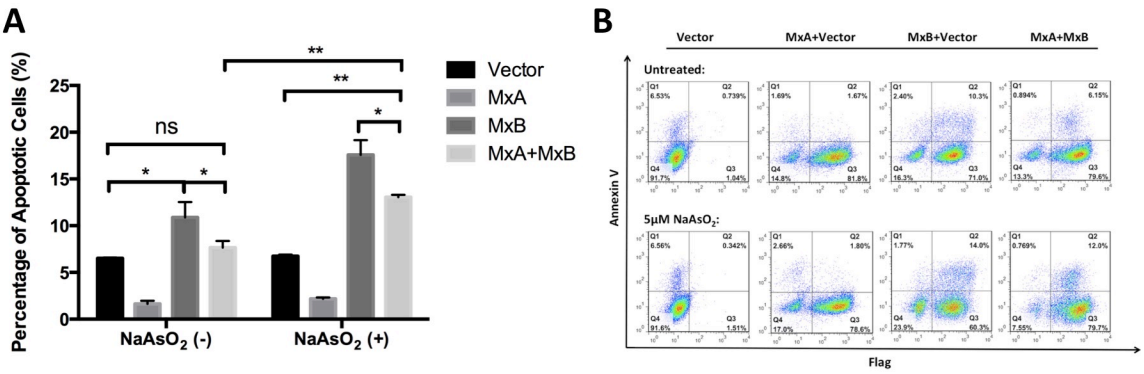


Figure 23. MxA reduces apoptosis in MxB-expressing cells. U87-CD4^{+}/CXCR4^{+} cells were transduced with the following retroviral vectors: 100μl MxA and 100μl pQCXIP2.0 (blank vector), 100μl MxB and 100μl pQCXIP2.0, or 100μl MxA and 100μl MxB. 200μl blank vector was transduced in the control group. The cells were kept in DMEM without puromycin or G418. 72 hours after transduction, 5μM sodium arsenite were added into the media of one set of the experimental groups and kept for 24 hours. The cells were stained with PE Annexin V and anti-flag antibody. (A) Summary of 3 independent experiments and (B) one group of representative original flow cytometry data were shown.

3.6 Depletion of IFN-induced endogenous MxB expression reduces oxidative stress-induced apoptosis in U87MG cells

To directly examine whether the endogenous level of MxB is sufficient in inducing apoptosis and promoting oxidative stress-induced apoptosis, MxB knockout experiment was performed in U87MG cells with CRISPR/Cas9 system. CRISPR/Cas9 with target-3 (T3) and target-4 (T4) guide RNA effectively knocked out MxB expression (Figure 24C). MxB-Knockout cell lines showed comparable basal levels of apoptosis ratio as the control cell line (Figure 24A and B). Without IFNα induction, treatment with sodium arsenite moderately increased the apoptosis ratio in all the groups (~1.4-fold) (Figure 24A and B). Likewise, without sodium arsenite, IFNα induction alone also increased apoptosis ratio in the control group (~1.7-fold), but not in T3 or T4 group (Figure 24A and B). Most strikingly, with both IFNα induction and sodium arsenite treatment, apoptosis ratios in all groups were significantly increased (Figure 24A and B), suggesting the existence of other interferon inducible proteins that have pro-apoptotic activities. This result is in consistence with previous studies showing that type I IFN induces apoptosis by up-regulating certain ISGs that either serve as or further induce downstream apoptotic effectors[236,237]. Moreover, compared to the control group that showed the highest increase in apoptosis ratio (~4-fold), apoptotic cell ratio in both T3 and T4 group were significantly reduced (~2.7-fold and ~2.1-fold, respectively) (Figure 24A and B), which indicated that knockout of IFNα-induced endogenous MxB expression reduced the sensitivity of U87MG cells to oxidative stress-induced apoptosis. In summary, results from this experiment confirmed that endogenous level of MxB promotes oxidative stress-induced apoptosis. Moreover, MxB may not be the only ISG that induces apoptosis

and promotes oxidative stress-induced apoptosis, as with IFNα induction, both of the MxB-KO groups still showed a significant increase in apoptotic cell ratio compared to the groups without IFN treatment.

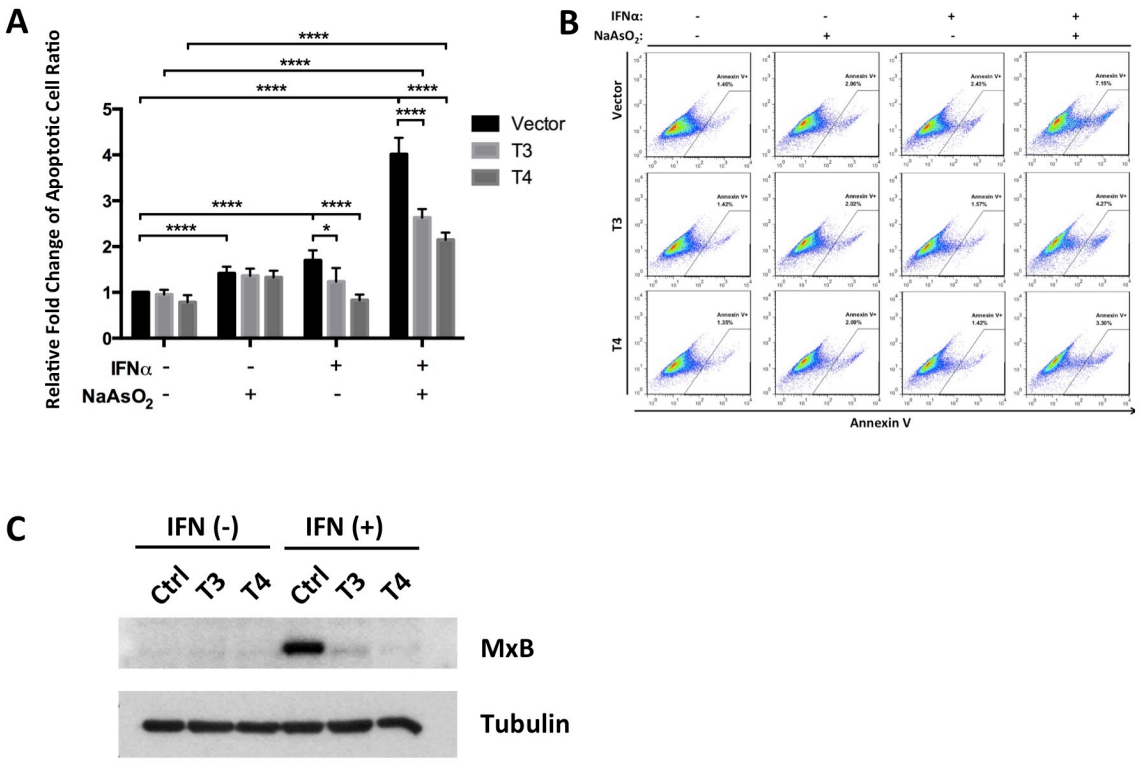


Figure 24. Knockout of MxB reduces oxidative stress-induced apoptosis. Stable MxB-knockout U87MG cell lines (T3 and T4) were treated with 500U/ml IFNα2b for 16hours. Cells were then treated with 5μM sodium arsenite for another 30 hours. Cell apoptosis ratio was detected by PE Annexin V staining and analyzed by flow cytometry. (A) Relative fold changes of apoptotic cell ratio were normalized according to the control cell line without IFNα or sodium arsenite treatment. (B) One group of representative original flow cytometry data are shown. (C) Endogenous MxB expressions and MxB knockout efficiency were confirmed by western blot.

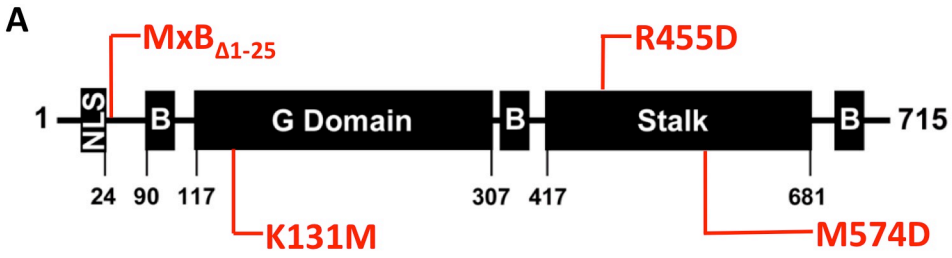
3.7 The N-terminal region, GTPase activity, and oligomerization of MxB are important for its pro-apoptotic activity

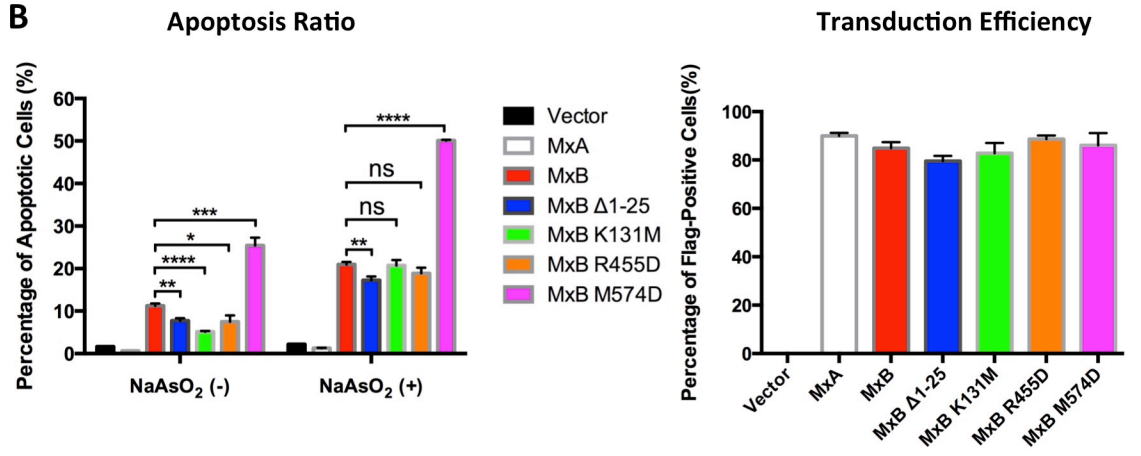
Next, in order to identify the functional regions that are important for the activity of MxB in inducing cell apoptosis and promoting oxidative stress-induced apoptosis, U87-CD4$^+$/CXCR4$^+$ cells were transduced to overexpress different MxB loss-of-function mutants.

The N-terminal region of MxB plays an essential role in its anti-HIV-1 activity[198]. An HIV-1 capsid-binding domain and a nuclear localization signal have been identified within the first 25 amino acids of MxB (Figure 25A)[196,199]. Deletion of this 1-25 amino acid of MxB causes a partial loss of its pro-apoptotic activity, as U87-CD4$^+$/CXCR4$^+$ cells overexpressing MxBΔ1-25 exhibited a moderately reduced apoptosis ratio compared to the wild type MxB in both conditions with or without sodium arsenite treatment (Figure 25B). This result indicated that the N-terminal region of MxB might be one, but not the only, determinant of its activity in inducing cell apoptosis.

A single amino acid substitution in MxB GTPase domain (K131M) causes the loss of its GTPase activity (Figure 25A)[244]. MxB K131M mutant also showed a reduced activity in inducing apoptosis in U87-CD4$^+$/CXCR4$^+$ cells (Figure 25B). Moreover, another MxB single point mutation R455D that occurs in the stalk interface 3 of MxB and thus blocks its higher-order oligomerization and reduces its dimerization[201], was also shown to cause a partial loss of its activity in inducing cell apoptosis (Figure 25A and B). Notably, neither of these two mutations alters the activity of MxB in promoting oxidative stress-induced apoptosis in this experiment (Figure 25B). These data indicated that, other than the N-terminal region, the GTPase activity and the oligomerization of MxB might also play crucial roles in MxB-induced cell apoptosis.

MxB M574D mutation occurs in the dimer interface (stalk domain interface 2) of MxB and completely blocks its dimerization and anti-HIV-1 activity[201]. Surprisingly, in this experiment, the M574D mutation significantly enhanced the activity of MxB in inducing apoptosis and promoting oxidative stress-induced apoptosis in U87-$CD4^+$/$CXCR4^+$ cells, resulting in a ~2-fold increase of the apoptosis ratio in both conditions with or without sodium arsenite treatment (Figure 25B). This result suggested that the role of MxB dimerization might differ from that of MxB oligomerization in cell apoptosis induction and promotion. Likely, the monomer form of MxB may have a higher activity in inducing apoptosis and promoting oxidative stress-induced apoptosis.


A
MxB Δ1-25
R455D
1
NLS
B
G Domain
B
Stalk
B
715
24
90
117
307
417
681
K131M
M574D


B
Apoptosis Ratio
Transduction Efficiency
Percentage of Apoptotic Cells (%)
NaAsO2 (-)
NaAsO2 (+)
Vector
MxA
MxB
MxB Δ1-25
MxB K131M
MxB R455D
MxB M574D
Percentage of Flag-Positive Cells(%)

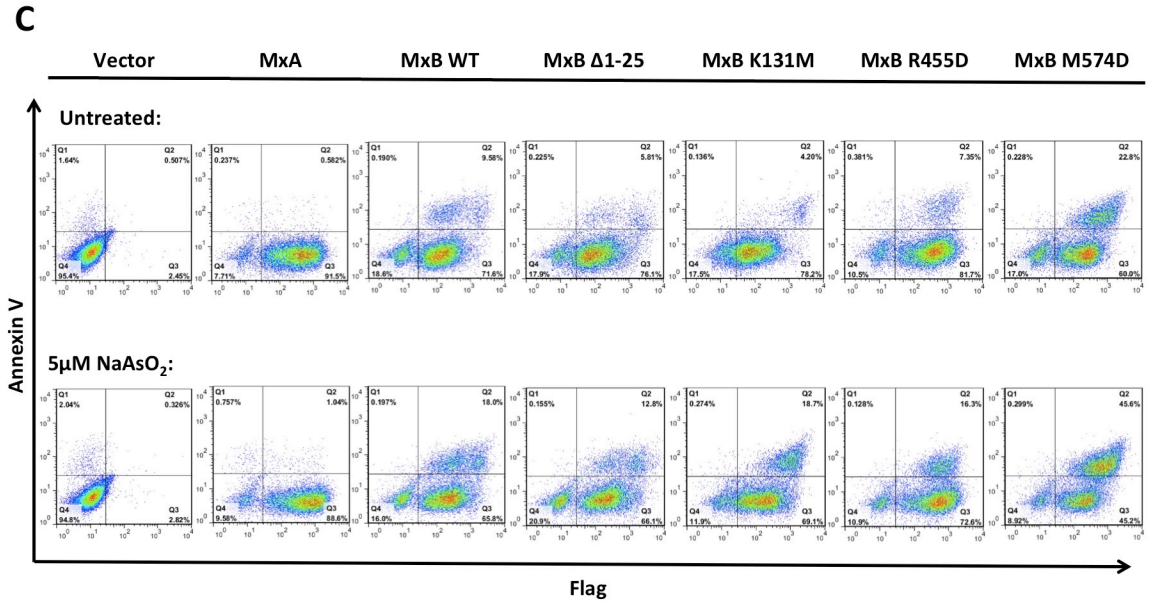

C
Vector
MxA
MxB WT
MxB Δ1-25
MxB K131M
MxB R455D
MxB M574D
Untreated:
5μM NaAsO2:
Annexin V
Flag

Figure 25. MxB Mutations alter its activity in inducing apoptosis and promoting oxidative stress-induced cell apoptosis. (A) Schematic model of wild type human MxB^{201}. The numbers of the amino acid residues at the boundaries of the MxB domains are indicated. The nuclear localization signal (NLS), stalk domain, GTPase domain (G Domain), and bundle-signaling element (B) are indicated. Sites of the mutations used in this study are labeled in red. (B) U87-$CD4^+$/$CXCR4^+$ cells were transduced with retroviral vectors that packaged RNAs encoding the following proteins: MxA, MxB, MxBΔ1-25, MxB K131M, MxB R455D and MxB M574D, under the same MOI of 1.6. 200μl blank vector was transduced in the control group. Cells were kept in DMEM without puromycin and G418. 72 hours after transduction, 5μM sodium arsenite ($NaAsO_2$) were added into the media and kept for 24 hours. The cells were stained with PE Annexin V and anti-flag antibody and were analyzed by flow cytometry. (C) One group of representative original flow cytometry data are shown.

Chapter 4-Discussion

In the present study, we demonstrate that the anti-HIV-1 host protein MxB induces cell apoptosis and promotes oxidative stress-induced apoptosis in both U87 and SupT1 cells. Knockout study confirmed the role of endogenous MxB expression on promoting cell apoptosis. We further show that MxA protein inhibits cell apoptosis and is sufficient to reduce the apoptosis ratio in MxB-expressing cells, indicating a possible role of MxA in balancing MxB-induced apoptosis under certain conditions. In addition, we identified MxB N-terminal domain, GTPase activity and oligomerization as important determinants for its pro-apoptotic activity.

Before the recent reveal of its anti-HIV-1 activity, MxB was believed to be non-antiviral over the decades. Due to the basal level expression and the subcellular localization of MxB, it has been hypothesized that MxB might have other cellular functions such as regulating nucleo-cytoplasmic transport and cell-cycle progression[196,203]. Inhibition of nuclear import and cell-cycle progression by mutated forms of MxB provided evidence that supports this hypothesis[203]. However, since previous studies didn't demonstrate a clear role that MxB plays other than being a host restriction factor, the cellular functions of MxB still remain poorly understood. This is the first study to report the activity of MxB in promoting cell apoptosis, which is likely to help unravel an unexpected role of MxB as a component in the type 1 PCD pathway.

It has long been known that IFNs play important roles in promoting apoptosis with both the mitochondrial[233] and the death-receptor pathways involved[234]. IFN-mediated induction of cell apoptosis occurs late (more than 48 hours post treatment), implicating the involvement of certain ISGs and possibly other intermediate cellular effectors[236].

Following this hypothesis, many ISGs have been identified to be involved in the IFN-induced apoptotic cell death pathway, such as Fas, caspase-4 and caspase-8[237]. In this study, we used CRISPR/Cas9 system to knockout the IFN-inducible endogenous MxB from U87MG cells, which leaded to a reduced induction of apoptosis when these cells were treated with IFNα (Figure 24). This result provides evidence that MxB promotes cell apoptosis and is likely to be an important intermediate cellular effector involved in the IFN-induced apoptosis pathway.

In the MxB-knockout U87MG cell lines, flag-tagged Cas9 protein and the target guide RNAs are constantly expressed due to the lentiviral vector-based transduction, which results in higher knockout efficiency. The remaining MxB expression upon IFNα induction might be a result of missense mutations that was introduced by Cas9/sgRNA edition followed by the non-homologous end joining (NHEJ) machinery of the cells. Thus, to generate a cell line with MxB being completely knocked out, single-cell clones will need to be isolated.

MxA has also been reported to enhance cell death under the conditions of influenza virus infection as well as apoptotic stimuli inducement by Nagata's group[239]. In Nagata's studies, Swiss mouse 3T3 (NIH 3T3) cell lines overexpressing human MxA protein exhibited significantly increased cell death ratio through both caspase-dependent and -independent mechanisms upon Influenza virus infection[239,240]. Similar as MxB, the C-terminal region of MxA that is responsible for its oligomerization has also been found to be important for its cell death promotion activity[240]. However, in our study, MxA was shown to inhibit cell apoptosis in both U87 cells and SupT1 cells. MxA doesn't promote cell apoptosis induced by oxidative stress in the two cell lines tested. This may suggest

that MxA mediates cell death in a cell type-dependent manner or a species-dependent manner. Compared to NIH 3T3 cell line that is derived from mouse embryonic fibroblast cells, U87 cells that is derived from human macrophages and SupT1 cells that is derived from human T cells may be more representative in studying the functions of human MxA protein, as they both have endogenous MxA expression upon IFN induction. Moreover, in order to further demonstrate the function of MxA in cell apoptosis regulation, an MxA knockout experiment may be performed in the future work of this study. By doing so, we would be able test how endogenous MxA interfere with MxB-promoted apoptosis when both of the proteins are induced by IFN.

In the study of the cell death promotion activity of MxA, MxA promotes cell death by enhancing endoplasmic reticulum (ER) stress signaling in influenza virus infected cells[241]. Similar as influenza virus, HIV-1 infection has also been shown to induce cell apoptosis through ER stress due to the expression of HIV-1 Tat and gp120 proteins[245-247]. However, in this study, HIV-1 infection didn't accelerate apoptosis in MxB-expressing cells. The major reason that may lead to this consequence is that, as MxB itself blocks HIV-1 replication before integration, the expression of viral proteins, including Tat and gp120, were impaired in MxB-expressing cells. Due to this, ER stress could not be significantly enhanced and, as a consequence, no accelerated apoptosis could be detected in the MxB-expressing cells. This is also supported by the result that very low percentages of p24-positive cells were detected in the MxB group (Figure 20B). MxA also inhibits influenza virus replication at an early stage, which leads to impaired viral protein expressions[248]. In Nagata's study, in order to compensate the MxA restriction, an extremely high dose of influenza virus (MOI=10) was used to infect the MxA-expressing

cells[241], which may not be representative to naturally occurred viral infections. Thus, further studies need to be done in order to answer the question whether MxA can enhance influenza virus-induced apoptosis *in vivo*. Nevertheless, to optimize the experimental condition of our study, higher virus doses will be used in the future. Transfecting the MxB-expressing cells with plasmids encoding the HIV-1 proviral DNA should also be a good method in this study, as it bypasses the early stages of HIV-1 replication thus avoids the MxB restriction.

It should also be noted that our data showed U87-$CD4^+$/$CXCR4^+$ cells and U87MG cells are not as sensitive to oxidative stress-induced apoptosis as Hela cells or SupT1 cells. Upon 24-hours sodium arsenite treatment, U87-$CD4^+$/$CXCR4^+$ cells showed no significant increase in apoptotic cell number (Figure 21 and Figure 23). Similarly, less than 1.5-fold change in apoptotic cell number was observed in U87MG cells 30 hours after sodium arsenite treatment (Figure 24). This is in agreement with the previous finding that both murine and human macrophages express a novel apoptosis inhibitory factor termed apoptosis inhibitor of macrophage (AIM), which inhibits apoptosis induced by multiple stimuli[249,250]. The expression of MxB significantly enhanced oxidative stress-induced apoptosis in both cell lines (Figure 21 and Figure 24), implying that the pro-apoptotic activity of MxB overcomes the AIM inhibition. Since our data suggest that AIM is sufficient enough to inhibit the apoptosis induced by multiple oxidative stresses, it's very interesting to see MxB overcomes this pretty strong inhibition. This might be better explained if MxB interacts with AIM and block its functions. To investigate this, we would possibly perform a Co-Immunoprecipitation (Co-IP) assay to test if MxB directly binds to AIM protein. This might be a promising future study direction to unravel

the roles of MxB and AIM in the apoptotic pathway in macrophages.

Taken together, our data demonstrate that MxB induces cell apoptosis and promotes oxidative stress-induced apoptosis whereas the closely related Mx protein MxA inhibits apoptosis. In addition, we identified the N-terminal domain, GTPase activity and oligomerization of MxB as important determinants for its activity in apoptosis promotion. Future studies would aim to illustrate the underlying mechanism of MxB-induced cell apoptosis and to unravel the possible link between the pro-apoptotic activity and the antiviral function of MxB.

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