RESTRICTION OF HIV BY TIM-FAMILY PROTEINS

AND ANTAGONISM BY NEF

A Dissertation presented to

the Faculty of the Graduate School

at the University of Missouri-Columbia

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Minghua Li

Dr. Shan-Lu Liu, Dissertation Supervisor

JULY 2016

The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

RESTRICTION OF HIV BY TIM-FAMILY PROTEINS AND ANTAGONISM BY NEF

presented by Minghua Li,

a candidate for the degree of Doctor of Philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

Dr. Shan-Lu Liu

Dr. Donald Burke

Dr. Marc Johnson

Dr. David Pintel

Dr. Guoquan Zhang

ACKNOWLEDGEMENTS

First and foremost, I would like to express my sincere gratitude to my mentor Dr. Shan-Lu Liu, who accepted me to join his team as the first Ph.D. student at Mizzou. I am very grateful for his continuous support and guidance throughout my five years of Ph.D. study. As a mentor, he has taught me not only how to design the experiments and analyze the data but also how to become a successful scientist and enjoy science. I appreciate all his contributions of time, ideas, and funding that have led to my Ph.D. experience enjoyable and productive. I will never forget the weekends he spent with me in the lab to discuss my research projects or revise manuscripts. I am also thankful for the excellent example he has set as a successful professor, with motivation, enthusiasm, patience, and broad knowledge. I believe that the Ph.D. experience in his laboratory will become a lifetime memory.

I would like to thank my thesis committee members, Drs. Donald Burke, Marc Johnson, David Pintel, and Guoquan Zhang, for their time and valuable input during my committee meetings and Virology Works in Progress (VWIP) meetings. Their comments and suggestions have promoted me to think my research projects thoroughly and sometimes from different angels. Also, their insightful comments in my comprehensive examination have been extremely helpful for my proposal writing.

I would like to thank all the past and present members of Dr. Liu lab. My sincere thanks firstly goes to Yi-Min Zheng for teaching me cell culture and making a wonderful lab environment for research. I would also like to thank Kun Li, who taught me cell-cell fusion assay and flow cytometry. I thank Chunhui Miao, Jingyou Yu and Fushun Zhang

for their assistance for my projects in the lab and their friendship. I thank Daniel Steitz, John Bryan, Justin Ehehardt, Lane Adams, Michael Wolski, Kristina Freund, Jacob Bly, and Mikko Viudez for their help in plasmids preparation and lab jobs.

I would like to thank Dr. Wendy Maury at University of Iowa for providing the TIM-1 construct and soluble proteins to initiate this project; Dr. Eric Freed and Sherimay Ablan at National Cancer Institute for sharing reagents as well as assistance in TEM analysis.

Last but not the least, I would like to thank my family for their love. I thank my wife Huili Jia for her continuous support to the family and my career development. I thank my two cute children, Michael and Katherine for the wonderful time and happiness they bring to us. I also thank my parents and my mother-in-law for their time to take care of my kids as well as housework. The love and encouragement from the family will be my spiritual support forever.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

6HB	Six-helix bundle
AGMK	African green monkey kidney
AHR	Airway hyperreactivity
AIDS	Acquired immune deficiency syndrome
AP	Adaptor protein
APCs	Antigen-presenting cells
APOBEC3G	Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G
ART	Antiretroviral therapy
bNAb	Broadly neutralizing antibody
CA	Capsid protein
CDC	Centers for Disease Control
cDNA	Complementary DNA
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
CRF	Circulating recombinant forms
CTL	Cytotoxic T-lymphocyte
DAI	DNA-dependent activator of interferon-regulatory factors
DCAF1	Damaged DNA binding protein 1-cullin 4-associated factor 1
DCs	Dendritic cells
DC-SIGN	Dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin

DDX41	DEAD (Asp-Glu-Ala-Asp) box polypeptide 41
dNTP	Deoxynucleoside triphosphate
dsDNA	Double-stranded DNA
DV	Dengue virus
EAE	Experimental autoimmune encephalomyelitis
EBOV	Ebola virus
ELISA	Enzyme linked immunosorbent assay
Env	Envelope glycoprotein
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport
FDA	Food and Drug Administration
FeLV	Feline leukemia virus
Gal-9	Galectin-9
Gag	Group-specific antigen
GAS	Gamma-activated sequence
GPCR	G protein-coupled receptor
GPI	Glycophosphatidylinositol
GTPase	Guanosine triphosphatase
HAV	Hepatitis A virus
HIV	Human immunodeficiency virus
HR	Heptad repeats
HTLV-III	Human T-cell leukemia virus type III
IFI16	IFNγ-inducible protein16

IFITM	Interferon-induced transmembrane protein
IFN	Interferon
IFNAR	IFN receptor
Ig	Immunoglobulin
IKK	IκB kinase
IL	Interleukin
IN	Integrase
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
JAK1	Janus kinase 1
kD	Kilodalton
KIM-1	Kidney injury molecule-1
LAV	Lymphadenopathy-associated virus
LTR	Long terminal repeat
MA	Matrix protein
mAb	Monoclonal antibody
MDDCs	Monocyte-derived dendritic cells
MDMs	Monocyte-derived macrophages
MHCI	Major histocompatibility class I
MILIBS	Metal ion-dependent ligand-binding site

MLV	Murine leukemia virus
MoMLV	Moloney murine leukemia virus
MPER	Membrane-proximal external region
mRNA	Messenger RNA
MVB	Multivesicular body
MX	Myxovirus resistance
MYD88	Myeloid differentiation primary response gene 88
NAb	Neutralizing antibody
NC	Nucleocapsid protein
NELF	Negative elongation factor
NES	Nuclear export signal
NF-κB	Nuclear factor κB
NLS	Nuclear localization signal
N-MLV	N-tropic murine leukemia viruses
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NPC	Nuclear pore complex
NRTI	Nucleoside-analog reverse transcriptase inhibitor
nt	Nucleotide
PAMP	Pathogen-associated molecular pattern
PBS	Primer binding site
pDCs	Plasmacytoid dendritic cells
PI	Protease inhibitor
PIC	Preintegration complex

Pol	Polymerase
PPT	Polypurine tract
PR	Protease
PRR	Pattern recognition receptor
PS	Phosphatidylserine
PtdIns(4,5)P_2	Phosphatidylinositol-4,5-bisphosphate
P-TEFb	Positive transcription elongation factor b
Rbx2	RING-box protein 2
RER	Rough endoplasmic reticulum
Rev	Regulator of expression of virion proteins
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I-like receptor
RRE	Rev response element
rRNA	Ribosomal RNA
RT	Reverse transcriptase
RTC	Reverse transcription complex
SAMHD1	Sterile alpha motif and histidine/aspartic acid domain-containing protein 1
SERINC	Serine incorporator
SHIV	Simian-human immunodeficiency virus
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SIV	Simian immunodeficiency virus

SIVcpz	SIV that infects chimpanzee
SIVcpz*Ptt*	SIV that infects chimpanzee *Pan troglodytes troglodytes*
SIVgor	SIV that infects gorilla
SIVsmm	SIV that infects sooty mangabey
SLFN11	Schlafen 11
ssRNA	Single-stranded RNA
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAF	TBP-associated factor
TAR	Trans-activation response region
Tat	Trans-activator of transcription
TBK1	Tank binding kinase 1
TBP	TATA-binding protein
TCR	T cell receptor
TFIID	Transcription factor II D
TIM	T-cell immunoglobulin and mucin domain protein
TLR	Toll-like receptor
TREX1	3’-repair exonuclease 1
TRIM5α	Tripartite motif-containing protein 5α
tRNA	Transfer RNA
TYK2	Tyrosine kinase 2
USF	Upstream stimulatory factor
VLP	Virus-like particle

ABSTRACT

T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins play important roles in immune regulation and viral infections. Recent studies indicate that TIM-1 promotes the entry of a wide range of enveloped viruses, likely by interacting with virions-associated phosphatidylserine (PS). TIM-family proteins are known to be expressed on human primary $CD4^+$ T cells, macrophages as well as dendritic cells, which are the major targets for HIV-1 replication. However, the functional roles of TIMs in HIV-1 infection are currently not known.

In this Ph.D. thesis work, I demonstrate that expression of TIM-family proteins, which include human TIM-1, TIM-3 and TIM-4, significantly restricts HIV-1 release from viral producer cells. Expression of TIM-1 leads to the accumulation of mature viral particles on the plasma membrane, therefore inhibiting HIV-1 production. Notably, TIM-1 mutants that are defective for PS binding fail to block HIV-1 release, indicating that the interaction of TIM-1 and PS is required for TIM's inhibitory effect. Similar to other well-documented host restriction factors such as tetherin, TIM-1 is incorporated into nascent HIV-1 virions. In addition to HIV-1, I also find that TIM-1 is able to inhibit the release of other viruses such as murine leukemia virus (MLV) and Ebola virus (EBOV). Importantly, knockdown of endogenous TIMs in human macrophages promotes HIV-1 production, suggesting that TIM-family proteins can function as general intrinsic inhibitors of viral release.

HIV-1 accessory proteins play a critical role in antagonizing host restriction factors. I show in my Ph.D. thesis work that compared to wildtype and other variants, Nef-deficient

HIV-1 particles are much more potently inhibited by TIM-1 for release. Consistent with this finding, ectopic expression of Nef efficiently overcomes the TIM-1 restriction of HIV-1. HIV-1 Nef does not appear to significantly downregulate TIM-1 expression on the cell surface, nor does it disrupt TIM-1 incorporation into HIV-1 virions. Interestingly, coexpression of SERINC3 and SERINC5 potentiates the ability of TIM-1 to inhibit HIV-1 release, and depletion of SERINCs in viral producer cells relieves the TIM-1 restriction of HIV-1 release. In addition to HIV-1 Nef, I find that the Nef proteins of simian immunodeficiency virus (SIV) and HIV-2 also antagonize the antiviral activity of TIM-1, suggesting an evolutionarily conserved role of the lentiviral Nef in counteracting TIMs.

Taken together, my Ph.D. thesis work has revealed a novel function of TIM-family proteins during HIV-1 infection, which strongly restricts viral release from the plasma membrane. Additionally, I demonstrate that lentivirus Nef proteins have evolved an efficient strategy to overcome the inhibitory effect of TIMs. The data I described in my Ph.D. thesis provide new insights into HIV-host interactions, particularly the interplay between TIMs, SERINCs and HIV-1 Nef.

CHAPTER 1 – INTRODUCTION

1.1 Discovery, Origins and Evolution of HIV

1.1.1 Discovery of HIV

Acquired Immune Deficiency Syndrome (AIDS) was first recognized as a new disease in 1981 when Center for Disease Control (CDC) reported five young homosexual men in Los Angeles infected with a rare disease, *Pneumocystis carinii* Pneumonia, due to severe immunodeficiency (1). This unusual syndrome was characterized by significant reduction of $CD4^+$ T cells in peripheral blood and association with opportunistic infection including mycobacterial infections, invasive fungal infections or rare cancer such as Kaposi's sarcoma and non-Hodgkin's lymphoma (2, 3). A few months later, additional reports described similar syndromes in other risk groups, such as blood transfusion recipients (4), sexual partners of infected individual (5) and infants (6), suggesting that AIDS was transmitted through blood and sexual contact (7).

The causative agent of AIDS was unknown until the isolation of the new pathogen in 1983. In early 1983, Montagnier group at the Pasteur Institute isolated a retrovirus-like virus from a patient with generalized lymphadenopathy of unknown origin (8). At that time, the new agent was named as lymphadenopathy-associated virus (LAV). LAV shared several common features with retrovirus, including reverse transcriptase (RT) activity and electron microscopy morphology. Strikingly, when it was compared with other retroviruses such as murine leukemia virus (MLV) and feline leukemia viruses (FeLV), LAV exhibited a high capacity to kill $CD4^+$ T-lymphocyte in human peripheral blood (9). Within the same year, Gallo and colleagues at the National Institutes of Health

isolated a similar human retrovirus, called human T-cell leukemia virus type III (HTLV-III), from a patient with AIDS (10). Importantly, they for the first time established the causative link between LAV-like retrovirus and AIDS by large-scale serological investigation (11). Later, the newly identified AIDS-associated human retrovirus was named as human immunodeficiency virus (HIV) and subsequently changed as HIV-1 to distinguish from HIV-2 (see below) (12).

In 1986, an HIV-1-related but distinct human retrovirus, now known as HIV-2, was isolated in patients originated from Guinea Bissau, Senegal and other West African countries (13). Unlike HIV-1, HIV-2 has lower cytopathic effect for human $CD4^+$ T-lymphocyte and lower mortality (14).

1.1.2 HIV Infections Worldwide

HIV became a major global public health issue since it was identified as the etiologic agent of AIDS in 1983. After its discovery more than three decades ago, around 60 million people have been infected with HIV-1 and more than 25 million people died (15). In 2014, an estimated 36.9 million people were living with HIV, and 1.2 million deaths were caused by HIV-related disease worldwide (16). Although HIV has spread world widely, developing countries, particularly Sub-Saharan Africa have the highest prevalence rates, with 25 million people living with HIV in 2012 (Figure 1.1). Furthermore, sub-Saharan Africa accounts for 70% of the total of new HIV infections globally.

Independent zoonotic transmission events from non-human primates to human resulted in diverse HIV lineages, including HIV-1 groups M (main), O (outlier), N (non-M, non-

O), and P and HIV-2 groups A-H (17). The epidemic area of each lineage is distinct. For example, HIV-1 group M prevails worldwide and causes global AIDS pandemic. Groups O, N and P are mainly restricted to West-central Africa such as Cameroon and only cause a few infections (18-20). According to HIV-1 sequence diversity in human, group M can be further classified into nine subtypes (A-D, F-H, J and K) and fifteen circulating recombinant forms (CRF). Nucleotide diversity within a subtype can be 3% to 10% for *gag* and 5% to 12% for *env*. Genetic variation between subtypes ranges from 15% to 20% for *gag* and 20% to 30% for *env* (2). Moreover, the distributions of different subtypes are distinct. All group M subtypes can be found in Central Africa such as the Democratic Republic of the Congo, Cameroon and Central African Republic (17). Subtype A has become the dominant subtype in eastern and central African countries. Subtype B predominates in Western Europe, North America, and Australia whereas subtype C mainly distributes in Africa and India (21).

Upon implementation of antiretroviral therapy (ART), HIV global incidence has decreased from 3.3 million in 2002 to 2.3 million in 2012 (21). However, HIV remains the leading contributor to the global disease burden. In 2010, HIV was the fifth leading cause of disability-adjusted life years worldwide. In addition to AIDS-related deaths, study also showed that HIV-1 infection can cause non-AIDS-related deaths, due to cancers, cardiovascular disease, and liver disease (22). Furthermore, compared to people without HIV, individuals infected with HIV were shown to have higher risk of myocardial infarctions (23).

Figure 1.1: Geographic distribution of HIV infections.

Diagram shows the estimated number of people who live with HIV globally in 2012 and the changes of incidence of new infections from 2001 to 2012. This figure is copied from (21) with permission (License Number: 3899030145231).

1.1.3 Origins and Evolution

HIV-1 and HIV-2 are the causative agent of AIDS, deriving from multiple zoonotic transmissions of simian immunodeficiency viruses (SIVs) infecting non-human primates in Africa (24). Four HIV-1 groups are originated from different SIV strains. According to phylogenetic analysis, HIV-1 groups M and N are in the same cluster and closely related to SIV infecting chimpanzee *Pan troglodytes troglodytes* (SIVcpz*Ptt*) in West-Central Africa (Figure 1.2) (17), suggesting that HIV-1 groups M and N originate from SIVcpz. In contrast, HIV-1 groups O and P are more related to SIV that was found in Western lowland gorillas (SIVgor) from Cameroon (17), indicating that they are of gorilla origin.

While HIV-1 spreads globally and cause AIDS pandemic, HIV-2 is predominately restricted to West Africa such as Guinea-Bissau and Senegal (25). Importantly, unlike HIV-1 infection, most individuals infected with HIV-2 do not progress to AIDS (26). The patterns of geographic distributions and disease course suggest that HIV-1 and HIV-2 have different origins. Indeed, HIV-2 was shown to originate from SIV identified in sooty mangabey (SIVsmm) in West Africa (27). Although SIVsmm does not cause disease in its natural host, the hunt of sooty mangabey as agricultural pests may have contribute to its cross-species transmission to human (24).

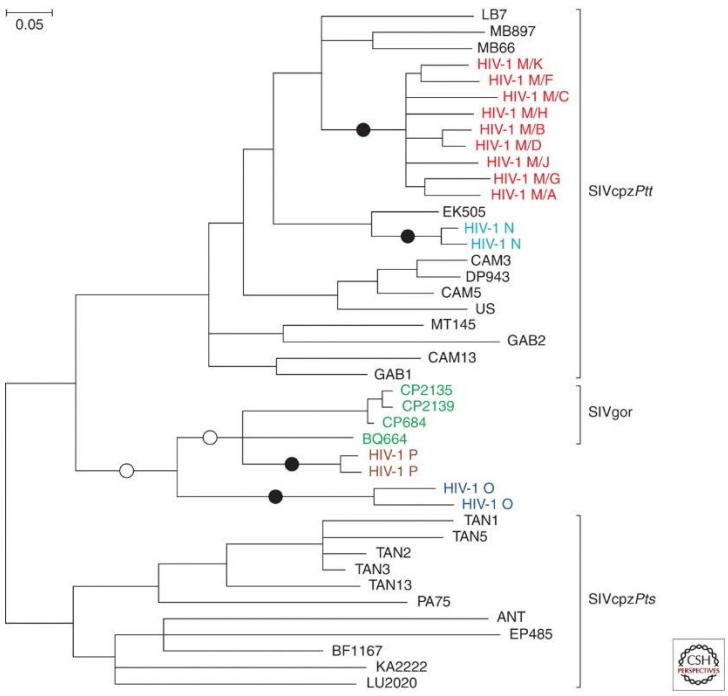

Figure 1.2: HIV-1 origins.

The phylogenetic tree of representative strains of SIVcpz, SIVgor, and HIV-1 is generated by the sequences of Pol gene. The sequences of SIVcpz and SIVgor are presented in black and green, respectively. The independent cross-species transmission events of four groups of HIV-1 are shown in different colors. Black circles indicate cross-species transmission to human has occurred. White circles indicate two possible transmission events from chimpanzee to gorilla occurred. The scale bar represents 0.05 nucleotide substitutions per site. This figure is copied from *Cold Spring Harb Perspect Med* 2011;1:a006841 (24) with permission (copyright to Cold Spring Harbor Laboratory Press).

1.2 HIV Replication

1.2.1 Taxonomic Classification

HIV is one member of the family *Retroviridae,* which consist of a large number of diverse viruses infecting vertebrate host. The viruses in the family *Retroviridae* replicate through a unique mode of life cycle. Upon entry into host cells, the viral genomic RNA is reverse transcribed into complementary DNA (cDNA) and subsequently is integrated into host chromosome. This integrated viral DNA, named as provirus, functions as the template for viral genes transcription and translation, resulting in generation of progeny virions.

The older classification of retroviruses were based on the virion morphology by electron microscopy. According to the new criteria from International Committee on Taxonomy of Viruses, all retroviruses belonging to the family *Retroviridae* are classified into two subfamilies, *Orthoretrovirinae* and *Spumaretrovirinae*. Within the *Orthoretrovirinae* subfamily, currently there are six genera including Alpharetroviruses, Betaretroviruses, Deltaretroviruses, Epsilonretroviruses, Gammaretroviruses, and Lentiviruses. *Spumaretrovirinae* subfamily only contains a single genus Spumavirus (Table 1.1) (28).

Table 1.1: Retrovirus genera

Subfamily	Genus	Species
Orthoretrovirinae	Alpharetrovirus	Avian leukosis virus (ALV) Rous sarcoma virus (RSV)
	Betaretrovirus	Jaagsiekte sheep retrovirus (JSRV) Mouse mammary tumor virus (MMTV) Mason-Pfizer monkey virus (M-PMV)
	Deltaretrovirus	Primate T-lymphotropic virus 1, 2, 3 (PTLV-1, 2, 3) Bovine leukemia virus (BLV)
	Epsilonretrovirus	Walleye dermal sarcoma virus (WDSV) Walleye epidermal hyperplasia virus 1, 2 (WEHV-1, 2)
	Gammaretrovirus	Feline leukemia virus (FeLV) Gibbon ape leukemia virus (GALV) Murine leukemia virus (MuLV) Reticuloendotheliosis virus (REV)
	Lentivirus	Bovine immunodeficiency virus (BIV) Caprine arthritis encephalitis virus (CAEV) Equine infectious anemia virus (EIAV) Feline immunodeficiency virus (FIV) Human immunodeficiency virus 1, 2 (HIV-1, 2) Simian immunodeficiency virus (SIV)
Spumaretrovirinae	Spumavirus	Bovine foamy virus (BFV) Equine foamy virus (EFV) Feline foamy virus (FeFV) Simian foamy virus (SFV)

1.2.2 Genomic Structure

HIV is an enveloped, single-stranded, positive-sense RNA virus with a diameter of around 120 nm. It contains two identical copies of genomic RNA, which encode nine viral genes including Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpr, Vpu (Vpx for HIV-2). The nucleocapsid (NC) protein bound to HIV single-stranded RNA and protects it from nucleases-mediated degradation. HIV RNA is enclosed by a conical capsid (CA) protein, surrounded by matrix (MA) protein. MA protein associates with host cell-derived lipid membrane containing viral envelope glycoproteins gp120 and gp41. Additionally, HIV particle also carries three viral enzymes: reverse transcriptase (RT), integrase (IN), protease (PR) (Figure 1.3) (29, 30), which are required for HIV replication and progeny virion maturation.

Translation of HIV mRNA results in three major structural proteins: group-specific antigen (Gag), polymerase (Pol) and envelope glycoprotein (Env). To form the mature and active products, these proteins are firstly synthesized as polyproteins, followed by viral or cellular proteases-mediated processing. For example, during or shortly after virion budding, the 55-kilodalton (kD) Gag precursor protein $Pr55^{Gag}$ is expressed from unspliced mRNA, and then cleaved into MA (p17), CA (p24), NC (p9) and p6 proteins as well as two spacer peptides, SP1 and SP2 by viral protease. The amino-terminal of myristoylated $Pr55^{Gag}$ is called MA domain, which binds to plasma membrane and recruits Env for incorporation into HIV virions. The central domain of Gag is CA, which functions to coordinate protein-protein interaction thus promoting Gag assembly to form viral conical core. The NC domain binds to and recruits HIV genomic RNA into HIV virions through two copies of zinc finger motif during assembly. The NC domain also

functions as a viral RNA chaperone during reverse transcription. The carboxy-terminus of $Pr55^{Gag}$ is p6 domain, which contains "late domain" that recruits the cellular endosomal sorting complex required for transport (ESCRT) pathway proteins, such as TSG101 and ALIX, to facilitate HIV virions efficient budding. The p6 domain also mediates interaction between Gag and an accessory protein Vpr, thus promoting Vpr incorporation into HIV particles (Figure 1.4) (31).

Along with Gag translation, a 160-kD GagPol polyprotein precursor, named $Pr160^{GagPol}$, is also expressed via ribosomal frameshifting event (32). During maturation, Pol polypeptide is cleaved from $Pr160^{GagPol}$ precursor by viral protease, and is subsequently cleaved into three viral enzymatic proteins: protease (PR), reverse transcriptase (RT), and integrase (IN). The dimeric aspartic PR is required for cleavage of Gag and GagPol precursors, resulting in mature viral particles. RT has both RNA-dependent and DNA-dependent polymerase activities. During reverse transcription, RT catalyzes DNA synthesis by using viral single-stranded RNA as the template. IN facilitates the integration of HIV reverse-transcribed DNA into host chromosome to form provirus.

Similar to Gag and Pol, HIV Env glycoprotein is first synthesized as a 160 kD precursor, named gp160. Upon cleavage by cellular protease furin, gp160 is converted into the gp120 surface (SU) subunit and gp41 transmembrane (TM) subunit, which are responsible for receptor binding and viral membrane fusion, respectively.

In addition to structural and enzymatic proteins, HIV also encodes several regulatory proteins such as trans-activator of transcription (Tat) and regulator of expression of virion proteins (Rev). Tat is expressed by spliced viral mRNA; it binds to the stem-loop secondary structure locating at the 5' long terminal repeat (LTR) of HIV genomic RNA,

named trans-activation response region (TAR). The binding of Tat to TAR efficiently activates transcription from the HIV LTR. Rev is a RNA binding protein, which recognizes and binds to a complex stem-loop secondary structure of mRNA, known as Rev response element (RRE), thus facilitating the unspliced and incompletely spliced viral RNAs export from nucleus to cytoplasm.

Unlike "simple" retroviruses that only encode Gag, Pol, and Env proteins, HIV-1 encodes four additional accessory proteins Nef, Vif, Vpr and Vpu. HIV-2 does not encode Vpu, but it contains another accessory protein Vpx instead. The accessory proteins are not always required for viral replication, but they are known to play important roles in viral pathogenesis.

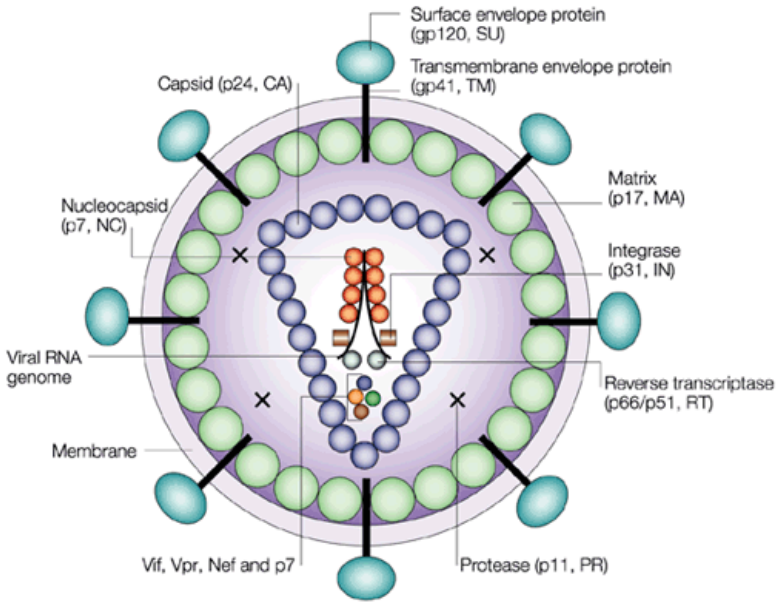

Figure 1.3: Diagram of HIV-1 mature virion.

HIV-1 envelope is derived from host cell membrane, which contains glycoproteins gp120 and gp41. The nucleocapsid (NC)-associated genomic RNA is encapsidated by capsid (CA) protein and surrounded by matrix (MA) protein. In addition, HIV-1 virion carries three virus-encoded enzymes: reverse transcriptase (RT), integrase (IN), and protease (PR). This figure is copied from (30) with permission (License Number: 3899040446499).

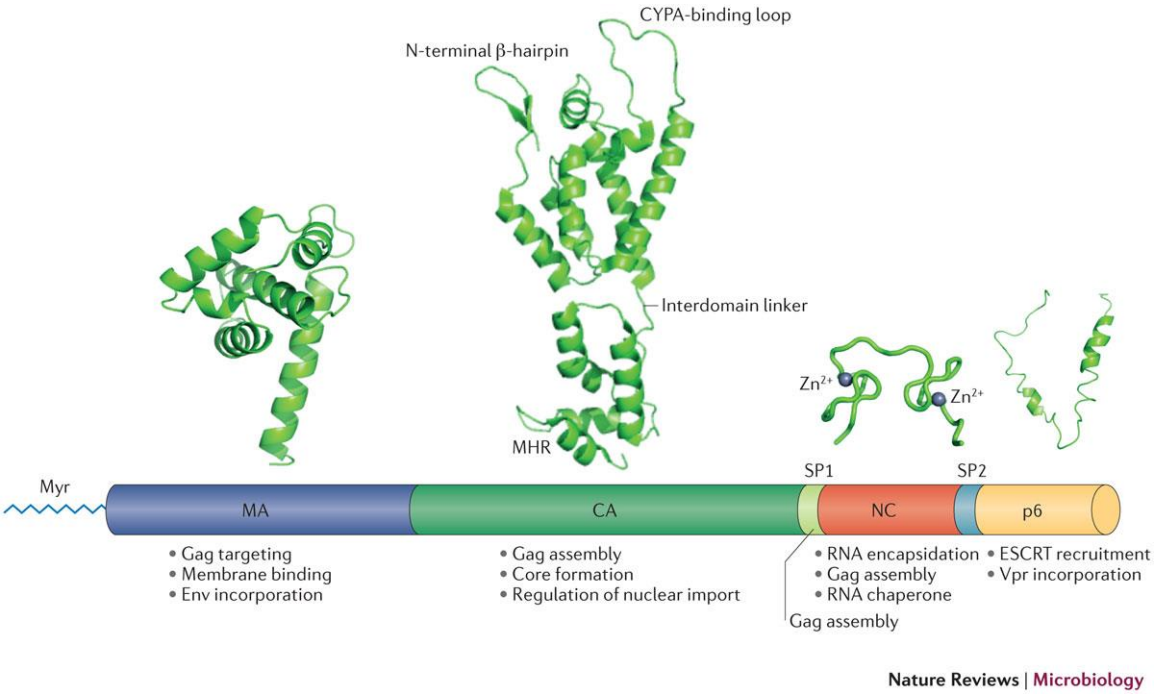

Figure 1.4: HIV-1 Gag structure and functions.

Upon virion budding, HIV-1 Gag precursor protein is cleaved into matrix (MA), capsid (CA), nucleocapsid (NC), p6 proteins, and two spacer peptides (SP1 and SP2). The N-terminal myristoylated domain is MA, which is responsible for Gag targeting and binding to plasma membrane as well as Env incorporation into virions. CA domain promotes Gag assembly, core formation, and regulation of nuclear import of viral DNA. The N-terminus of CA contains an N-terminal β-hairpin and a proline-rich loop that binds the host protein cyclophilin A (CYPA). The C-terminus of CA contains the major homology region (MHR). Spacer peptide 1 (SP1) participates Gag assembly. NC domain recruits genomic RNA into HIV virions through two copies of zinc finger motif. NC domain also serves as a RNA chaperone. p6 domain recruits the host endosomal sorting complex required for transport (ESCRT) pathway proteins to facilitate HIV-1 release. p6 domain is also involved in the Vpr incorporation into HIV virions. This figure is copied from (31) with permission (License Number: 3899040717671).

1.2.3 Overview of HIV Replication

HIV replication cycle initiates by binding of HIV Env to the primary receptor CD4 and coreceptor CXCR4 or CCR5 on the surface of target cells. Following fusion of the viral and cellular membranes either on the plasma membrane or in the endosome, partially uncoated HIV particles release their contents into cytoplasm and generate complementary DNA from viral genomic RNA through reverse transcription. The double-stranded DNA (dsDNA) is then translocated from the cytoplasm into the nucleus and integrated into host chromosomal DNA to form provirus. The provirus functions as template for viral RNA synthesis via RNA polymerase II (pol II). Unspliced or partially spliced HIV transcripts are transported to cytoplasm, followed by viral proteins translation. The resultant translation products, such as Env gp160 precursor, Gag, and GagPol polyprotein precursor, traffic to the plasma membrane. The assembly of Gag, GagPol polyproteins, Env, and viral RNA results in the formation of spherical immature viral particles. During or immediately upon virus release, processing of Gag and Pol by HIV PR results in mature HIV virions.

1.2.4 Receptor Binding and Viral Entry

HIV Env glycoprotein plays critical roles in receptor binding and viral entry. Env harbors specific domains required for interacting with CD4 receptor and coreceptor, which facilitates the fusion of viral envelope and host cellular membrane. HIV Env-mediated entry can be generally divided into the following four steps (Figure 1.5). The first is to bind the host attachment factors, such as α4β7 integrin (33), dendritic cell-specific intercellular adhesion molecular 3-grabbing non-integrin (DC-SIGN) (34), and heparan sulfate proteoglycans (35). These non-specific interactions bring Env more close to CD4 receptor and other coreceptors on the cell surface, thus enhancing their specific binding.

The second step of HIV entry is the binding of HIV Env to the host primary receptor CD4. CD4 is a member of the immunoglobulin (Ig) superfamily, which is expressed on the surface of immune cells such as monocytes, macrophages and dendritic cells. CD4 is a 55-kD protein, which contains a cytoplasmic domain, a single transmembrane domain, and four extracellular domains, named D1 to D4 (36). CD4 normally functions to assist the communication between T cell receptor (TCR) and the antigen-presenting cells. The HIV Env glycoprotein consists of gp120 trimer and gp41 heterodimers. The gp120 surface glycoprotein contains five conserved domains (C1-C5) and five variable loops (V1-V5), and is responsible for receptor binding. The variable loops, particularly V3 loop, is most critical for gp120-mediated receptor binding activity (37). The binding of Env gp120 to CD4 leads to conformational change in V1/V2 and V3 loops and formation of a so-called bridging sheet consisting four β-sheets, which are required for co-receptor binding and HIV entry.

The third step of HIV entry is coreceptor binding, which is required for efficient HIV Env-mediated membrane fusion and entry. CXCR4 and CCR5 are two major coreceptors belonging to seven-transmembrane G protein-coupled receptors (GPCRs) superfamily (38). Based on coreceptor usage, HIV isolates have been classified into two major tropisms. The HIV virus that uses CXCR4 to infect human $CD4^+$ T cell lines is denoted X4 virus, while the virus that uses CCR5 to infect monocyte-derived macrophages (MDM) is named as R5 virus. Some dual-tropic HIV strains exist, which use both CXCR4 and CCR5 coreceptors for entry. The coreceptor binding exposes the fusion peptide in the gp41 region, which is responsible for viral fusion.

The last step of HIV entry is membrane fusion, which is mediated by gp41. The functional domains of gp41 include the ectodomain, the membrane-spanning domain and the cytoplasmic domain. The ectodomain contains N-terminal hydrophobic region (fusion peptide) and two heptad repeats (HR1 and HR2), all of which are required for membrane fusion (39, 40). The cytoplasmic domain consists of two helical domains (helix-1 and helix-2), which modulate HIV Env expression on the cell surface (41). CXCR4 or CCR5 coreceptor binding results in exposure of gp41 fusion peptide, which subsequently inserts into the lipid bilayer of the host cells. This brings the viral and cellular membranes together to facilitate the formation of six-helix bundle (6HB), which are derived from the N-terminal helical region (HR-N) and the C-terminal helical region (HR-C) of gp41 trimer (42). The 6HB drives the generation and expansion of the fusion pore (43), allowing HIV to deliver its contents into cytoplasm.

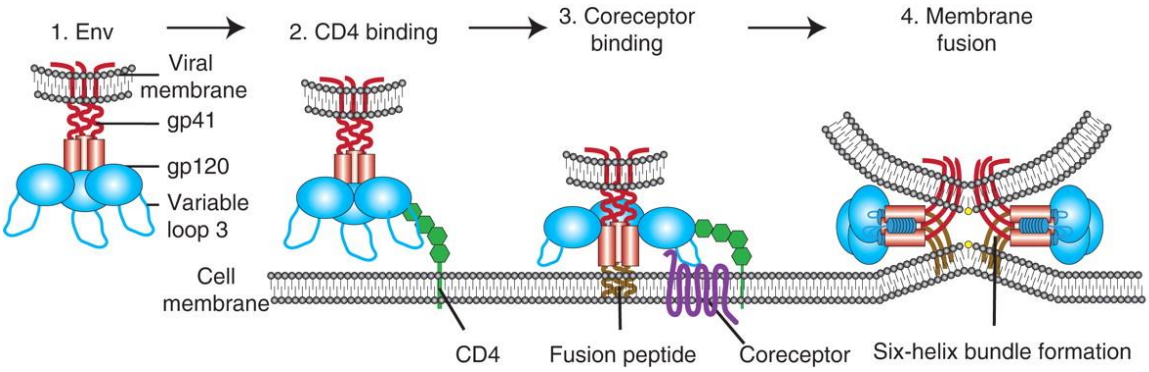

Figure 1.5: Overview of HIV entry.

HIV entry can be generally divided into four steps. (1) HIV Env consisting of gp120 and gp41 first binds to the target cells by host attachment factors. (2) HIV Env binds to primary receptor CD4, leading to the conformational change of Env. (3) This allows the binding of Env to HIV coreceptors CXCR4 or CCR5, resulting in the exposure of the fusion peptide in gp41. (4) Fusion peptide inserts into host membrane, which induces the formation of six-helix bundle (6HB). 6HB directs the formation of the fusion pore, therefore allowing HIV deliver its content into host cytoplasm. This figure is copied from *Cold Spring Harb Perspect Med* 2012;2:a006866 (37) with permission (copyright to Cold Spring Harbor Laboratory Press).

1.2.5 Reverse Transcription

One key feature in HIV life cycle is reverse transcription, which converts its single-stranded RNA genome into double-stranded DNA by RT. HIV RT has two enzymatic activities, one is DNA polymerase activity which synthesizes DNA by using either RNA or DNA templates; another is RNase H activity that removes RNA from DNA-RNA intermediates. During the reverse transcription, HIV plus-stranded genomic RNA and host $tRNA^{Lys3}$ function as the template and the primer, respectively.

The 5' UTR of HIV genomic RNA contains the secondary structure, named the primer binding site (PBS). Upon binding of host $tRNA^{Lys3}$ to PBS, the RT-mediated reverse transcription is initiated from the 3'-OH of $tRNA^{Lys3}$, leading to the generation of minus-strand DNA (Figure 1.6 step A). The newly synthesized DNA strand then extends to the 5' end of the viral genome, results in the formation of RNA-DNA intermediates. The RNase H activity of RT degrades the RNA template from this RNA-DNA hybrid to release the single-stranded DNA fragment, known as minus-strand strong-stop DNA (Figure 1.6 step B). The minus-strand strong-stop DNA then transfers to the 3' end of HIV genome and binds to the repeated (R) region present at the both 5' and 3' end of the viral genome, allowing to continue the extension of minus-strand DNA to the 5' end of the genome, coupled with RNA degradation by RNase H (Figure 1.6 step C). The polypurine tract (PPT) near to viral U3 region, which is not cleaved by RNase H, functions as the primer for plus-strand DNA synthesis (Figure 1.6 step D). Upon plus-strand DNA synthesis copies the first 18 nucleotides of the tRNA, the tRNA primer is removed by RNase H (Figure 1.6 step E). Subsequently, the plus-strand DNA transfers to the 3' end of minus-strand DNA and proceed to synthesize (Figure 1.6 step F). The plus-

and minus-strand DNAs continue to extend, resulting in the formation of complete double-stranded viral DNA (Figure 1.6 step G) (44).

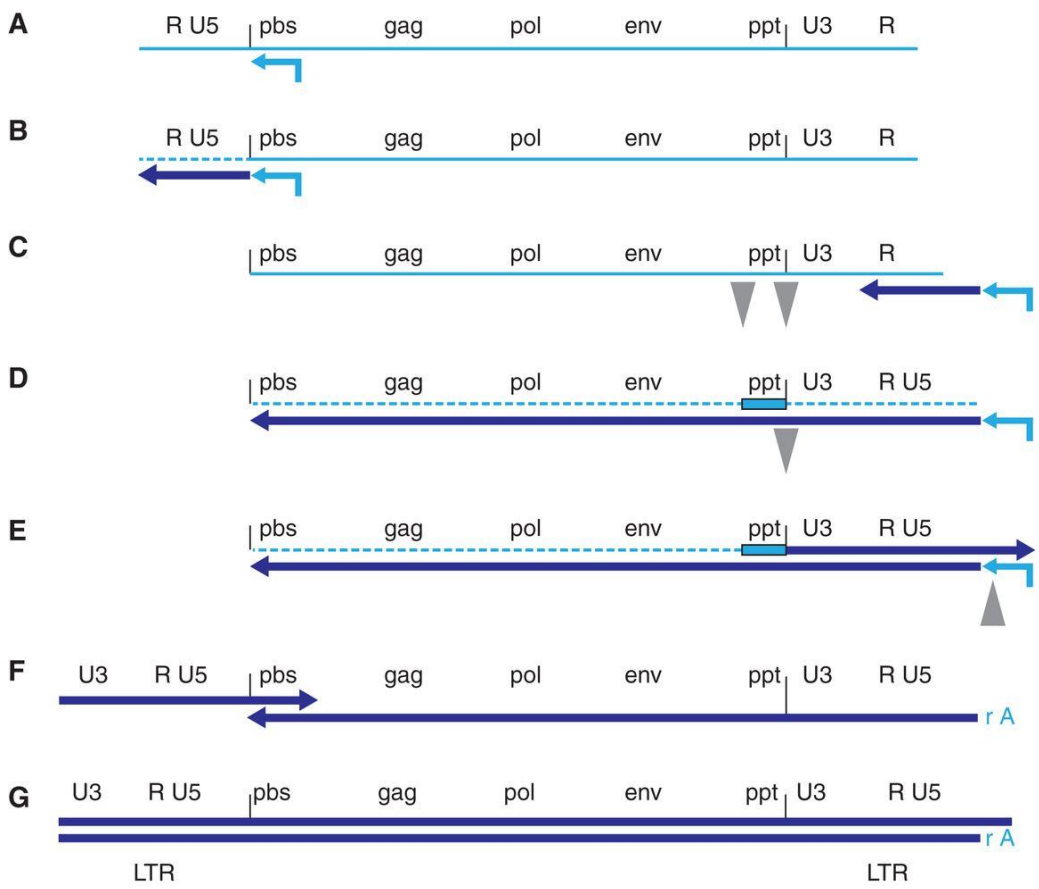

Figure 1.6: HIV reverse transcription.

HIV-1 converts genomic single-stranded RNA into double-stranded DNA. (A) Cellular tRNA functions as a primer and binds to the primer binding site (PBS) on 5' UTR of HIV genomic RNA (light blue). (B) Reverse transcription is initiated from the 3'-OH of tRNA by RT, leading to the generation of minus-strand DNA (dark blue). Upon the extension of newly synthesized DNA strand, RNase H activity of RT degrades the RNA template (dashed line). (C) Minus-strand DNA transfers between the R sequences at both ends of the genome, allowing the synthesis of minus-strand DNA to continue. (D) RNase H degrades the RNA template from RNA-DNA hybrid. The polypurine tract (PPT), adjacent to U3 region, is resistant to RNase H cleavage and functions as the primer for the synthesis of plus-strand DNA. (E) The extension of plus-strand DNA continues until the first 18 nucleotides of the tRNA are copied, allowing the removal of tRNA primer by RNase H. (F) Plus-strand DNA transfers to the 3' end of minus-strand DNA. (G) The extension of both strands results in the synthesis of the complete double-stranded viral DNA. This figure is copied

from *Cold Spring Harb Perspect Med* 2012;2:a006882 (44) with permission (copyright to Cold Spring Harbor Laboratory Press).

1.2.6 Integration

In addition to reverse transcription, another key feature of HIV replication, which distinguishes it from other viruses, is its ability to integrate the reverse-transcribed viral cDNA into the host chromosome. This integrated proviral DNA, named as provirus, replicates along with the host DNA and functions as templates for the transcription of viral RNAs and translation of viral proteins. The integration process is mediated by the HIV IN protein, a 32-kD molecule that is resulted from the HIV GagPol polyprotein precursor via RP cleavage.

The first step of HIV integration is 3' processing. In most cases, two nucleotides are removed from the blunt 3' end of both strands of linear viral DNA by IN (Figure 1.7 steps I and II). The second step is DNA strand transfer. After 3' processing, IN remains bound to both ends of HIV DNA and form the preintegration complexes, which translocates into the nucleus. In the nucleus, IN mediates the cleavage of the cellular target DNA, allowing the processed 3' end of the viral DNA to bind to the cleaved target DNA via the covalent attachment (Figure 1.7 step III). The third step is gap repair. The DNA strand transfer step leads to a five-base, single-strand gap at the junction between HIV viral DNA and host target DNA. This gap, as well as the two-nucleotide overhang at 5' end of viral DNA, are repaired by cellular enzyme-mediated repair machinery, resulting in the completion of the integration process (Figure 1.7 steps IV and V) (45, 46).

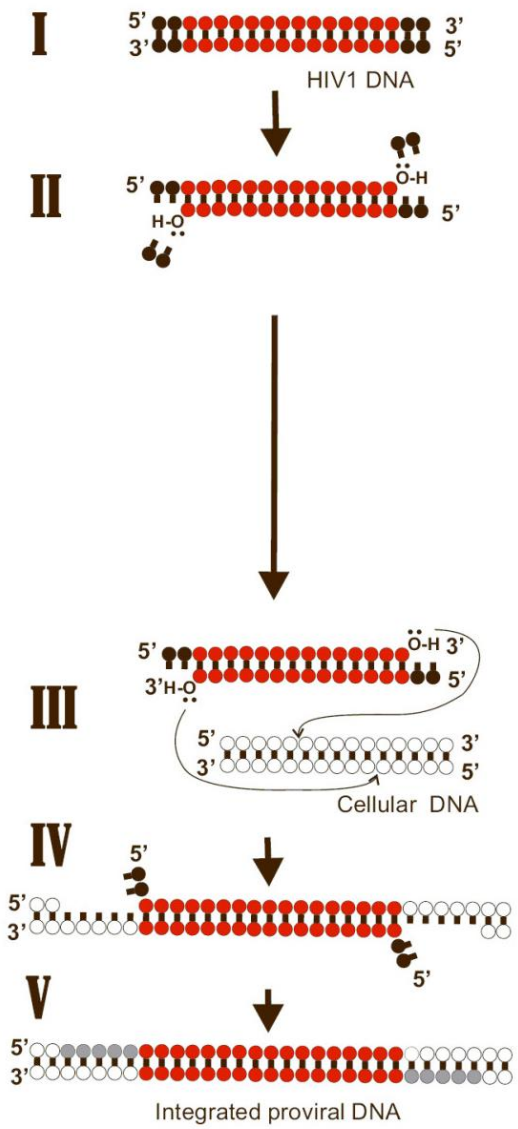

Figure 1.7: HIV-1 integration.

HIV-1 integrates viral DNA into host DNA. (I) Donor DNA that becomes integrated is shown in red. (II) Integrase mediates the removal of two nucleotides from 3' end of both DNA strands. (III) Integrase binds to both ends of viral DNA, leading to the formation of preintegration complexes that then transports to the nucleus. In the nucleus, integrase mediates the cleavage of cellular target DNA (white). (IV) The 3' end processed viral DNA binds to the cleaved host DNA, which forms a five-base, single-strand gap. (V) This

gag is repaired by cellular machinery. This figure is copied from (46) with permission (https://creativecommons.org/licenses/by/4.0).

1.2.7 Viral DNA Transcription and Regulation

The transcription of the integrated HIV DNA is mediated by the viral long terminal repeat (LTR), which is generated during reverse transcription. The HIV LTR is around 630 base pair (bp) at both 5' and 3' ends of the proviral DNA. It has been divided into three distinct regions, namely U3, R and U5, based on their locations (Figure 1.8) (47).

The 5' terminus of HIV-1 LTR is the U3 region (unique 3' sequence), which is approximately 454 bp in length. The U3 region of 5' LTR contains the promoter required for HIV transcription as well as cis-acting DNA elements required for cellular transcription factor binding. These regulatory elements subsequently recruit the cellular RNA pol II to the start site of HIV transcription, which is located at the R region. The central region of LTR is the 96 bp R region (repeated sequence). Transcription begins at the first nucleotide in the R region. The R region of 3' LTR contains the polyadenylation signal. The U5 region (unique 5' sequence) located at 3' end of LTR is an 84 bp segment which contains the Tat binding site and HIV packaging sequences (2).

The U3 region of LTR plays a critical role in HIV RNA synthesis. It consists of three regulatory domains required for HIV transcription: the core promoter region, the core enhancer region and the modulatory region (48). The core promoter region is a highly optimized promoter containing a TATA box, three SP1 binding elements, and an active initiator sequence (49). These elements facilitates the engagement of the transcription factor II D (TFIID), the TATA-binding protein (TBP), and the TBP-associated factors (TAF) to TATA box (50). The bound TFIID then recruits RNA pol II to the promoter, resulting in the initiation of transcription (51). The core enhancer region comprises of two NF-κB binding motifs and activator protein 1 (AP1) elements (Figure 1.9). The negative

modulatory region contains the binding site for the upstream stimulatory factor (USF). The transcription from HIV 5' LTR generates a 9-kb primary transcript, which encodes all nine viral genes. This primary transcript can be either spliced or packaged into HIV virions as the genomic RNA without modification (32).

The regulation of HIV DNA transcription is mediated by its Tat protein. HIV Tat is a small nuclear protein of 101 aa containing three domains: the cysteine-rich domain, the core domain and the TAR-binding domain (52). HIV Tat is essential for the transactivation of LTR-driven genes expression. Tat primarily promotes the elongation of HIV transcription, allowing the generation of the full-length transcripts (53). In the absence of Tat expression, the elongation of HIV transcription is strongly inhibited by the negative elongation factor (NELF) (54). The binding of Tat to its cofactor positive transcription elongation factor b (P-TEFb) and other elongation factors including CDK9 and CycT1 leads to formation of a complex. When the transcription goes through the TAR element, the Tat/P-TEFb complex as well as NELF are engaged into the transcription elongation complex by the interaction with TAR RNA. The binding of Tat to P-TEFb results in the conformational change of CDK9 kinase and its activation (55). This activated CDK9 then phosphorylates the CTD of RNA pol II, Spt5 and NELF-E. The phosphorylation of NELF-E causes its dissociation from TAR element (56), thus removing its inhibitory effect on HIV transcription. Furthermore, the resultant hyperphosphorylated RNA pol II and Spt5 efficiently promote HIV transcription by stabilizing the transcription complex (57).

HIV transcripts have different species. The unspliced 9 kb primary transcripts can express the Gag and GagPol precursors, or function as the genomic RNA and are

packaged into HIV virions (32). Primary HIV transcripts can be spliced into more than 40 alternative mRNAs in the nucleus (57). The completely spliced mRNAs do not contain introns and can be expressed to generate Rev, Nef, and two-exon forms of Tat. Other viral mRNA is incompletely spliced, which express Env, Vif, Vpu, Vpr and a single-exon form of Tat (32). Normally, the cellular unspliced and incompletely spliced mRNA are degraded in the nucleus. To prevent viral mRNA from degradation, HIV encodes Rev protein to facilitate export of the intro-containing mRNA from nucleus into cytoplasm. HIV Rev is a 13-kD RNA binding protein (58), which binds to a viral RNA secondary structure named RRE. An effector domain of Rev contains the nuclear export signal (NES) (59), which is recognized by its receptor, known as CRM1. In the presence of RAN GTPase, the interaction between Rev and CRM1 mediates HIV mRNA export through the nuclear pore complexes (NPC) (57). Once HIV mRNAs are translocated into cytoplasm, the cellular translation machinery is recruited, which initiates the translation of viral genes.

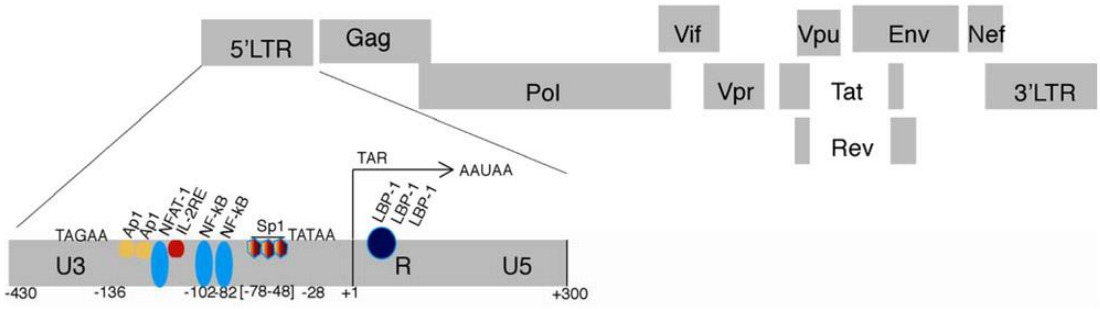

Figure 1.8: The LTR promoter of HIV.

Diagram shows the genomic organization of HIV-1 and highlights the 5' LTR structure. The HIV LTR consists of three regions, U3, R and U5. The upstream weak transcriptional start TAGAA, the transcription factors AP1, NFAT-1, IL-2RE, NF-kB, the TATA box, the binding sites of Sp1 and LBP-1, Tat activating region (TAR), and the downstream AAUAA poly-adenylation sites are shown. This figure is copied from (48) with permission (https://creativecommons.org/licenses/by/3.0).

1.2.8 Assembly, Release and Maturation

Following biosynthesis, HIV Gag recruits viral genomic RNA and then traffics to the plasma membrane for assembly. The resultant immature viral particle then incorporates Env glycoprotein and recruits the host ESCRT pathway to promote release by membrane scission. During or shortly after HIV budding, the viral protease cleaves Gag polyprotein and drive its maturation, resulting in the infectious HIV virions (Figure 1.9).

Assembly

HIV assembly begins with the synthesis of Gag polyprotein and its subcellular trafficking to the viral assembly site, the plasma membrane. In the cytoplasm, the Gag polyprotein is firstly translated into precursors, including $Pr55^{Gag}$ and $Pr160^{GagPol}$. The Gag polyprotein precursor $Pr55^{Gag}$, consisting of MA, CA, NC and p6 domains, is synthesized from unspliced viral mRNA. The GagPol polyprotein precursor $Pr160^{GagPol}$, which contains MA, CA, NC, PR, RT and IN, is generated through a programmed ribosomal frameshifting event (31).

Upon its synthesis, the Gag polyprotein then targets to the inner leaflet of the plasma membrane for assembly. This process is mediated by a specific phospholipid named phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2). PtdIns(4,5)P_2 is highly enriched in the inner leaflet of the plasma membrane and is responsible for Gag targeting by directly interacting with MA domain of the Gag (60). The binding of PtdIns(4,5)P_2 to MA causes the exposure of the N-terminal myristate of MA, thus facilitating its insertion into plasma membrane (61). During its targeting to the plasma membrane, Gag interacts with HIV genomic RNA through NC domain that binds to the packaging signal (ψ element) near

the 5’ UTR region of the viral RNA (62). The NC domain of Gag contains two Cys-Cys-His-Cys zinc-finger-like motifs, which stabilize the interaction of NC and RNA (63).

At the plasma membrane, the Gag polyprotein, GagPol precursor, as well as HIV full-length genomic RNA assemble into an immature HIV particle. The Gag molecules in the immature particle are packed radially, with the N-terminus of MA domain bound to the inner leaflet of the viral membrane and the C-terminus of the p6 domain facing towards the interior of the viral particle (64). The assembly of immature HIV particle is mainly mediated by the CA domain of Gag. The protein-protein interactions within CA domain, particularly the C-terminal domain of CA (CA_{CTD}) and SP1 plays a critical role in HIV assembly (65).

Following its synthesis, HIV Env glycoprotein traffics from the rough endoplasmic reticulum (RER) to the Golgi apparatus and ultimately reaches at the plasma membrane via the cellular secretory pathway. The assembly of immature HIV particle also requires Env glycoproteins for co-packaging. However, the mechanism by which HIV Env is incorporated into the nascent virion remains elusive. The potential mechanisms include: passive incorporation (HIV Env expressed on the cell surface is incorporated into virions passively as a component of the plasma membrane); Gag-Env co-targeting (both Gag and Env target to a specific site that locates at the plasma membrane, such as lipid raft); direct Gag-Env interaction (The direct interaction between the MA domain of Gag and the cytoplasmic tail of gp41 of Env recruits Env into virions); indirect Gag-Env interaction (The MA domain of Gag and the cytoplasmic tail of gp41 of Env may interact indirectly through a host bridging protein, allowing the recruitment of Env into HIV virions) (66).

Release

The release of immature viral particles from the cell surface is driven by the host cellular ESCRT machinery. Normally, ESCRT pathway proteins play roles in catalyzing the membrane fission reaction to release vesicles into multivesicular bodies (MVBs) (67). In the same manner, HIV Gag hijacks the host ESCRT machinery via its p6 domain to facilitate the release of viral particle.

The p6 domain of HIV Gag consists of two "late domain" for ESCRT protein binding (68). The primary late domain called "PTAP", which interacts with the TSG101 subunit of the ESCRT-I complex (69). The second late domain is "YPXL" (Tyr-Pro-X-Leu) that binds to the ALIX subunit of ESCRT (70). The binding of Gag "late domain" to the TSG101 and ALIX subunits of ESCRT factors leads to the recruitment of the ESCRT-III proteins, including the CHMP1, CHMP2, and CHMP4. These downstream factors then induce the polymerization of the protein into filaments within the "neck" of the budding virus (71). Additionally, CHMP2 binds to and recruits VPS4 ATPases, which promotes the membrane fission reaction by destabilizing the hemi-fission intermediates (72). By using the energy of ATPase, VPS4 dissociates the filaments and releases the ESCRT-III proteins into the cytoplasm from the plasma membrane (73).

Maturation

During or immediately following HIV release from the plasma membrane, the immature viral particle undergoes maturation process to generate the infectious virions. HIV maturation is triggered by HIV encoded PR, which cleaves Gag and GagPol polyprotein precursors into MA, CA, NC, p6, PR, RT and IN proteins at ten different sites (74). These fully processed proteins will reassemble to form the conical core. The MA targets to the inner leaflet of the viral membrane, giving rise to a matrix shell without

long-range order. Approximately 1200 copies of the CA forms the outer capsid shell with conical shape. The nucleocapsid, which associates with HIV genomic RNA, is surrounded by the capsid (75).

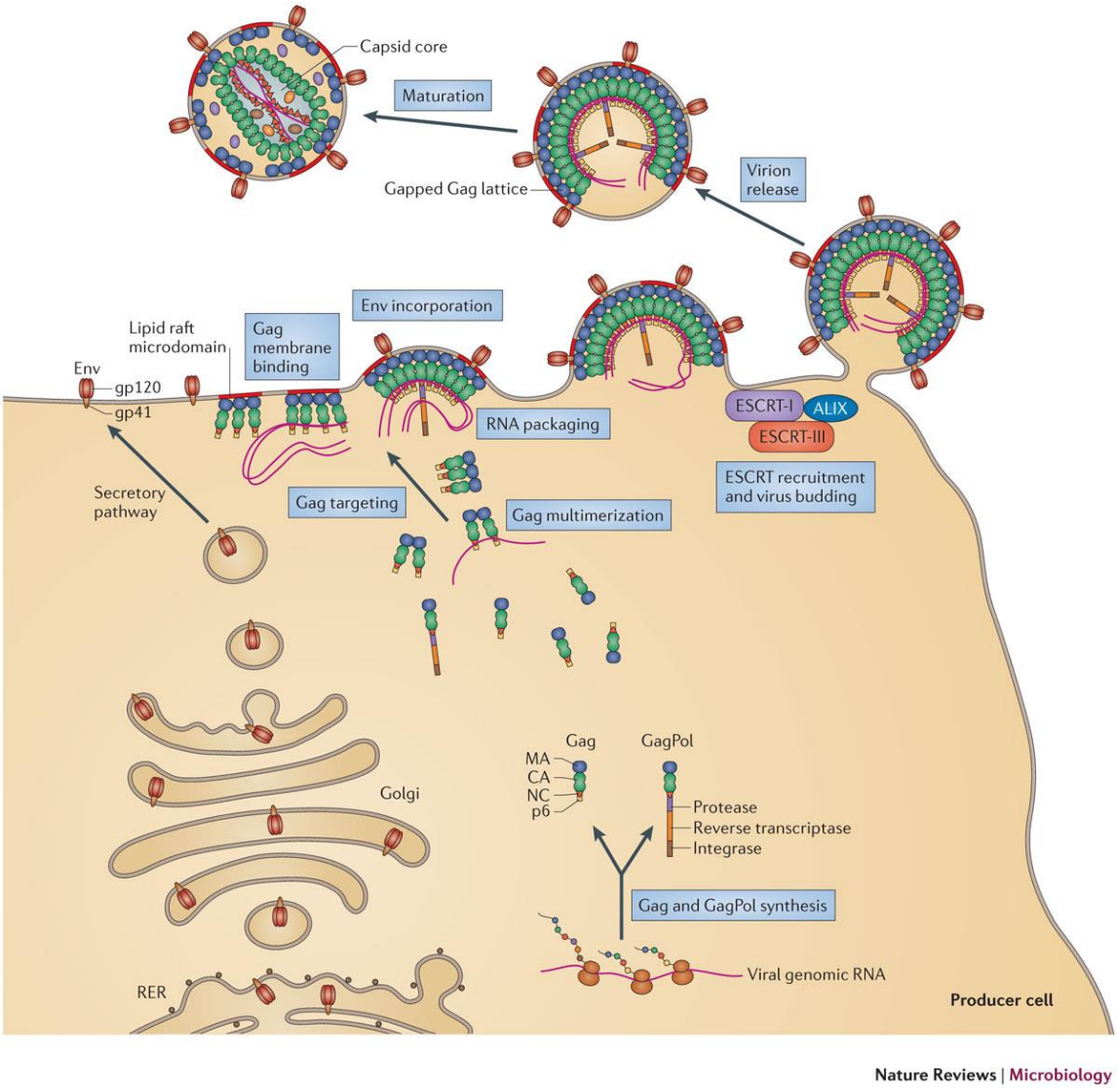

Figure 1.9: HIV assembly, release and maturation.

Upon synthesis, HIV-1 Env glycoproteins traffic from the rough endoplasmic reticulum (RER) to the Golgi and then to the plasma membrane. In the cytoplasm, the Gag precursor polyprotein is synthesized from full-length viral RNA while the GagPol precursor polyprotein is synthesized through programmed frameshifting. Gag recruits the viral genomic RNA and traffics to the plasma membrane. Gag then binds to the plasma membrane in lipid raft microdomain via the interaction with the phospholipid phosphatidylinositol-(4,5)-bisphosphate. The assembling particle incorporates Env and then recruits

cellular endosomal sorting complex required for transport (ESCRT) pathway proteins to drive the membrane scission reaction that leads to the release of virions. During or immediately following HIV budding, the Gag and GagPol polyprotein precursors are cleaved by virus-encoded protease, leading to the formation of mature viral particles. This figure is copied from (31) with permission (License Number: 3899120368176).

1.3 HIV Transmission and Pathogenesis

1.3.1 Transmission Routes

Generally, HIV-1 is transmitted by sexual contact across a mucosal surface, by percutaneous inoculation, and by mother-infant exposure (76). The sexual transmission, including heterosexual and homosexual mode, is the major route for HIV-1 transmission. In 2010, the heterosexual transmission accounts for 70% of HIV-1 infection globally (77, 78).

For sexual transmission, the mucosal surface of genital tract is the main invasion site for HIV-1 infection. The individual can be infected by certain body fluids such as semen, cervicovaginal and rectal secretions as well as blood when he/she has sex with a HIV-1 infected partner (79). In 2010, more than 22 million infection is caused by sexual contact (78). Another important transmission route is the percutaneous inoculation, including needle sharing, contaminated blood transfusion, and health-care-related accidents (79). This transmission mode is responsible for 2.6 million HIV-1 infection worldwide in 2010 (78). The mother-to-child transmission takes place at the placenta via contaminated maternal blood (79). In 2010, approximately 480,000 HIV-1 infection results from the maternal-infant transmission (78).

1.3.2 Acute Infection

During HIV infection, the virus directly kills cells that are required for host immune response, leading to the depletion of $CD4^+$ T cells and the development of AIDS. The time course of disease from acute infection to AIDS varies greatly among the infected individuals. Some person progresses as rapidly as 6 months (80), while others maintain

normal $CD4^+$ T level and exhibit no obvious immune deficiency even 25 years post-infection (76). The time course of typical HIV infection is generally divided into four stages (Figure 1.10) (81).

1. The first 1-2 week after initial infection is called eclipse phase. At this stage, HIV can replicate and spread to other target tissues and organs without the interruption by the host immune response. Viremia is undetectable. Neither $CD4^+$ T cells decline nor symptoms are observed.

2. The 2-4 weeks after infection is the acute infection phase. The level of viremia is relatively high in this phase, with up to 10^7 copies of viral RNA per milliliter of blood (81). Due to large portion of $CD4^+$ T cells in the blood and lymph nodes that are infected by HIV, the transient reduction of $CD4^+$ T cells can be observed. When viremia reaches at the peak, the host immune response is induced, which subsequently suppresses virus replication by more than 100-fold (81). The resultant immune response includes neutralizing antibodies that target to all viral proteins, as well as $CD8^+$ T cells-mediated clearance of HIV-1 infected cells.

3. The chronic infection phase varies enormously from one person to another. In some cases this phase begins at 1 year after the initial infection whereas in others it may last up to 20 years. The level of viremia in this phase remains constant or increases slowly, with 1-100,000 copies/mL (81). Accordingly, the count of $CD4^+$ T cells maintains steady or decline gradually. Patients in this phase usually exhibit no apparent symptom.

4. Along with the disease progress, the number of $CD4^+$ T cells declines to 200 cells/μL (81). As a result, the host immune response cannot be maintained to effectively control

the HIV-1 infection, leading to increase in viremia as well as the appearance of opportunistic infections. Eventually, HIV infection will cause death of the infected individual without treatment.

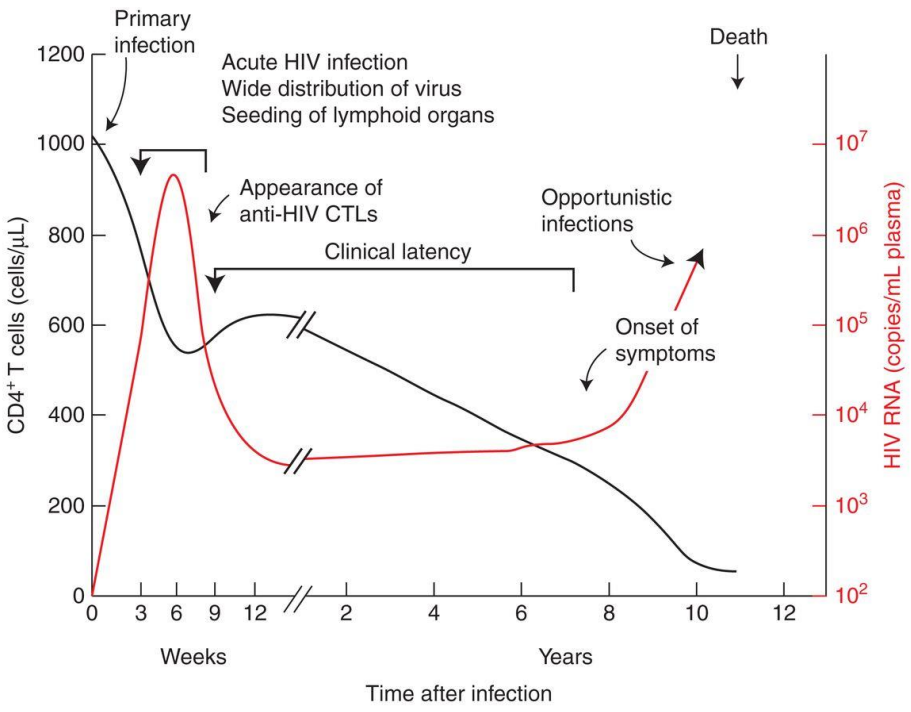

Figure 1.10: Time course of typical HIV infection.

$CD4^+$ T cells number and HIV RNA in the plasma from HIV-1 patients are shown. The first 1-2 week after initial infection, HIV can replicate and spread to other tissues, but neither viremia nor decline of $CD4^+$ T cells can be detected. The 2-4 week after infection is the acute infection phase. HIV replicates rapidly, leading to the relatively high viremia. The decline of $CD4^+$ T cells can be observed. When viremia reaches at the peak, the host immune responses including CTL are activated, which suppress virus replication. The following chronic infection phase varies from one patient to another. In this phase, the viremia and $CD4^+$ T cells largely remain constant. No apparent symptom is observed. Along with the disease progress, the number of $CD4^+$ T cells dramatically decreases, leading to the attenuation of host antiviral immune responses. As a result, the viremia increases, accompanied with the appearance of opportunistic infections and other symptoms. Eventually, HIV infection may cause death in the absence of treatment. This figure is copied from *Cold Spring Harb Perspect Med* 2013;3:a012526 (81) with permission (copyright to Cold Spring Harbor Laboratory Press).

1.3.3 Latency

In addition to the acute infection, HIV can latently infect the long-lived resting memory $CD4^+$ T cells, resulting in the establishment of a stable reservoir (82). Although the latent reservoir contains the replication-competent provirus, it is transcriptionally inactive, therefore no viral proteins or enzymes are produced (76). Due to this state of non-productive infection, HIV-infected cells are resistant to the antiviral drug treatment and the host immune response. HIV latency can be established in the central and transitional memory T cells (83, 84) as well as in the naive T cells (85). Moreover, monocyte-macrophages (86) and astrocytes (87) are the potential targets for HIV latency.

Because HIV does not efficiently infect the resting memory $CD4^+$ T cells (88), it is not clear how HIV can establish a stable latent reservoir in these cells. One leading theory is the conversion of HIV-infected activated $CD4^+$ T cells to resting memory $CD4^+$ T cells. The activated $CD4^+$ T cells serve as the main target that are highly susceptible to HIV infection. During HIV infection, although the majority of the activated $CD4^+$ T cells die (89), some HIV-infected population can still survive long time to revert back to the resting state (90), leading to the formation of long-lived resting memory $CD4^+$ T cells.

The mechanisms by which the HIV latency is maintained in resting T cells is unclear. Many factors that suppress HIV transcription may contribute to the maintenance of the latent state. One factor is the epigenetic changes in the chromatin. The condensed structure in the chromatin around provirus impedes recruitment of key transcription factors, thus suppressing HIV gene expression (91). Another factor is the DNA methylation. Two CpG islands near the state site of HIV transcription can be methylated in primary $CD4^+$ T cells model of HIV-1 latency (92), suggesting that the DNA

methylation may promote HIV latency. Furthermore, the transcriptional interference also plays a critical role in latency. If HIV provirus and the upstream host gene share the same polarity, the pol II-mediated elongation of host gene occludes HIV 5' LTR promoter, thus promoting HIV latency (93). In contrast, if HIV provirus and host gene are in the opposite polarity, the pol II initiation collides with each other, resulting in the suppression of both gene expression (94). Moreover, efficient HIV transcription relies on the host transcription factors such as NF-κB, NFAT, and AP-1. In resting $CD4^+$ T cells, sequestration of NF-κB and NFAT in the cytoplasm results in shortage of those factors in the nucleus (90), therefore reinforcing viral latency.

1.4 HIV Treatment and Prevention

1.4.1 Antiviral Drugs

Since HIV has been identified as the pathogen of AIDS, much efforts have been made to search for the agents that target viral reverse transcriptase (RT) (3), the key enzyme for productive HIV infection. In 1987, Zidovudine (AZT), an RT inhibitor, became the first antiretroviral drug approved by Food and Drug Administration (FDA) (95). To date, more than 25 drugs against HIV infection have been approved by FDA. These drugs are generally divided into 6 classes that target and inhibit HIV replication at different steps of the life cycle (95): (1) coreceptor antagonists, (2) fusion inhibitors, (3) nucleoside-analog reverse transcriptase inhibitors (NRTIs), (4) non–nucleoside reverse transcriptase inhibitors (NNRTIs), (5) integrase inhibitors, (6) protease inhibitors (PIs) (Table 1.2).

The first step of HIV life cycle, i.e. viral entry, is targeted by coreceptor antagonists and fusion inhibitors. Maraviroc (MVC), a small-molecule CCR5 antagonist, binds to the

hydrophobic transmembrane region of CCR5 and induces conformational changes in the second extracellular loop of the receptor, therefore disrupting the interaction of HIV gp120 and CCR5 (96). The final step of HIV entry is membrane fusion that requires formation of a six-helix bundle (6HB). The helical inhibitors enfuvirtide (T-20) blocks the 6HB formation and inhibits the fusion event between cellular and viral membranes (97).

NRTIs function as prodrugs that require cellular kinase-mediated phosphorylation in order to generate an active form (98). NRTIs lacking a 3'-OH group at the sugar moiety inhibits the formation of a 3'-5'-phosphodiester bond between the NRTIs and incoming 5'-nucleoside triphosphates, thus leading to the termination of HIV DNA synthesis (95, 99). Currently, 7 NRTIs have been approved by FDA, including: abacavir (ABC), didanosine (ddI), emtricitabine (FTC), lamivudine (3TC), stavudine (d4T), tenofovir disoproxil fumarate (TDF), zidovudine (AZT).

NNRTIs are noncompetitive inhibitors that bind to a hydrophobic pocket in RT distinct from the dNTP binding site (100). The binding of NNRTIs induces conformational changes of the binding site for substrate, thus reducing RT activity (101). Currently, there are 5 approved NNRTIs: delavirdine (DLV), efavirenz (EFV), etravirine (ETR), nevirapine (NVP), rilpivirine (RPV).

FDA-approved drugs that target HIV integrase include dolutegravir (DTG), elvitegravir (EVG), and raltegravir (RAL). These inhibitors bind to the complex between the viral integrase and the viral DNA and sequester the active site of Mg^{2+}, resulting in the termination of HIV DNA strand transfer reaction (102).

HIV-encoded protease, which is required for generation of infectious HIV virions, is targeted by protease inhibitors (PIs). Unlike other entry and RT inhibitors, PIs do not prevent host cells from HIV infection, rather they lead to the production of uninfectious HIV virions (103). PIs function as analogs of the cleavage sites in the Gag and Gag-Pol polyprotein precursors, thus disrupting viral protease-mediated maturation (76). Currently, 8 PIs have been approved by FDA: atazanavir (ATV), darunavir (DRV), fosamprenavir (FPV), indinavir (IDV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV), tipranavir (TPV).

By 2015, approximately 15.8 million HIV-infected people were treated with antiretroviral therapy (ART) globally. Currently, the standard antiretroviral therapy regimens combine two NRTIs with a NNRTIs, protease inhibitor, or integrase inhibitor (21). Compared to monotherapy, the combination of antiretroviral agents has higher efficacy and durability. Importantly, the combination of antiretroviral therapy has been shown to significantly suppress HIV replication and decrease the viral load in the blood to an undetectable level (104).

Table 1.2: FDA-approved HIV medicines

Drug Class	Brand Name	Generic Name	FDA Approval Date
Entry Inhibitors	Selzentry	maraviroc (MVC)	August 6, 2007
Fusion Inhibitors	Fuzeon	enfuvirtide (T-20)	March 13, 2003
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)	Ziagen	abacavir (ABC)	December 17, 1998
	Videx	didanosine (ddI)	October 9, 1991
	Emtriva	emtricitabine (FTC)	July 2, 2003
	Epivir	lamivudine (3TC)	November 17, 1995
	Zerit	stavudine (d4T)	June 24, 1994
	Viread	tenofovir disoproxil fumarate (TDF)	October 26, 2001
	Retrovir	zidovudine (AZT)	March 19, 1987
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Rescriptor	delavirdine (DLV)	April 4, 1997
	Sustiva	efavirenz (EFV)	September 17, 1998
	Intelence	etravirine (ETR)	January 18, 2008
	Viramune	nevirapine (NVP)	June 21, 1996
	Edurant	rilpivirine (RPV)	May 20, 2011
Integrase Inhibitors	Tivicay	dolutegravir (DTG)	August 13, 2013
	Vitekta	elvitegravir (EVG)	September 24, 2014
	Isentress	raltegravir (RAL)	October 12, 2007
Protease Inhibitors (PIs)	Reyataz	atazanavir (ATV)	June 20, 2003
	Prezista	darunavir (DRV)	June 23, 2006
	Lexiva	fosamprenavir (FPV)	October 20, 2003
	Crixivan	indinavir (IDV)	March 13, 1996
	Viracept	nelfinavir (NFV)	March 14, 1997
	Norvir	ritonavir (RTV)	March 1, 1996
	Invirase	saquinavir (SQV)	December 6, 1995
	Aptivus	tipranavir (TPV)	June 22, 2005

1.4.2 Broadly Neutralizing Antibodies

To generate specific antibodies against HIV, host humoral immune response occurs within weeks of the initial infection. The antigen-antibody complexes as the early B cell response can be detected within 1 week of viremia (105). Subsequently, anti-gp41 antibodies can be detected, which is followed by anti-gp120 antibodies that mainly target the V3 loop of the HIV gp120 subunit (106). However, these early antibodies have no detectable effect on HIV replication (105) until the appearance of neutralizing antibodies (NAbs) months later. NAbs bind to the envelope glycoproteins of cell-free HIV and block the interaction between HIV envelope and host receptor CD4 and/or CCR5, therefore preventing targets cells from viral infection (106). Alternatively, following binding of gp120 to CD4, NAbs block the fusion event between viral and cellular membrane (107).

HIV has evolved a variety of mechanisms to overcome the recognition of NAbs. For examples, HIV envelope is highly glycosylated, which prevents the binding of NAbs (76). Due to its error-prone RNA-dependent DNA polymerase, HIV replication gives rise to the unprecedented antigenic diversity (108). Additionally, HIV-specific NAbs themselves can drive the evolution of viral envelope glycoprotein within an infected individual, resulting in generation of escape mutants (109). As a result, the overall effect of NAbs on viremia is minimal (110).

Although NAbs have the potential to neutralize the autologous virus, they are unable to target diverse strains of HIV isolated from other HIV-infected patients (111). During HIV infection, approximately 10-25% of HIV infected subjects can generate the so-called "broadly neutralizing antibodies (bNAbs)" that are able to neutralize a significant number of divergent HIV isolates (112). Before 1999, only 4 bNAbs have been identified with

neutralization activity (113). Along with the development of new technologies such as multiparameter flow cytometry (114) and high-throughput microneutralization assay (115), more than 23 new bNAbs have been recently isolated and characterized. Based on structure studies, bNAbs typically recognize and bind to the conserved regions of HIV envelope. For examples, 2F5 and 4E10 target the membrane-proximal external region (MPER) of gp41; PG9 and PG16 target the V1V2-glycan; 2G12 binds to the glycan array on the gp120 outer domain; b12 and VRC01-03 recognize the CD4 binding site (106, 116).

By passive infusion, NAbs are shown to protect non-human primates from infection of a chimeric simian-human immunodeficiency virus (SHIV) bearing HIV-1 Env (117, 118). Although the effect of these NAbs on controlling HIV infection in human is still unknown (106), the development of means to induce broadly cross-reactive neutralizing antibodies remains a major goal of HIV-1 vaccine design.

1.4.3 Vaccine Trials

Vaccination is one of the most powerful medical interventions in controlling and preventing viral infection such as smallpox, polio, measles, and yellow fever. Since the first HIV vaccine trial opened in 1987, more than 187 separate trials have been conducted (119). Unfortunately, there is currently no safe and effective vaccine available against HIV infection.

The challenges in developing an HIV vaccine are caused by several factors. First, the genetic diversity of HIV both within and among subtypes remains the major obstacle for vaccine development (119). HIV group M consists of nine subtypes and more than 35

circulating recombinants globally. The amino acid sequence diversity within a subtype can be 20% while between subtypes can be up to 35% in Env (120). Furthermore, because of its error-prone RNA-dependent DNA polymerase, HIV replication generates tremendous amounts of mutants, allowing HIV to escape recognition of host immune response (121). Second, human body is unable to constitute the protective immunity against HIV because natural immune responses fail to prevent HIV infection or eradicate the virus (21, 119). Although passive administration of neutralizing antibodies can protect nonhuman primates from infection of SHIV (122), their role in controlling the established HIV infection in human remains elusive (106). Broadly neutralizing antibodies likely have the potential to neutralize diverse HIV strains, but these only exist in a rare population of HIV-infected individuals (~10%) (123). Moreover, HIV-elicited T cell responses, including cytotoxic T-lymphocyte (CTL) cell activity, are incapable of eradicating viral infection although they can suppress HIV infection (124). Finally, the establishment of a stable reservoir in resting memory $CD4^+$ T cells acts as another barrier for antiviral treatment as well as host immune response (125).

Due to safety concern, inactivated vaccines, which are one of the conventional strategies for vaccine development, are not considered for HIV (119). Additionally, HIV inactivation can reduce the antigenicity and only elicit modest neutralization activity (126). These considerations have directed newer approaches for HIV vaccine development that utilize chemically synthesized HIV Env protein subunits (AIDSVAX B/B) or peptide fragments (LIPO-5). HIV-1 proteins expressed via recombinant viral vectors (MRKAd5 HIV-1 gag/pol/nef (B)) or via naked plasmid DNA have also been utilized (119).

Because of the challenges in HIV vaccine development, only 5 candidates have been proceeded to an efficacy test (119); the majority of them have been eliminated due to lack of an efficacy. One phase III trial, RV114, in Thailand provided the first evidence that an HIV vaccine could elicit effective protection in human (127). Over a period of six months, volunteers received the prime-boost vaccination of two vaccines (ALVAC-HIV vCP1521 and AIDSVAX B/E gp120). ALVAC-HIV vCP1521 contains a canarypox vector that expresses HIV Env, Gag and Pol genes. AIDSVAX B/E consists of an engineered gp120 subunit of HIV envelope glycoprotein (128). After 42 months of follow-up post-vaccination, RV144 trial showed 31.2% reduction in HIV acquisition (129). The valuable information from RV144 trial helps determine which immune response is required and also inform the future HIV vaccine design.

1.5 Innate Immunity to HIV-1 Infection and Viral Countermeasures

1.5.1 Immune Sensing to HIV-1 Infection

The innate immune response against microbes' invasion is initiated by recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) that activate downstream type I interferon (IFN) induction and proinflammatory cytokines secretion (130, 131). Pathogen-derived nucleic acids as PAMPs can be detected by a variety of host innate immune sensors. The membrane-associated Toll-like receptor (TLR) family proteins detect pathogen's nucleic acids in the endosomes and lysosomes (132), whereas other cytosolic nucleic acid sensors recognize RNA or DNA in the cytoplasm or nucleus. For example, RIG-I-like receptor (RLR) family members detect pathogen-derived RNA in the cytosol (133) while cytoplasmic DNA is mainly sensed by cyclic GMP-AMP (cGAMP) synthase (cGAS) (134), IFNγ-inducible protein16 (IFI16)

(135), DEAD (Asp-Glu-Ala-Asp) box polypeptide 41 (DDX41) (136) and DNA-dependent activator of interferon-regulatory factors (DAI) (137).

Multiple PRRs have been shown to involve HIV-1 immune sensing, including cGAS, IFI16 and TLR7 (138) (Figure 1.11). During HIV replication, the newly generated cDNA resulting from reverse transcription can serve as a potential ligand for immune sensing by cGAS and IFI16. cGAS is shown to directly bind to HIV DNA and activate type I IFNs induction in human monocytic THP-1 cells and monocyte-derived dendritic cells (MDDCs) (139). Upon binding to HIV DNA, cGAS catalyzes the production of 2'3'-cGAMP from ATP and GTP (140). The resultant cGAMP subsequently functions as a second messenger that binds to and activates the endoplasmic reticulum (ER)-associated adaptor protein stimulator of interferon genes (STING) (141, 142). STING then activates the protein kinases IκB kinase (IKK) and the tank binding kinase (TBK1), resulting in the further activation of the nuclear factor κB (NF-κB) and the interferon regulatory factor 3 (IRF3) respectively. The activated NF-κB and IRF3 then translocate into nucleus and induce transcription of type I IFNs and other cytokines (143-145). Similar to cGAS, HIV sensing by IFI16 also dependents on the activation of STING-TBK1-IRF3 signaling axis. Additionally, IFI16 activation leads to cell death in quiescent tonsillar $CD4^+$ T cells via caspase 1-mediated pyroptosis (146).

In addition to the recognition of HIV DNA in the cytoplasm by DNA sensors, HIV genomic RNA can be sensed by TLR7 in the endosomes of plasmacytoid dendritic cells (pDCs) (147). The binding of TLR7 to HIV single-stranded RNA (ssRNA) results in recruitment of adaptor protein myeloid differentiation primary response gene 88 (MYD88). The MYD88 protein then activates NF-κB and interferon regulatory factor 7

(IRF7), leading to the induction of type I IFNs. Significantly, disruption of TLR7 sensing has been shown to inhibit IFN production in SIV-infected macaques, highlighting the critical role of TLR7-mediated immune sensing *in vivo* (148). Except pDCs, HIV likely avoids IFNs induction in many of its infected cells (149). The host 3'-repair exonuclease 1 (TREX1) may play a role in HIV immune escape. In the absence of TREX1, HIV reverse transcribed-DNA functions as a ligand for immune recognition by the cytosolic DNA sensors. However, in the presence of TREX1, it degrades and removes excess viral DNA in the cytosol, therefore inhibiting HIV immune sensing (150).

Through an autocrine and a paracrine manner, the binding of type I IFNs to type I IFN receptor (IFNAR) on the cell surface initiates IFN signaling. The interaction between IFN and IFNAR leads to the activation of the intracellular receptor-associated protein kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2). These activated kinases then phosphorylate the signal transducer and activator of transcription (STAT) family proteins to form STAT1 homodimer or STAT1-STAT2 heterodimer that subsequently translocate into the nucleus. STAT1-STAT2 heterodimer binds to IFN regulatory factor 9 (IRF9) to form the ISG factor 3 (ISGF3) complex that binds to IFN-stimulated response elements (ISREs), whereas STAT1 homodimer binds to gamma-activated sequence (GAS). The binding of STAT dimers to ISREs and GAS initiates the transcription of hundreds of IFN-stimulated genes (ISGs) (145, 151-153).

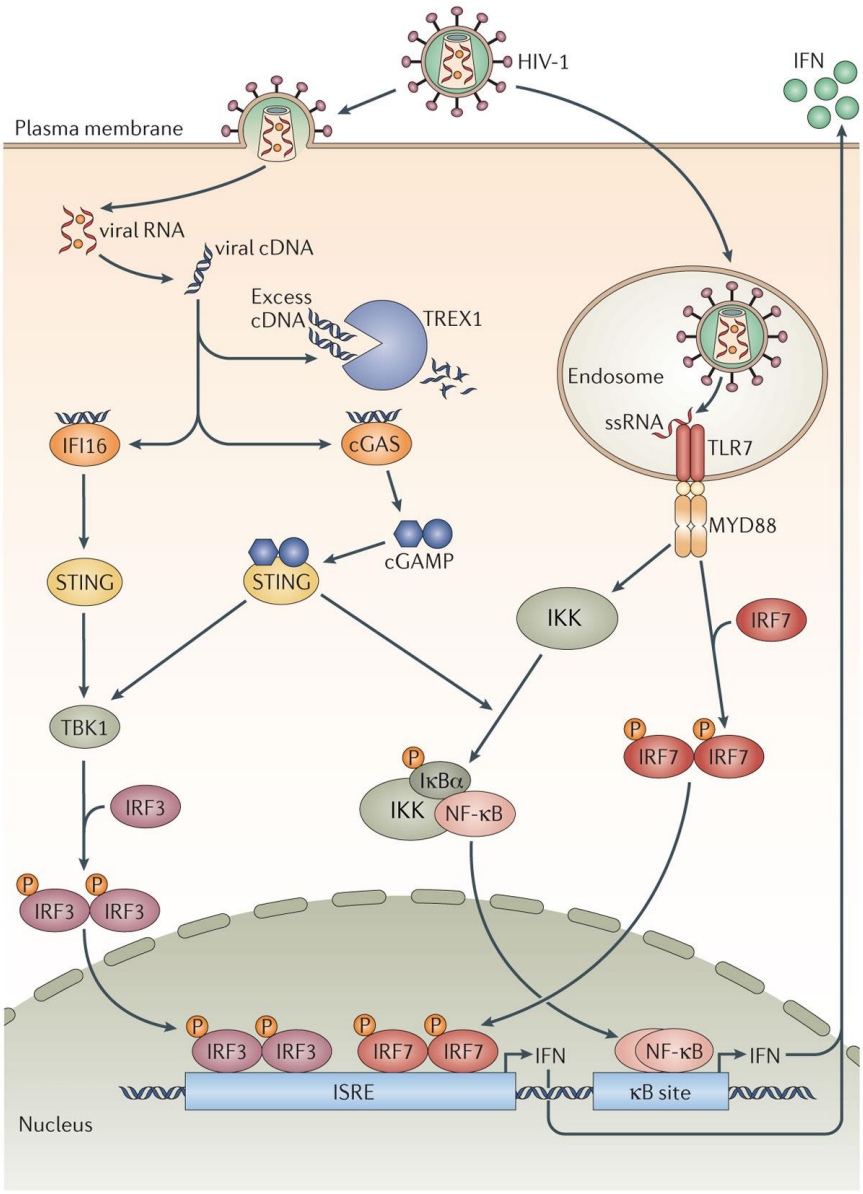

Figure 1.11: Intracellular sensing of HIV-1 infection.

Upon entry into the target cells, HIV-1 converts genomic RNA into cDNA by reverse transcription. The viral DNA functions as ligands for the recognition of cytoplasmic DNA sensors such as cyclic GMP-AMP (cGAMP) synthase (cGAS) and interferon-γ (IFNγ)-inducible protein 16 (IFI16). Following viral cDNA detection, IFI16 activates stimulator of IFN genes (STING). Subsequently, STING activates TANK-

binding kinase 1 (TBK1), leading to the phosphorylation of the IFN regulatory factor 3 (IRF3). Phosphorylated IRF3 translocates into nucleus and engages IFN-stimulated response elements (ISREs), inducing the transcription of type I IFNs. cGAS binds to viral DNA and catalyzes the production of cGAMP. cGAMP acts as a second messenger that binds to and activates STING. STING then activates the inhibitor of NF-κB (IκB) kinase (IKK) complex and TBK1, leading to the activation of nuclear factor-κB (NF-κB) and IRF3, respectively. Activated NF-κB and IRF3 enter into nucleus and induce the expression of type I IFNs. The cellular 3′-repair exonuclease 1 (TREX1) degrades viral DNA in the cytoplasm, therefore preventing host immune recognition. In addition to cytosolic sensing, HIV-1 single-stranded RNA (ssRNA) can be detected by Toll-like receptor 7 (TLR7) in endosomes. TLR7-mediated immune sensing results in the activation of myeloid differentiation primary response gene 88 (MYD88). MYD88 then activates IRF7 and NF-κB, leading to the induction of type I IFNs. This figure is copied from (138) with permission (License Number: 3899120814532).

1.5.2 Restriction Factors

HIV restriction factors are host-encoded, dominantly acting, and cell-intrinsic proteins that potently restrict HIV replication and spread (138). Restriction factors normally express constitutively while their expression can be further increased by IFNs (154). Restriction factors function at different steps of HIV life cycle including entry, uncoating, reverse transcription, nuclear import, translation, and release (Figure 1.12).

IFITMs

Human interferon-induced transmembrane (IFITM) family proteins consist of four members, IFITM1, IFITM2, IFITM3 and IFITM5 and they play distinct roles in cell development, cell adhesion, cell growth control as well as bone mineralization (155, 156). Recent studies showed that human IFITMs have antiviral activities that restrict entry of a wide range of enveloped viruses, such as dengue virus, Ebola virus, influenza A virus and West Nile virus (157). By using shRNA screen in SupT1 cells, IFITM1, IFITM2 and IFITM3 have been identified as inhibitory factors for HIV replication (158). Subsequent experiments showed that overexpression of IFITM2 and IFITM3 inhibit HIV entry, suggesting that IFITMs may function at the early stages of HIV life cycle (158). Importantly, IFITMs inhibit viral membrane fusion, possibly through decreasing membrane fluidity and altering curvature, therefore restricting enveloped viruses from entering into target cells (159). In addition to the restriction of cell-free HIV entry, IFITMs also suppress HIV cell-to-cell infection. IFITMs have been shown to be incorporated into HIV-1 virions, which allows them to inhibit HIV-1 spread by restricting viral membrane fusion in new target cells (160). However, recent study from our lab

demonstrated that IFITMs interact with HIV-1 Env and subsequently impair Env processing in viral producing cells, therefore inhibiting viral infection (161).

TRIM5α

Tripartite motif-containing protein 5α (TRIM5α) is one of the tripartite motif (TRIM)-containing family proteins (162). TRIM5α consists of N-terminal RING domain, B-box domains, central coiled-coil domain and C-terminal SPRY domain harboring the recognition site for HIV capsid (163). Following viral entry into the target cells, TRIM5α directly binds to HIV-1 capsid lattice and accelerates the fragmentation of reverse transcription complex (RTC), therefore inhibiting the synthesis of viral cDNA (164). Interestingly, TRIM5α is unable to restrict retroviruses that are found in the same host species but potently inhibits retroviruses that are found in other species (165). For examples, human TRIM5α efficiently suppress the reverse transcription of N-tropic murine leukemia viruses (N-MLV) but does not suppress HIV-1 (166). By contrast, HIV replication is effectively restricted by TRIM5α derived from Old World monkey (164). This suggests that TRIM5α may provide a barrier for the cross-species transmission of primate lentiviruses (165).

SAMHD1

Sterile alpha motif and HD domain-containing protein 1 (SAMHD1) is a recently identified restriction factor that suppresses HIV-1 replication in myeloid cells, such as monocyte derived-dendritic cells (MDDCs), monocyte-derived macrophages (MDMs) and resting T cells (167, 168). SAMHD1 is a 626 aa protein that consists of a sterile α motif (SAM) domain and an HD domain. The SAM domain is a protein-protein or

protein-RNA interaction domain while the HD domain has putative hydrolase activity (169). SAMHD1 functions as a 2'-deoxynucleoside 5'-triphosphate (dNTP) triphosphohydrolase that depletes the intracellular dNTP pool required for HIV reverse transcription in myeloid cells, therefore disrupting the synthesis of viral cDNA (170). Moreover, SAMHD1 has also been shown to possess the RNase activity that is responsible for the degradation of viral RNA, thus preventing HIV infection (171).

APOBEC3

Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) is one of a family of proteins that possess the polynucleotide (RNA or DNA) cytidine deaminase activity (163). APOBEC3G catalyzes the deamination of cytidine to uridine in the single stranded DNA substrate, thus altering the nucleotide sequence and introducing an unnatural base (172). In the absence of Vif, APOBEC3G is incorporated into nascent HIV-1 virions and function in target cells (173). Following HIV-1 entry into new targets cells, APOBEC3G associates with viral RTC and deaminates cytidine residues to uridine in the newly synthesized minus strand cDNA (174). This editing causes guanosine-to-adenosine hypermutation in viral plus strand cDNA, thereby compromising HIV-1 genetic integrity. In addition to hypermutation, APOBEC3G has also been shown to directly suppress HIV reverse transcription by interfering the translocation of RT and viral RNA template (175).

MX2

Human myxovirus resistance 1 (MX1) and myxovirus resistance 2 (MX2) are members of the IFN-inducible dynamin-like guanosine triphosphatase (GTPase) superfamily that

plays roles in cellular processes that require membrane remodelling, such as vesicular transport and cytokinesis (176). Human MX1, which shares 63% amino acid identity with MX2, is a well-documented inhibitor of a variety of RNA and DNA viruses, including influenza A virus, La Crosse encephalitis virus, and hepatitis B virus (177). By contrast, the antiviral activity of MX2 is unclear until two independent groups identified MX2 as a post-entry inhibitors of HIV-1 infection. Compared to MX1, MX2 can locate to the nucleus via the nuclear localization signal (NLS) and this localization is required for MX2-medicated inhibition of diverse HIV-1 strains (178). In the presence of MX2, both the accumulation and integration of viral cDNA in the nucleus are suppressed, suggesting that MX2 inhibits nuclear import and integration of HIV-1 cDNA (178, 179).

SLFN11

Schlafen 11 (SLFN11) is a member of Schlafen (SLFN) family that is involved in multiple functions in mammals, including control of T cell development, induction of immune response and regulation of viral infection (180). Recently, SLFN11 has been identified as a HIV-1 restriction factor that suppresses viral protein synthesis (181). SLFN11 functions as a cytoplasmic RNA-binding protein that selectively inhibits the translation of HIV-1 mRNAs with a codon bias that differ from human mRNAs (138). Moreover, SLFN11 has also been shown to directly bind to tRNA and overcomes the changes of tRNA pool induced by HIV-1 infection through either sequestering or degrading tRNA (181). Importantly, elevated expression of SLFN11 has been found in $CD4^+$ T cells from HIV-1 elite controllers, suggesting that SLFN11 may play a critical role in HIV-1 pathogenesis *in vivo* (182).

Tetherin

Tetherin (also known as BST2 or CD317) is an IFN-inducible protein that potently inhibits HIV-1 release at late stages of the viral life cycle (183). Tetherin is an unusual type II transmembrane protein that has an N-terminal transmembrane domain and a C-terminal glycophosphatidylinositol (GPI) lipid anchor (184). This unique topology enables tetherin to interact with both cellular and viral lipid bilayers (185). Tetherin is expressed at the plasma membrane, which is the budding site of HIV virions, as well as within several endosomal compartments (186). In the absence of Vpu, tetherin is incorporated into HIV-1 virions and causes the retention of nascent viral particles on the plasma membrane of the infected cells by forming protein bridges between viral and cellular membranes (187). In addition to the inhibition of cell-free virus release, tetherin has been shown to restrict HIV-1 cell-to-cell transmission from human primary macrophages to $CD4^+$ T cells (188).

SERINCs

Serine incorporator (SERINC) family proteins contain 10-12 putative transmembrane domains and are conserved from yeast to mammals (189). Human SERINC family consists of five members and shares 17% homology in amino acid sequence. Although SERINCs have been reported to play a role in biosynthesis of serine-derived lipids such as phosphatidylserine (190), their functions are largely unknown. Recent studies have identified that both SERINC3 and SERINC5 function as restriction factors that suppress HIV-1 infectivity but are countered by HIV-1 Nef protein (191, 192). In the absence of Nef, SERINCs are incorporated into nascent HIV-1 virions and inhibit HIV-1 infectivity through impairing virion fusion with the target cells. By contrast, in the presence of Nef,

SERINCs traffic to the endosomal compartment for degradation. The removal of SERINCs from cell surface prevents their incorporation into HIV-1 virions (191, 192).

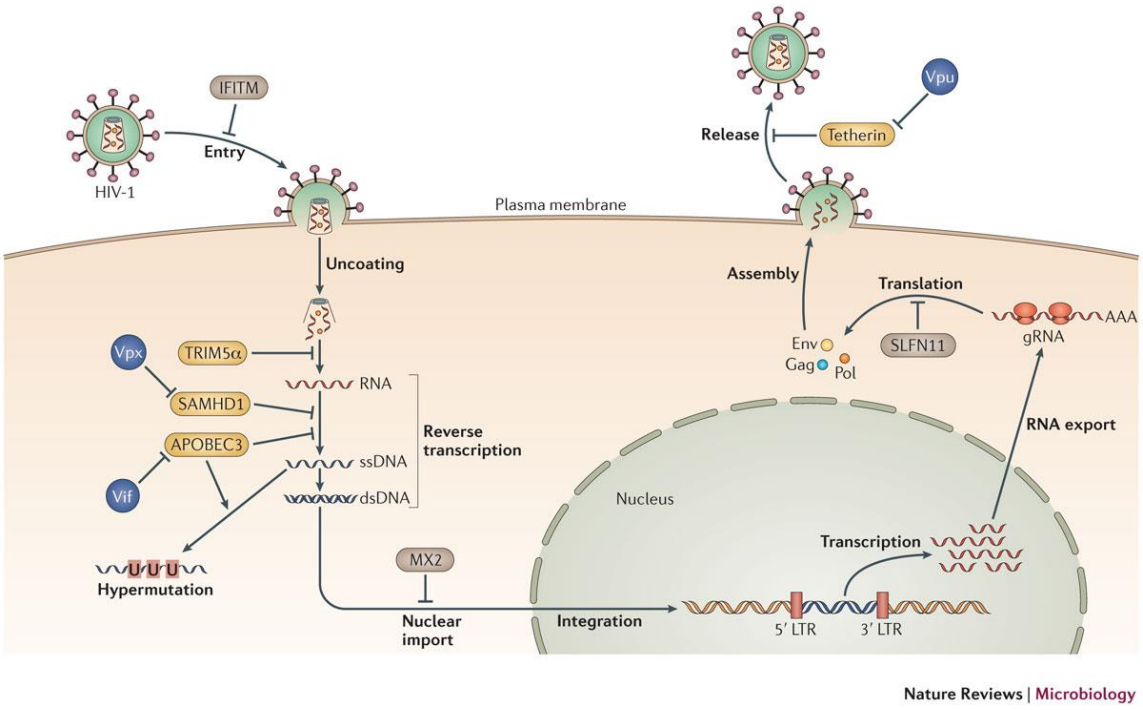

Figure 1.12: HIV-1 restriction factors and viral countermeasures.

HIV-1 restriction factors inhibit different stages of HIV-1 life cycle. Interferon-induced transmembrane proteins (IFITMs) inhibit viral entry through restricting viral membrane fusion. The tripartite motif-containing protein 5α (TRIM5α) binds to HIV-1 capsid lattice and accelerates the fragmentation of reverse transcription complex (RTC), thereby inhibiting the synthesis of viral cDNA. SAM and HD domain-containing protein 1 (SAMHD1) depletes the intracellular dNTP pool required for HIV reverse transcription, therefore disrupting viral cDNA synthesis. APOBEC3 (apolipoprotein B mRNA-editing enzyme, catalytic polypeptide-like 3) causes hypermutation in viral cDNA during HIV-1 reverse transcription, thus compromising HIV-1 genetic integrity. Myxovirus resistance 2 (MX2) inhibits nuclear import and integration of HIV-1 cDNA. Schlafen 11 (SLFN11) suppresses the synthesis of viral proteins. Tetherin restricts HIV-1 release from the surface of infected cells. HIV employs accessory proteins to counteract host restriction factors. HIV-1 Vif antagonizes APOBEC3, Vpu antagonizes tetherin, and Vpx from HIV-2 antagonizes SAMHD1. This figure is copied from (138) with permission (License Number: 3899121067462).

1.5.3 Accessory Proteins and Their Antagonistic Strategies

One feature of primate lentiviruses that distinguishes them from other retroviruses is the expression of accessory proteins (193). In addition to viral structural proteins and enzymes, HIV-1 encodes four accessory proteins: Nef, Vif, Vpu and Vpr. Earlier studies showed that these proteins are largely dispensable for HIV-1 replication in many cell types. In fact, HIV-1 replication in the cell culture systems often acquires inactive mutations or deletions in these genes (2). However, HIV-1 accessory proteins are strongly expressed *in vivo* and associated with viral pathogenesis and disease progression (193). Notably, numerous studies have shown that HIV-1 accessory proteins antagonize host restriction factors and facilitate HIV-1 infection and spread, indicating that viral accessory proteins play an essential role in HIV-1 immune evasion (172, 194).

Nef

HIV-1 Nef is a 27-kD membrane-associated protein that interacts with a variety of cellular partners such as clathrin adaptor protein (AP) complexes and dynamin-2 (163). The most documented function of Nef is downmodulation of several cell surface molecules in helper T cells and macrophages, two major targets of HIV-1 infection; these include HIV-1 receptor CD4, major histocompatibility class I (MHCI), and other cell surface receptors such as CD8, CD28, and CD80 (195-199). Nef was shown to interact those molecules and promote their internalization via clathrin-mediated endocytosis, thus removing them from the plasma membrane (2). The downregulation of cell surface proteins by Nef helps prevent superinfection as well as evade host immune response (200, 201). Significantly, Nef has been shown to play a critical role in pathogenesis *in vivo*. For example, SIV harboring a deletion in Nef gene is much less pathogenic in rhesus

macaques (202). Additionally, no apparent or slow progression of AIDS was observed from patients infected with Nef-deleted HIV-1 (203). Similar to HIV-1 group M Vpu, Nef proteins from HIV-1 group O and most SIVs have been shown to counteract tetherin by either downregulation or intracellular sequestration of tetherin, thereby removing tetherin from cell surface and promoting viral release (204-206). In addition to tetherin, most recent studies showed that Nef overcomes SERINCs inhibition by redirecting them to a Rab7-positive endosomal compartment and thus preventing SERINCs incorporation into HIV-1 viral particles (191, 192). In my Ph.D. work, I investigate the potential role of Nef in antagonizing TIM-family proteins-mediated inhibition of HIV-1 release.

Vif

HIV Vif is a 23-kD cytoplasmic protein that is expressed in all lentiviruses except EIAV (2). For HIV-1 infection, the requirement of Vif has been shown to be producer-cell dependent (207). For example, Vif is dispensable in certain cell types such as 293T, HeLa, SupT1 and Jurkat cells. However, Vif is required for HIV-1 replication in primary $CD4^+$ T cells and monocyte-derived macrophages (2). This observation suggested that these primary cells express a HIV-1 restriction factor that is counteracted by Vif. By comparing mRNA expression profiles in permissive cells and nonpermissive cells, such a restriction factor, human APOBEC3G has been identified as a potent inhibitory factor that suppresses the replication of Vif-deficient HIV-1 (172). Vif directly interacts with APOBEC3G and recruits it to an E3 ubiquitin ligase complex consisting of the scaffold protein cullin 5 (CUL5), the substrate adaptors elongin B (ELOB), elongin C (ELOC), and RING-box protein 2 (Rbx2) (208). This leads to the polyubiquitylation and

proteasomal degradation of APOBEC3G, therefore preventing its incorporation into the nascent HIV-1 virions (208, 209).

Vpu

Vpu is a 14-kD integral membrane protein that contains an N-terminal transmembrane domain and a C-terminal cytoplasmic tail (210). Vpu is only expressed in HIV-1 but not in HIV-2 or SIVs, and its two major functions are well-documented: degradation of CD4 and enhancement of HIV release (2). Similar to Nef, Vpu binds to the cytoplasmic tail of CD4 and then recruits it to the cullin1-Skp1 ubiquitin ligase complex, thereby triggering the polyubiquitylation and proteasomal degradation of CD4 (211). In addition to CD4, Vpu has been shown to downmodulate the cell surface expression of MHCI molecules, thus preventing HIV-1 infected cells from host immune recognition and killing by cytotoxic T cells (212). The enhancement of HIV release by Vpu is achieved by antagonizing the restriction of tetherin. Vpu interacts with tetherin and reduces the cell surface expression of tetherin either by interfering with its trafficking to the plasma membrane or by promoting its internalization (213, 214). Moreover, Vpu has also been shown to bind to tetherin and promote its ubiquitylation and subsequent endolysosomal degradation (215).

Vpr

HIV-1 Vpr is a 14-kD protein that is incorporated into mature HIV-1 virions (216). One major function of Vpr is its ability to arrest HIV-infected cells in the G2 phase of the cell cycle through recruitment of a cullin-RING ubiquitin ligase (193). Vpr has been shown to interact with damaged DNA binding protein 1-cullin 4-associated factor 1 (DCAF1) and

engage cullin4A-DDB1 complex (217). In addition to G2 arrest, Vpr also promotes HIV infection in primary cells, such as macrophages through unknown mechanisms (218). Most recent study suggested that HIV-1 Vpr may facilitate HIV-1 replication and spread by antagonizing a macrophage-specific restriction factor (219). In the absence of Vpr, HIV-1 replication in macrophages induces more IFNs that promotes Env degradation in the lysosomes and reduces the release of Env-containing viral particles. By contrast, Vpr expression suppresses IFN induction and restores HIV-1 Env expression and virions production (219). Notably, expression of DCAF1 is required for Vpr-mediated antagonism, suggesting that Vpr counteracts a macrophage-specific restriction factor through the interaction with DCAF1 (219).

1.6 T-cell Immunoglobulin and Mucin Domain (TIM) Family Proteins

1.6.1 Structures and Expression Patterns

The T-cell immunoglobulin and mucin domain (TIM) family was first discovered in 2001 by using a mouse model of asthma (220, 221). To date, eight members (TIM-1-TIM-8) of TIM-family proteins have been identified in mouse while three members (TIM-1, TIM-3 and TIM-4) in human (222). TIM-family proteins are type I cell-surface glycoproteins that are comprised of N-terminal immunoglobulin-like domain, a mucin domain of varying length with both O-linked and N-linked glycosylations, a single transmembrane domain, and C-terminal cytoplasmic tail (Figure 1.13). Additionally, within the cytoplasmic domain, human TIM-1 and TIM-3 contain a tyrosine phosphorylation motif that may play a role in TIM proteins signaling (222).

TIM-1, TIM-3 and TIM-4 were shown to be the pattern recognition receptors of phosphatidylserine (PS) (223). PS is an important phospholipid that is normally located in the inner leaflet of the plasma membrane; however, when cells undergo apoptosis or during viral infection, PS flips and relocalizes to the outer leaflet of the plasma membrane (224, 225). TIMs have been shown to bind to and recognize the exposed PS on the cell surface through its PS binding pocket within the immunoglobulin variable (IgV) domain that consists of two anti-parallel β-sheets. This conserved binding pocket, named as metal ion-dependent ligand-binding site (MILIBS), harbors several conserved amino acids that coordinate with bivalent metal ions, such as calcium (222). Although TIM-1, TIM-3 and TIM-4 all bind to PS, their affinities for PS are distinct. Binding studies indicated that the affinities of TIM-1 and TIM-4 for PS are similar, which are higher than that of TIM-3 (226, 227).

Although TIM proteins share similar structures, their expression patterns and functions are diverse. TIM-1 is mainly expressed in T-helper 2 (Th2) cells and acts as a costimulatory factor for T cell activation (228). In mice, TIM-1 is expressed in the activated $CD4^+$ T cells rather than naive $CD4^+$ T cells. After differentiation, TIM-1 is preferentially expressed in Th2 cells (228). In human, TIM-1 is also expressed in tubular epithelial cells after kidney injury (229). TIM-3 is predominately expressed in T-helper 1 (Th1) and cytotoxic T cell type 1 (Tc1) cells and functions as an inhibitory factory that leads to apoptosis of Th1 and Tc1 cells (230). TIM-3 is also expressed in dendritic cells (DCs) and involved in the phagocytosis of apoptotic cells (231). Additionally, human TIM-3 is expressed in the activated $CD4^+$ T cells, Th1 cells, and $CD8^+$ T cells (232). In contrast to TIM-1 and TIM-3, TIM-4 is not expressed in T cells, but is preferentially

expressed in antigen-presenting cells (APCs), including macrophages and DCs and mediates the phagocytosis of apoptotic cells (227).

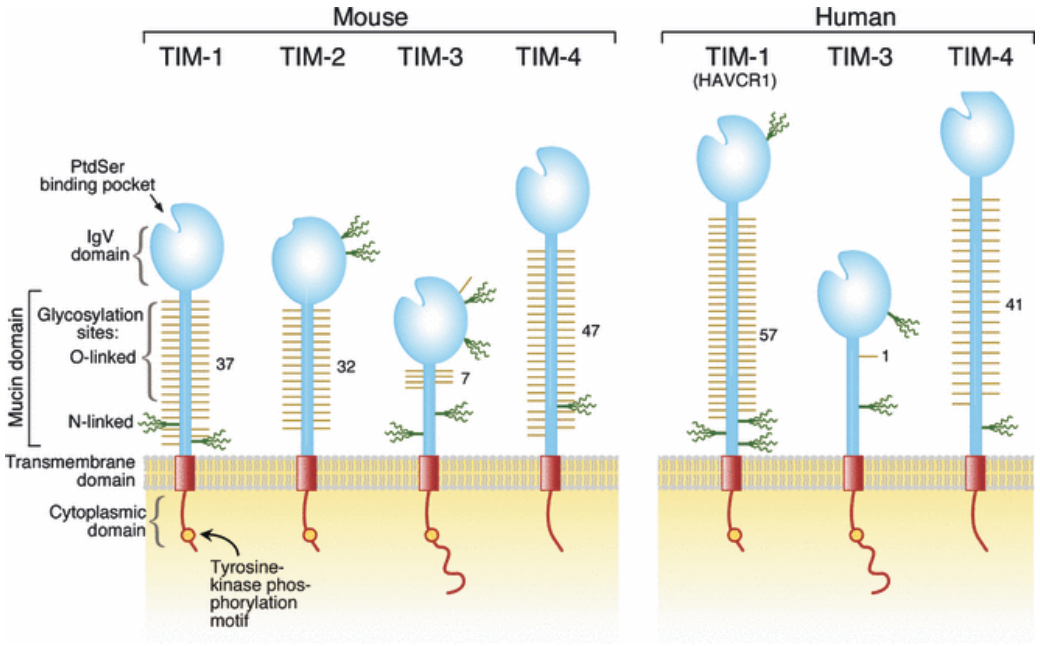

Figure 1.13: TIM-family proteins structures.

Schematic representation of T-cell immunoglobulin and mucin domain (TIM) family structures is shown. Four members of TIM-family proteins have been identified in mouse (TIM-1, TIM-2, TIM-3 and TIM-4), while three members in human (TIM-1, TIM-3, and TIM-4). TIM-family proteins are type I cell-surface glycoproteins, which consist of N-terminal immunoglobulin-like (IgV) domain harboring PS binding site, a mucin domain including both O-linked and N-linked glycosylations, a single transmembrane domain, and C-terminal cytoplasmic domain. Except for TIM-4, other members also contain a tyrosine phosphorylation motif in their cytoplasmic domain. This figure is copied from (222) with permission (License Number: 3899121486229).

1.6.2 Functions in Innate Immune Regulation

Overall, TIM family proteins play important roles in host immune regulation, including allergy, asthma, transplant tolerance, autoimmunity, and viral infection (222).

TIM-1

Costimulatory function of TIM-1. By using an agonist anti-mouse TIM-1 monoclonal antibody (mAb), cross-linking of TIM-1 on T cells was shown to promote naive T cell proliferation and cytokine production. In addition to naive T cells, TIM-1 also increases the production of interleukin (IL)-4 by differentiated Th2 cells (228). Most importantly, TIM-1 play a costimulatory role *in vivo*. Administration of agonist anti-mouse TIM-1 mAb together with antigen enhances antigen-specific T cell response (228). Moreover, during influenza vaccination, TIM-1 mAb can function as a potent adjuvant that enhances the host antiviral immune response (233).

TIM-1 and asthma. Human chromosome 5q23-35 has been shown to associate with asthma susceptibility in several studies (234). To identify genes that are responsible for asthma in this region, a series of congenic mice were generated based on asthma-susceptible BALB/c background that differs from the asthma-resistant strain DBA/2. One of these congenic lines, named C.D2 Es-HBA (HBA), exhibited the DBA/2 phenotype with low Th2 cytokine, high IFN-γ, and low airway hyperreactivity (AHR); it carried a chromosomal segment with homology to human chromosome 5q23-35. The offsprings of HBA that were backcrossed to BALB/c mice were used to identify TIM family genes as asthma susceptibility genes (220). Consistently, TIM-1 mAb was shown to decrease

allergen-induced airway inflammation, further supporting the role of TIM-1 in asthma development (235).

TIM-1 and transplant tolerance. By using transplant model, one study showed that administration of TIM-1 mAb 3B3 *in vivo* promoted Th17 and Th1 responses and overcame the protective effects of anti-CD154 mAb, leading to allograft rejection (236). Conversely, another study revealed that administration of a different TIM-1 mAb RMT1-10 suppressed cardiac allograft rejection of MHC-mismatched mouse. The resultant transplant tolerance was associated with decreased Th1 response but increased Th2 response (237). The reasons why different TIM-1 mAbs may exhibit opposite effects is still unknown. Many factors may be involved, such as the binding affinity of TIM-1 mAbs for TIM-1 and also their ability to block TIM-1 and PS interaction (222).

TIM-1 and kidney injury. TIM-1 (also known as kidney injury molecule-1 (KIM-1)) is highly upregulated on the surface of injured kidney epithelial cells. Following injury in kidney tubules, TIM-1 specifically recognizes the exposed PS presenting on the apoptotic cells surface and subsequently facilitates the phagocytosis of the apoptotic cells by neighboring epithelial cells that express TIM-1 (238). In addition to rat injured kidney epithelial cells, TIM-1 has also been shown to be expressed in human rental cell carcinomas as well as in a monkey kidney cell line, COS cells (239, 240).

TIM-3

Co-inhibitory function of TIM-3. TIM-3 is preferentially expressed in T cells and act as a co-inhibitory factor for T cells proliferation. Administration of TIM-3 mAb (8B.2C12) accelerates the disease progression of experimental autoimmune

encephalomyelitis (EAE) and causes the activation of macrophages (241). Moreover, TIM-3 has been shown to bind to galectin-9 and induce the death of Th1 cells, thereby suppressing Th1 cells response (230). Additionally, administration of TIM-3 Ig fusion protein was shown to stimulate Th1 cells proliferation and IFN-γ production (242).

TIM-3 and phagocytosis of apoptotic cells. Several studies indicated that both human and mouse TIM-3 can function as receptors for PS and play a critical role in clearance of apoptotic cells *in vivo* (222). Indeed, $CD8^+$ DCs expressing TIM-3 recognize and engulf the apoptotic cells, and this process was inhibited by addition of a TIM-3 mAb that disrupts the interaction of TIM-3 and PS. Moreover, treatment of TIM-3 mAbs in mice results in the increased number of apoptotic cells in splenic follicles as well as anti-dsDNA antibodies in blood (231), suggesting an important role of TIM-3 in clearance of dead cells.

TIM-4

TIM-4 and phagocytosis of apoptotic cells. By using a library of monoclonal antibodies against macrophages in mouse, TIM-4 has been shown to recognize and bind to the exposed PS and mediate phagocytosis of apoptotic cells (227). Indeed, treatment of anti-TIM-4 mAb in mice significantly inhibits the engulfment of apoptotic cells by macrophages (227). Interestingly, TIM-4-mediated phagocytosis seems to be independent of the known engulfment signaling pathways such as ELMO1/Dock180/Rac or GULP pathways; rather, it depends on the actin cytoskeleton, myosin-II motor proteins, as well as ATP-dependent cellular processes (243).

1.6.3 Role of TIMs in Viral Infection

In addition to immune regulation, recent studies have shown that TIM-family proteins play a critical role in viral infection. In particular, TIMs have been shown to enhance a wide range of enveloped viruses entry by interaction of virion-associated PS.

TIM-1 and HAV

TIM-1 was originally discovered as the cellular receptor for hepatitis A virus (HAV), a non-enveloped picornavirus that causes human acute liver disease, in both African green monkey and human (240, 244). By using a protective monoclonal antibody 190/4 that inhibited the binding of HAV to susceptible African green monkey kidney (AGMK) cells, TIM-1 (also known as HAVcr-1) was isolated and identified as a binding receptor for HAV. Notably, transfection of mouse Ltk- cell line that is normally resistant to HAV infection with TIM-1 was shown to gain limited susceptibility; and TIM-1-mediated HAV infection was further blocked by the treatment of protective antibody 190/4. This confirmed the functional role of TIM-1 in HAV entry (240).

Role of TIMs in enveloped virus entry

By comparing the gene expression pattern and Ebola virus (EBOV) transduction profile in 54 human cell lines, TIM-1 was identified as a receptor or factor that promotes EBOV infection (245). Consistent with this notion, overexpression of TIM-1 in poorly permissive 293T cells increased EBOV infection up to 30-fold. Conversely, knockdown of endogenous TIM-1 in Vero cells dramatically reduced EBOV infectivity (245). Importantly, ARD5, a monoclonal antibody against IgV domain of TIM-1 can significantly suppress EBOV infection, therefore providing a potential agent for antiviral therapy (245).

In addition to EBOV, a subsequent study using gain-of-function cDNA screen showed that both TIM family and TAM family proteins can function as receptors or factors for Dengue virus (DV) entry (246). Ectopic expression of TIM-1 in poorly susceptible 293T cells enhances DV infectivity by 500-fold. By contrast, addition of TIM-1 mAb or silence of endogenous TIM-1 in A549 cells strongly suppresses DV infection (246). Importantly, TIM-1 has been shown to directly interact with PS presenting on the surface of DV virions and TIM-1 mutants defective for PS binding failed to enhance DV entry. These results suggested that TIM family proteins enhance DV entry by interaction with virions-associated PS (246).

Recent studies have extended the effect of TIM family proteins on virus entry to other enveloped viruses of seven distinct families (247). Overexpression of TIM-1 has been shown to promote the infection of pseudotyped viruses that bear a wide range of viral envelope glycoproteins, including filovirus, flavivirus, New World arenavirus and alphavirus families (247). PS-binding deficient TIM-1 variants and treatment of liposomes containing PS failed to promote virus entry, suggesting that the interaction of TIM-1 and PS is critical for TIM-1-medicated enhancement of virus entry. Interestingly, TIM-1 only has modest or no effect on the entry of some viruses including Lassa fever virus, influenza A virus and SARS coronavirus. This may be related to the presence of the high-affinity receptor (Lassa fever virus) and/or TIM-1-mediated internalization to a compartment that is unsuitable for productive infection (influenza A virus and SARS coronavirus) (247).

1.6.4 Functions in HIV/AIDS

TIM-1 polymorphisms and AIDS

TIM-1 polymorphisms have been repeatedly reported to associate with human asthma and allergy (248). One study also suggested that TIM-1 polymorphisms is related to the disease progression of AIDS (249). The polymorphic exon 4 of TIM-1 from 246 HIV-1 infected female in Thailand has been sequenced and seven haplotypes of TIM-1, D3-A, D4, D3-C, D1, W-A, W-C, and D3-C*, have been identified (249). Notably, compared with other haplotypes, HIV infected patients carrying D3-A haplotype was associated with relative slower progression of AIDS with higher $CD4^+$ cell count, lower proportion of AIDS symptoms, as well as better survival prognosis. Interestingly, D3-A haplotype exhibited lower expression of TIM-1 than other haplotypes (249). Because TIM-1 has been shown to be expressed on $CD4^+$ Th2 cells and play a role in the regulation of Th1/Th2 balance, this study suggested that TIM-1 polymorphisms may influence AIDS progression through modulating Th1/Th2 responses (249).

TIM-3 and HIV infection

In addition to immune regulation, TIM-3 has been identified as an exhaustion marker on T cells and play a role in chronic HIV-1 infection (250). Compared to the healthy controls, the proportion of $CD8^+$ T cells expressing TIM-3 was shown to be increased by around 2-fold in HIV-1 infected individuals (251), which correlates positively with HIV-1 viral load and CD38 expression but negatively with $CD4^+$ T cells count. It was also found that the upregulation of TIM-3 in HIV-1-specific $CD8^+$ T cells can result in poor cell proliferation and reduced cytokine production in response to antigen (251). Consistent with this finding, block of TIM-3 signaling by mAb promotes the proliferation and cytokine production, suggesting that TIM-3 likely functions as a therapeutic target for HIV-1-induced T cell dysfunction (251).

By contrast, another study showed that TIM-3 may prevent HIV-1 infection in activated $CD4^+$ T cells by interaction with its ligand Galectin-9 (Gal-9) (252). During HIV-1 infection, soluble Gal-9 binds to TIM-3 on the surface of activated $CD4^+$ T cells and lead to downregulation of HIV-1 coreceptors required for viral entry, including CXCR4, CCR5, and $\alpha4\beta7$. Furthermore, the binding of Gal-9 to TIM-3 also results in the upregulation of the cyclin-dependent kinase inhibitor p21 that has been previously reported to suppress HIV-1 replication in $CD4^+$ T cells from elite controllers (253). By these means, TIM-3/Gal-9 pathway therefore renders TIM-3-expressing $CD4^+$ T cells less susceptible to HIV-1 infection (252).

Although TIM-family proteins have been shown to be related to HIV-1 infection and AIDS progression, their functional roles in HIV-1 replication is largely unknown. By using lentiviral transduction, I find that cells transduced by HIV-1 vector encoding TIM-1 exhibited extremely lower transduction efficiency compared to cells transduced by vector expressing GFP control. This provides the first hint that expression of TIM-1 in viral producer cells may limit HIV-1 production. In Chapter 2, I explore the role of TIM-family protein in release of HIV-1 as well as other viruses and investigate the underlying mechanisms. The possible roles of HIV-1 accessory proteins, in particular Nef, in antagonizing TIMs, is described in Chapter 3.

CHAPTER 2 – TIM-FAMILY PROTEINS INHIBIT HIV-1 RELEASE

Minghua Li[a], Sherimay D. Ablan[b], Chunhui Miao[a], Yi-Min Zheng[a], Matthew S. Fuller[a], Paul D. Rennert[c], Wendy Maury[d], Marc C. Johnson[a], Eric O. Freed[b], and Shan-Lu Liu[a]

[a]Department of Molecular Microbiology and Immunology, Bond Life Sciences Center, University of Missouri, Columbia, MO 65211; [b]Virus-Cell Interaction Section, HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, MD 21702; [c]Department of Immunotherapy, SugarCone Biotech LLC, Holliston, MA 01746; and [d]Department of Microbiology, University of Iowa, Iowa City, IA 52245

Proceedings of the National Academy of Sciences USA, 2014 Sep 2;111(35):E3699-707.

Author contributions: Minghua Li, Eric O. Freed, and Shan-Lu Liu designed research; Minghua Li, Sherimay D. Ablan, Chunhui Miao, Yi-Min Zheng, and Matthew S. Fuller performed research; Paul D. Rennert and Wendy Maury contributed new reagents/analytic tools; Minghua Li, Sherimay D. Ablan, Yi-Min Zheng, Wendy Maury, Marc C. Johnson, Eric O. Freed, and Shan-Lu Liu analyzed data; and Minghua Li, Eric O. Freed, and Shan-Lu Liu wrote the paper.

2.1 Abstract

Accumulating evidence indicates that T cell immunoglobulin and mucin domain (TIM) proteins play critical roles in viral infections. Herein, we report that the TIM family proteins strongly inhibit HIV-1 release, resulting in diminished viral production and replication. Expression of TIM-1 causes HIV-1 Gag and mature viral particles to accumulate on the plasma membrane. Mutation of the phosphatidylserine (PS) binding sites of TIM-1 abolishes its ability to block HIV-1 release. TIM-1, but to a much lesser extent PS-binding deficient mutants, induces PS flipping onto the cell surface; TIM-1 is also found to be incorporated into HIV-1 virions. Importantly, TIM-1 inhibits HIV-1 replication in CD4-positive Jurkat cells, despite its capability of up-regulating CD4 and promoting HIV-1 entry. In addition to TIM-1, TIM-3 and TIM-4 also block the release of HIV-1, as well as that of murine leukemia virus (MLV) and Ebola virus (EBOV); knockdown of TIM-3 in differentiated monocyte-derived macrophages (MDMs) enhances HIV-1 production. The inhibitory effects of TIM family proteins on virus release are extended to other PS receptors, such as Axl and RAGE. Overall our study uncovers a novel ability of TIM family proteins to block the release of HIV-1 and other viruses by association with virion-associated PS. Our work provides new insights into a virus-cell interaction that is mediated by TIMs and PS receptors.

2.2 Introduction

The T cell immunoglobulin and mucin domain (TIM) proteins play essential roles in cellular immunity (254, 255). Certain human pathologies, in particular allergic diseases, are associated with TIM protein dysfunctions and polymorphisms (256-258). Viral infection has recently been linked to TIM proteins, with some TIMs acting as key factors

for viral entry. Human TIM-1 was initially discovered as the receptor for hepatitis A virus (HAV), and has been recently shown to function as a receptor or entry cofactor for Ebola virus (EBOV) and Dengue virus (DV) (258-261). TIM-1 polymorphisms have been reported to be associated with severe HAV infection in humans (262). More recent studies revealed that TIM-family proteins promote entry of a wide range of viruses, possibly by interacting with virion-associated phosphatidylserine (PS), highlighting a more general role of TIMs in viral infections (263, 264).

TIM-family proteins are classical type I transmembrane proteins, with the N-terminus containing the variable Ig-like (IgV) domain extending from the plasma membrane and the C-terminal tail largely mediating intracellular signaling oriented towards the cytosol (255, 265). Human genes encode three TIM proteins, i.e., TIM-1, TIM-3, and TIM-4, whereas the mouse genome encodes eight TIM members, with TIM-1, TIM-2, TIM3 and TIM-4 proteins expressed. Despite significant sequence variations, the IgV regions of all TIM proteins contain a PS binding site that is absolutely conserved (255). Notably, the functions of TIM family proteins differ greatly, depending on cell type-specific expression as well as the interactions of these TIMs with other molecules, including TIM family members (255). Human TIM-1 is predominantly expressed in epithelial and T helper 2 (T_H2) cells, and is involved in cell proliferation and apoptotic body uptake, whereas human TIM-3 is expressed in activated T helper cells (T_H1), and functions as a negative co-stimulatory signal, often resulting in immune tolerance and apoptosis (266, 267). Human TIM-4 has been found to be mainly expressed in macrophages and dendritic cells (DCs), and possibly acts as a ligand for TIM-1, thereby facilitating T cell activation (268, 269).

TIM-1 has been reported to be expressed in activated $CD4^+$ T cells (266, 270), which are the major targets of HIV-1 infection. However, it is currently unknown if TIM-1 plays a role in HIV-1 replication and infection, although reduced TIM-3 expression on NK cells has been reported to be associated with chronic HIV-1 infection (271). Here we report that TIM-1 inhibits HIV-1 release, resulting in decreased virus production. Notably, TIM-1 mutants deficient for PS binding are incapable of blocking HIV-1 release. Similar to human TIM-1, we show that human TIM-3 and TIM-4 also potently inhibit HIV-1 production. The inhibitory effect of TIM family proteins as well as some PS receptors can be extended to murine leukemia virus (MLV) and Ebola virus (EBOV). Our study has revealed a novel and general function of TIMs, and likely other PS receptors, in the release of HIV-1 and other viruses.

2.3 Materials and Methods

Plasmids. The human TIM-1 gene was cloned into pCIneo vector with a FLAG tag at the N-terminus (pCIneo-F-TIM-1), or cloned into pLenti-GFP-Puro vector (Addgene) or pQCXIP retroviral vector (Clontech) with a FLAG tag at the C-terminus. The TIM-1 mutants (N114A, D115A, ND114/115AA) were generated by using PCR-based mutagenesis based on pCIneo-F-TIM-1. The plasmids encoding human TIM-3, TIM-4, Axl and RAGE have been previously described (263). The HIV-1 NL4-3, HIV-1 Gag-GFP, and HIV-1 Gag constructs were obtained from the NIH AIDS Reagent Program. The HIV-1 molecular clone pLAI was a gift from Michael Emerman (Fred Hutchinson Cancer Research Center). The HIV-1 Fyn10 constructs (272-274), MLV Gag-Pol (275), pNL4-3 29/31KE(272, 276), pNL4-3/$PTAP^-$ (277), and pNL4-3 PR^- (277)) have been previously described. The EBOV VP40-GFP construct was kindly provided by Kartik

Chandran (Albert Einstein College of Medicine). The plasmids encoding TIM-3 shRNA, as well as scramble shRNA, were purchased from Sigma.

Cells and Reagents. HEK293, 293T, HTX (a subclone of HT1080), A549, and the HeLa/TZM indicator cells were grown in Dulbecco's modified Eagle's (DMEM) medium, supplemented with 0.5% penicillin/streptomycin and 10% fetal bovine serum (FBS). SupT1, THP-1, CEM-T4, JLTRG, MT-2, and Jurkat cells were obtained from the NIH AIDS Reagent Program and maintained in RPMI and 10% FBS. PBMCs were isolated from healthy human donors, and maintained in RPMI medium containing 10% FBS and supplemented with phytohemagglutinin (PHA-P) and interleukin-2 (IL-2). MDMs were differentiated from PBMCs and maintained in RPMI medium containing 10% FBS. HEK293 and Jurkat cells stably expressing TIM-1 were established by transducing these cells with HIV-1 or MLV vector expressing TIM-1. The antibodies against HIV-1 Gag, HIV-1 gp41 and CD4 were also from the NIH AIDS Reagent Program. The anti-TIM-1 and TIM-3 antibody was purchased from R&D Systems. The antibody against MLV Gag was produced from hybridoma (R187) from ATCC. The anti-GFP antibody was purchased from Santa Cruz. The secondary anti-mouse immunoglobulin G proteins conjugated to FITC or HRP were purchased from Sigma. Annexin V apoptosis detection kit was purchased from Santa Cruz. Subtilisin A was purchased from Sigma.

Virus Production and Infection. For infectious or pseudotyped virus production, 293T cells were transfected with HIV-1 proviral DNAs encoding NL4-3 or LAI, plus plasmids encoding genes of interest (TIM-1, TIM-3, etc). Alternatively, 293T cells were transfected with HIV-1 lentiviral or MLV vectors, HIV-1 or MLV Gag-Pol, and viral glycoproterins (VSV-G, Env, etc.). For virus-like particle (VLP) production, 293T cells

were transfected with plasmids encoding HIV-1 Gag, MLV Gag-Pol, or EBOV VP40-GFP in the presence or absence of TIMs. In all cases, 24 h post-transfection, the supernatants were harvested and passed through a 0.2 μm filter or by centrifuge to remove the cell debris. Viruses were used to infect target HeLa-TZM-bl cells in the presence of polybrene (8 μg/ml). For Jurkat cells and PBMCs, spinoculation at 1,500 g for 1h at 4°C was applied. At 48 h post-infection, the luciferase activity was measured by following the manufacturer's instructions. If applicable, viral supernatants were concentrated by ultracentrifugation at 32,000 rpm for 2 h at 4°C. The purified virus was dissolved in PBS and detected by Western blotting.

Subtilisin A Protease Stripping. HEK293T cells were transfected with HIV-1 proviral pNL4-3 DNA with or without TIM-1 expression plasmid. Twenty-four post-transfection, viral particles released into supernatants were harvested. The cells were treated either with buffer alone (50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$) or Buffer containing 1 mg/ml subtilisin A for 15 min at 37°C. Subsequently, DMEM medium containing 5 mM PMSF was added and incubated at 37°C for another 15 min. Following a wash with PBS, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.1 % SDS), and the supernatants were collected, concentrated using sucrose cushion purification, and analyzed by Western blotting.

HIV-1 Replication and RT Measurement. For HIV-1 replication, parental Jurkat or Jurkat cells stably expressing TIM-1 were transfected with HIV-1 proviral pNL4-3 DNA using Lipofectamine 2000 (Invitrogen). Forty-eight hours post-transfection, the supernatants were harvested every 2 days, and the HIV-1 replication was determined by measuring HIV-1 reverse transcriptase (RT) activity at different time points. Briefly, 10

µl clarified supernatants containing virions were incubated with 40 µl reaction cocktail at 37°C for 3 h. The cocktail contains 50 mM Tris-HCL pH 7.9, 5 mM $MgCl_2$, 0.5 mM EGTA, 0.05% Triton X-100, 2% ethylene glycol, 150 mM KCL, 5 mM DTT (dithiothreitol), 0.3 mM GSH (reduced glutathione), 0.5 U/ml poly (rA) oligo (dT), and 0.1 µCi/µl ^{3}H dTTP (Perkin Elmer). The reaction was stopped by adding 10% cold TCA at 4°C for 30 min, and the mixture was transferred to Millipore MultiScreen Glass Fiber FC plate. Following wash twice with cold 10% TCA and cold ethanol, the membranes were inserted into Beta Gamma vials and read in Microbeta counter.

HIV-1 Infection of MDMs. MDMs were differentiated from PBMCs and maintained in RPMI medium containing 10% FBS. MDMs were transiently transduced by lentiviral vectors bearing VSV-G and encoding TIM-3 shRNA or scramble shRNA. Twenty-four hours post-transduction, MDMs were infected with HIV-1 NL4-3/KFS pseudotypes (defective for Env expression) bearing VSV-G. Six hours after infection, cells were washed 3 times with PBS, and the viral release was monitored by RT activity 24 h post-infection.

TEM and Live Cell imaging. TEM was performed as described previously (278). The NL4-3 29/31KE MA mutant, which redirects virus assembly to a late endosomal compartment (276) was used as a control for viewing HIV-1 intracellular assembly. For live cell imaging, 293 cells were co-transfected with HIV-1 Gag-GFP, along with plasmids encoding TIM-1. Twenty-four hours post-transfection, cells were seeded to glass-bottom Microwell dishes (MatTek) and imaged using a fluorescent microscope (Olympus).

Western Blotting. The experiments were performed as previously described (279, 280). Briefly, 293T cells were transfected with plasmids encoding HIV-1 proviral DNAs or HIV-1 Gag, along with plasmids expressing TIMs or PS receptors. Forty-eight hours post-transfection, cells were collected, washed once by PBS, and lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.1 % SDS). The supernatants from culture were harvested and the virus was purified by ultracentrifugation. The cell lysates and purified viral particles were dissolved in sample buffer, separated on 10% SDS/PAGE, and detected by using anti-HIV-1 p24, anti-MLV p30 (ATCC, R187), anti-TIM-1, anti-gp41, or anti-GFP (for VP40) antibodies.

TIM-1 Incorporation into HIV-1 Virions. HIV-1 virions were produced by transfection of 293T cells with pNL4-3 proviral DNA in the presence or absence of TIM-1 N114A. Virions were concentrated by ultracentrifugation and the purified particles were incubated overnight at 4°C with rProtein A (GE Healthcare) beads pre-conjugated with 5 μg anti-TIM-1 antibody. Beads were washed three times with PBS and bound virions were resolved on SDS/PAGE followed by Western blotting with anti-HIV-1 gp41 or anti-TIM-1 antibodies.

Flow cytometry. Transfected or normal cells, including MDMs, were washed twice with cold PBS plus 2% FBS, and incubated with anti-TIM-1, TIM-3, or anti-CD4 for 1 h. For Annexin V and PI staining, transfected cells were incubated with FITC conjugated Annexin V or PI, and analyzed by flow cytometry.

2.4 Results

TIM-1 Diminishes HIV-1 Production. While using an HIV-1 lentiviral vector to generate a TIM-1-expressing stable cell line for unrelated studies, we serendipitously found that HIV-1 vectors encoding TIM-1 yielded abnormally low transduction levels. We observed that the population of puromycin-resistant colonies in cells transduced by an HIV-1 lentiviral vector encoding TIM-1 (pLenti-puro-TIM-1) was significantly lower than that transduced by the vector control encoding GFP (pLenti-puro-GFP) (Figure 2.6). This provided the first hint that TIM-1 may impair HIV-1 production. To determine if the inefficient transduction was caused by the TIM-1 protein, we produced HIV-1 GFP pseudoviral particles bearing different viral envelope glycoproteins in the presence of a TIM-1 mammalian expression plasmid, pCIneo-TIM-1. Again, the resulting viral titers were considerably lower than those produced in the absence of TIM-1, regardless of the pseudotyping envelope used. Ultimately, we were able to establish a stable 293 cell line expressing TIM-1, and confirmed that TIM-1 profoundly diminishes HIV-1 production, resulting in cell supernatants with ~100-fold reduced viral infectivity (Figure 2.1A).

To further investigate the effect of TIM-1 on infectious HIV-1 production, we transiently transfected 293T cells with NL4-3 or LAI proviral DNA, but in the presence of relatively low doses of TIM-1. TIM-1 decreased HIV-1 Gag in purified NL4-3 and LAI particles in a dose-dependent manner (Figure 2.1B and C, bottom panels). The RT activity of the harvested viruses in TIM-1-expressing cells was also accordingly reduced, correlating with reduced viral infectivity (Figure 2.1D and E). Intriguingly, we observed that the reduced viral release caused by TIM-1 correlated with the increased levels of mature HIV-1 capsid (CA, p24) associated with the viral-producer cells (Figure 2.1B and C, compare the p24 changes in top and bottom panels). Altogether, our data revealed that

expression of human TIM-1 inhibits cell-free HIV-1 production, which is accompanied by an increase in mature HIV-1 Gag in viral producer cells.

TIM-1 Blocks HIV-1 Gag Release. Because expression of TIM-1 led to an accumulation of mature HIV-1 p24 in the cell (Figure 2.1B and C), we asked if the TIM-1 inhibition of HIV-1 production was related to viral protease (PR) activity. To address this issue, we transfected 293T cells with a plasmid encoding the codon-optimized HIV-1 Gag lacking PR, and measured the production of HIV-1 virus-like particles (VLPs) in the presence or absence of TIM-1. As shown in Fig. 1F, the levels of purified VLPs formed by the full-length HIV-1 Gag ($Pr55^{Gag}$) were also reduced by TIM-1, similar to that of infectious HIV-1. In addition, we treated transfected 293T cells with the HIV-1 protease inhibitor Saquinavir; again, HIV-1 release was strongly inhibited by TIM-1 (Figure 2.1G).

We next explored what functional domains of Gag might be involved in the TIM-1 inhibition of HIV-1 release. To this end, we co-transfected 293T cells with plasmids encoding TIM-1, along with HIV-1 proviruses encoding Gag lacking MA (Fyn10ΔMA) or NC (Fyn10ΔNC) (273, 274, 281). As a control, the parental Gag chimera (Fyn10FullMA) was examined in parallel. In all of these constructs, the N-terminal Gag has a 10-aa sequence derived from the Fyn kinase, thus permitting them to associate with the cell membrane in a phospholipid phosphatidylinositol-(4,5)-bisphosphate [$PI(4,5)P_2$]-independent manner (273, 281). We found that TIM-1 blocked release of all these HIV-1 Gag mutants (Figure 2.7A). TIM-1 also inhibited release of three additional Gag-Pol proviral mutants, i.e., HIV-1 $PTAP^-$ (defective in budding due to loss of late domain) (282), HIV-1 PR^- (defective in maturation due to loss of protease) (282), as well as 29/31KE (known to assemble in intracellular compartments because of mutations in MA)

(272, 276) (Figure 2.7B). Similar to WT NL4-3, the amount of cell-associated p24 was also increased for the PTAP$^-$ and 29/31KE mutants in TIM-1-expressing cells compared to control cells not expressing TIM-1 (Figure 2.7B). Collectively, these results show that TIM-1 does not act by binding MA or NC or by disrupting interactions between Gag and ESCRT machinery. The results also suggest that TIM-1 inhibitory activity is not linked to dysregulation of PR activity.

TIM-1 Retains HIV-1 Virions on the Cell Surface. To identify the site of Gag accumulation in TIM-1-expressing cells, we performed live-cell imaging and transmission electron microscopy (TEM). In the absence of TIM-1, HIV-1 Gag-GFP was diffusely distributed in the cytosol of 293T cells, with abundant VLPs released into extracellular spaces which showed vigorous Brownian movements (Figure 2.2A). By contrast, in TIM-1-expressing cells Gag-GFP was predominantly concentrated in cell-cell contact areas, with little movement of VLPs observed (Figure 2.2A).

TEM was then carried out to further visualize HIV-1 particle production in TIM-1-expressing 293T cells. We observed markedly increased accumulation of mature HIV-1 particles on the surface of cells expressing TIM-1 (Figure 2.2B). This phenotype is reminiscent of that observed with tetherin, an IFN-inducible cellular restriction factor that blocks HIV-1 release (283, 284). No apparent accumulation of HIV-1 particles in intracellular compartments was observed (Figure 2.8A and B), a pattern that was different from that observed with the 29/31KE mutant (272, 276).

We then asked if the block in HIV-1 release induced by TIM-1 could be abolished by treating virus-producer cells with subtilisin A, a serine endoproteinase previously shown to partially relieve the HIV-1 release block imposed by tetherin (283, 285). Subtilisin A

substantially increased HIV-1 production in TIM-1 expressing cells, although viral particle production from subtilisin A-treated control cells (not expressing TIM-1) was also increased (Figure 2.2C, left panel; compare the middle two lanes with the right two lanes). No obvious HIV-1 p24 was recovered from the control buffer-treated cells expressing or not expressing TIM-1 (Figure 2.2C, left two lanes). Furthermore, levels of cell-associated p24 in the presence of TIM-1 were greatly decreased by subtilisin A treatment compared to cells treated with buffer control (Figure 2.2C, right panel). Together, these results suggest that the elevated levels of mature p24 associated with TIM-1-expressing virus-producer cells are largely due to the accumulation of mature HIV-1 particles on the cell surface.

The Phosphatidylserine-Binding Capability of TIM-1 Is Essential for its Inhibition of HIV-1 Release. TIM-1 is a receptor for PS, a phospholipid that is normally present in the inner leaflet of the plasma membrane; PS can be redistributed to the outer leaflet upon apoptosis or under some pathological conditions, including viral infection (269, 286). Given that PS is important for HIV-1 assembly and has been reported to be enriched in HIV-1 particles (287-290), we first examined if the PS-binding activity of TIM-1 is critical for its inhibition of HIV-1 release. We created three TIM-1 mutants, in which the two critical PS-binding residues of TIM-1, i.e., N114 and D115, were changed to Ala alone or in combination (N114A, D115A and ND114/115AA). We found that all three TIM-1 mutants exhibited a significantly decreased ability to inhibit HIV-1 release compared to WT TIM-1 in 293T cells (Figure 2.3A and B). The expression of these TIM-1 mutants on the cell surface was confirmed by flow cytometry, with patterns similar to a previous report (Figure 2.3C) (261). The inefficient block of HIV-1 release by PS

binding-deficient TIM-1 mutants was further confirmed by TEM, showing a phenotype that is indistinguishable from that of control cells not expressing TIM-1 (Figure 2.8 C-E).

We next evaluated if TIM-1 is incorporated into HIV-1 virions by taking advantage of these PS-binding deficient mutants not efficiently inhibiting HIV-1 release. We attempted to immunoprecipitate HIV-1 virions using an antibody against TIM-1, and were able to detect HIV-1 gp41 in the anti-TIM-1 pulldown product for cells expressing both TIM-1 N114A and NL4-3 (Figure 2.3D, last lane of top panel). A faint gp41 band was also observed in purified virions derived from cells expressing NL4-3 alone, likely due to pulldown of the low endogenous TIM-1 in 293T cells (Figure 2.3D, lane 6 of top panel). Interestingly, we found that the TIM-1 expression level in 293T cells co-transfected with NL4-3 was always higher than that in 293T cells transfected with TIM-1 alone, resulting in enhanced TIM-1 incorporation in purified virions, the signal of which was significantly higher than the background (Figure 2.3D, compare the last 4 lanes of middle panel). Collectively, these results demonstrate that TIM-1 is incorporated into HIV-1 virions.

As divalent calcium (Ca^{2+}) is required for binding of TIM-1 to PS (286), we asked if inhibition of the association between TIM-1 and PS by EGTA could abolish the TIM-1-induced block in HIV-1 release. Indeed, we observed that addition of EGTA to 293T cells expressing TIM-1 markedly restored HIV-1 production, as measured by RT activity (Figure 2.3E). In addition, ARD5, a monoclonal antibody against the IgV domain of TIM-1 (291), also partially overcame the TIM-1-mediated impairment of HIV-1 production (Figure 2.3F).

Viral infection has been shown to induce PS flipping to the outer leaflet of the plasma membrane, accounting for, at least in part, PS exposure on the surface of viral particles (292-294). We thus examined if expression of TIM-1 induces PS redistribution on the cell membrane, and if so, whether or not this would be important for the TIM-1 inhibition of HIV-1 release. We observed that TIM-1 induced apparent PS flipping to the cell surface, as evidenced by Annexin V binding (Figure 2.3G). Of note, all three PS mutants, which were inefficient at inhibiting HIV-1 release, showed a background level of PS flipping (Figure 2.3G). This result indicates that expression of TIM-1 induces PS redistribution to the outer leaflet of the cell membrane.

Despite Enhanced Entry, TIM-1 Inhibits HIV-1 Replication in CD4$^+$ T Cells. To investigate the role of TIM-1 in HIV-1 replication in CD4$^+$ T cell lines, we transfected Jurkat or Jurkat cells stably expressing TIM-1 with proviral NL4-3 DNA, and measured HIV-1 replication kinetics. TIM-1 substantially inhibited HIV-1 replication in Jurkat cells, as evidenced by decreased RT activity (Figure 2.4A). The effect of TIM-1 in Jurkat cells was not as pronounced as was observed in 293T cells, possibly due to the relatively low level of TIM-1 expression in Jurkat cells (Figure 2.4B). As TIM-1 has been shown to promote entry of a wide range of viruses (261, 263, 264), we tested if TIM-1 also enhances HIV-1 entry by transducing Jurkat cells with HIV-1 lentiviral vectors bearing NL4-3 Env. Indeed, we found that TIM-1 enhanced entry mediated by HIV-1 Env, but not by VSV-G, into Jurkat cells by ~3 fold (Figure 2.4C). Interestingly, we observed that the CD4 level in TIM-1 expressing Jurkat cells was consistently higher than that of parental Jurkat cells (Figure 2.4D). Hence, the relatively low level of TIM-1 expression, the upregulation of CD4 expression, as well as enhanced HIV-1 entry all likely have

contributed to the relatively modest inhibitory effect of TIM-1 on HIV-1 replication in Jurkat cells.

To determine the role of endogenous TIM-1 in HIV-1 replication, we screened a panel of human cell lines, including T-cell and monocytoid lines, for TIM-1 expression. Unfortunately, none of these cell lines, except the human lung epithelial A549 cell line, expressed a significant level of TIM-1 as determined by flow cytometry (Figure. 2.9A and 4B). We treated 293, A549 and several other cell lines with IFN-α2b, but did not observe a significant increase in TIM-1 expression (Figure 2.9C), suggesting that TIM-1 is not IFN inducible.

TIM-3 and TIM-4, as well as Additional PS Receptors Inhibit Release of HIV-1, MLV and EBOV. Human TIM family proteins consist of TIM-1, TIM-3 and TIM-4. We observed that, similar to TIM-1, human TIM-3 and TIM-4 also strongly inhibited HIV-1 release, as shown by the profound reduction of virion-associated Gag, which inversely correlated with increased cell-associated p24 (Figure 2.5A). Consistently, the RT activity and viral infectivity was significantly reduced by TIM-3 and TIM-4 (Figure 2.5B).

Interestingly, we observed that monocyte-derived macrophages (MDMs) express an abundant level of TIM-3 (Figure 2.5C). We thus tried to knock down the endogenous TIM-3 in MDMs of two healthy donors using lentiviral shRNA (Figure 2.5C), followed by infection with NL4-3 pseudotypes (pNL4-3-KFS construct containing a frameshift mutation in Env (295)) bearing VSV-G. The use of VSV-G-pseudotyped HIV-1 increased infection efficiency and bypassed the TIM-1-induced upregulation of CD4, which would otherwise complicate the analysis. Despite the relatively low TIM-3 knockdown efficiency (Figure 2.5C), we consistently observed an enhanced HIV-1

production in MDMs transduced by TIM-3 shRNA compared to that of shRNA control (Figure 2.5D).

We next evaluated if TIM-family proteins affect other viruses. We first expressed human TIM-1 in 293T cells transfected with plasmids encoding Moloney murine leukemia virus (MoMLV) Gag-Pol or EBOV VP40 fused with GFP. TIM-1 indeed inhibited the production of both MoMLV Gag and EBOV GFP-VP40, in a TIM-1 dose-dependent manner (Figure 2.5E and F). Similar effects were also found for human TIM-3 and TIM-4, although the effect of TIM-3 appeared to be less on MoMLV (Figure 2.5G and H). Interestingly, TIM expression also increased levels of cell-associated CA (p30) for MoMLV as it does for HIV-1 (Figure 2.5A, E and G) suggesting that HIV-1 and MoMLV are inhibited by TIMs via a similar mechanism.

We then tested other PS receptors, i.e., Axl and RAGE, and found that their effect on viral release depended on the tested viruses. While RAGE inhibited the release of both HIV-1 and EBOV, it did not significantly reduce MoMLV release, though some accumulation of cell-associated MoMLV CA (p30) was observed (Figure 2.10B). By contrast, Axl strongly blocked EBOV production, but it showed no obvious inhibition of HIV-1 and MoMLV release (Figure 2.10). These differential effects of RAGE and Axl on HIV-1, MoMLV and EBOV could be due to their binding capabilities to PS present in individual viruses (see Discussion). Taken together, these results indicate that TIMs, as well as some other PS receptors, can inhibit viral release.

2.5 Discussion

There is an increased appreciation that the PS-binding proteins or PS receptors, such as the TIMs, or the Gas6/TAM complex, play important roles in innate sensing and viral infections (261, 263, 264, 296-298). These proteins, either secreted into culture media (Gas6) or expressed on the cell surface (TIMs and TAMs), can interact with PS present on the surface of infectious virions thus enhancing viral entry (261, 263, 264, 297). Here, we have documented a novel function of TIM-family proteins, which inhibits the release of HIV-1, MLV and EBOV from viral-producer cells. TIM-1 appears to accomplish this function by accumulating viral particles on the cell surface through associating with virion-associated PS. Consistent with this hypothesis, TIM-1 mutants deficient for PS binding are unable to efficiently block HIV-1 release, and EGTA, which depletes Ca^{2+} that is required for the association of TIM-1 and PS, overcomes the TIM-1-mediated inhibition of HIV-1 release. In addition, we showed that TIM-1 is incorporated into HIV-1 virions, further supporting the role of TIM-1 and PS interaction in blocking HIV-1 release. Consistently, other PS receptors, including TIM-3, TIM-4, RAGE and Axl, share with TIM-1 the ability to inhibit the release of HIV-1, MLV and EBOV, although the degree of inhibition is in some cases virus-dependent. Cumulatively, our study provides new insights into the roles of TIM family proteins in virus production.

One striking observation in this study is that expression of TIM-1 diminishes HIV-1 production, but increases cell-associated p24 in viral-producer cells. This accumulation of cell-associated CA is also seen with MoMLV. We excluded the possibility that the increased mature Gag in the cell is due to excessively or prematurely activated HIV-1 protease activity, because release of an HIV-1 PR^- mutant was similarly blocked by TIM-1. In addition, the PR inhibitor, Saquinavir, failed to abolish the TIM-1 phenotype. Our

TEM data convincingly demonstrated that mature HIV-1 particles accumulate on the surface of viral producer cells, which can be stripped off by treatment of cells with the protease subtilisin A. Thus, the biochemical and EM phenotypes of TIM-1 on HIV-1 release are similar to those of tetherin, which blocks the release of HIV-1 and other viruses from the plasma membrane (299). However, given that TIM-1 is not significantly detected in HEK293, A549, Jurkat and peripheral blood mononuclear cells (PBMCs) treated with IFN-α2b (Figure 2.9C), we thus suggest that, unlike tetherin, TIM-1 is not a classical IFN-stimulated gene (ISG). TIM-1 is also distinct in terms of IFN inducibility from viperin, another ISG that inhibits HIV-1 release in a virus strain-specific manner (300).

The mechanisms by which TIM-1 and tetherin inhibit HIV-1 release are fundamentally different. Tetherin achieves this function by adopting a unique membrane topology and possibly an "axial" configuration, in which pairs of the N-terminal transmembrane domains or pairs of C-terminal GPI anchors are attached to assembling virion particles, while the remaining pairs of transmembrane domains are inserted into the virus-producing cell membrane (187, 301). By contrast, we propose that TIM-1 accomplishes this task by association of its PS-binding domain located in the IgV domain with the PS exposed on the envelopes of the budding virions. Consistent with this model, PS has been shown to be exposed on the surface of HIV-1 as well as some other viruses (292-294). Furthermore, we show here that TIM-1 mutants, which are deficient in PS binding, cannot effectively block HIV-1 release, do not induce PS flipping, yet are incorporated into HIV-1 virions. Hence, TIM-1 and PS present on the surface of viral producer cells and infectious virions may form a network of interactions, resulting in chains of

aggregates of HIV-1 particles on the cell surface (Figure 2.11). Exactly how the expression of TIM-1 induces PS flipping at the plasma membrane and how TIM-1 and PS in the virion-cell complex interact to block HIV-1 release remain to be elucidated.

TIM-1 is mainly expressed in T_H2 and various epithelial cells, and is involved in immune activation, tissue injury, and engulfment of PS-containing apoptotic cells (255). While we have observed that TIM-1 induces PS flipping, we did not find apparent cell death in TIM-1-expressing cells, suggesting that cell death, if any, should not significantly contribute to the marked reduction of viral production. In Jurkat cells stably expressing a relatively low level of TIM-1, we observed that, despite its ability to promote HIV-1 entry and upregulate CD4 expression, TIM-1 inhibits HIV-1 replication in Jurkat cells. These results argue that, at least in Jurkat cells, the inhibition of HIV-1 release by TIM-1 dominates over its enhancement of viral entry, resulting in diminished viral replication. Consistent with this model, shRNA knockdown of TIM-3 in MDMs enhances HIV-1 release. Future study should focus on determining the role of TIMs *in vivo* using relevant animal models.

Despite apparent differences in molecular structure and immune regulation, all three human TIMs, i.e., TIM-1, TIM-3 and TIM-4, potently inhibit HIV-1 release. This may not be so surprising, given that all of these TIMs contain an IgV domain, in which the PS binding pocket, known as the metal ion-dependent ligand-binding site (MILIBS), is absolutely conserved (255). In the published mouse TIM crystal structures, it has been demonstrated that the Asn and Asp residues (equivalent to human TIM-1 N114 and D115) within MILIBS are crucial for a series of intra- and intermolecular interactions and are involved in metal ion-dependent binding to PS (286, 302, 303). In this work, we

demonstrated that mutations of human TIM-1 at positions N114 and D115 resulted in a remarkable loss of inhibition of HIV-1 release. Also, we found that mouse TIM-4 exhibited an even stronger phenotype than human TIM-1 in inhibiting HIV-1 release, the mechanism of which will be explored in future studies. Interestingly, Axl and RAGE, which are PS receptors not belonging to the TIM family, also block virus release, yet in a virus-dependent manner. It is therefore reasonable to speculate that the differential inhibition of virus release by Axl and RAGE is associated with their capabilities to bind PS present in different viruses. Similarly, the effects of TIMs and PS receptor on viral release can also be cell-type dependent, a phenomenon that has been noted for TIM-mediated promotion of viral entry (264).

Our discovery that human TIMs, as well as Axl and RAGE, function as inhibitory factors to block virus release adds another layer of complexity to the increasingly appreciated role of TIMs in viral replication, innate sensing, and viral infection (255, 265). It will be important to examine the functions of different primate TIMs in the replication of other lentiviruses, and explore their potential roles in retrovirus-host co-evolution. Given the strong positive selection that has been shown for TIM-1 (304), and possibly other TIM proteins and PS receptors, primate TIMs would have conserved yet distinct antiviral effects on divergent primate lentiviruses. It might be informative to analyze the enrichment of PS in divergent lentiviruses that are produced from different primate cells, including macrophages and dendritic cells, and investigate their possible relationships to the antiviral activities of TIMs and PS receptors. By extension, the polymorphisms of TIMs in the human population, which have been shown to be

associated with asthma and some viral diseases (262, 305, 306), could hold important links to AIDS pathogenesis and disease progression.

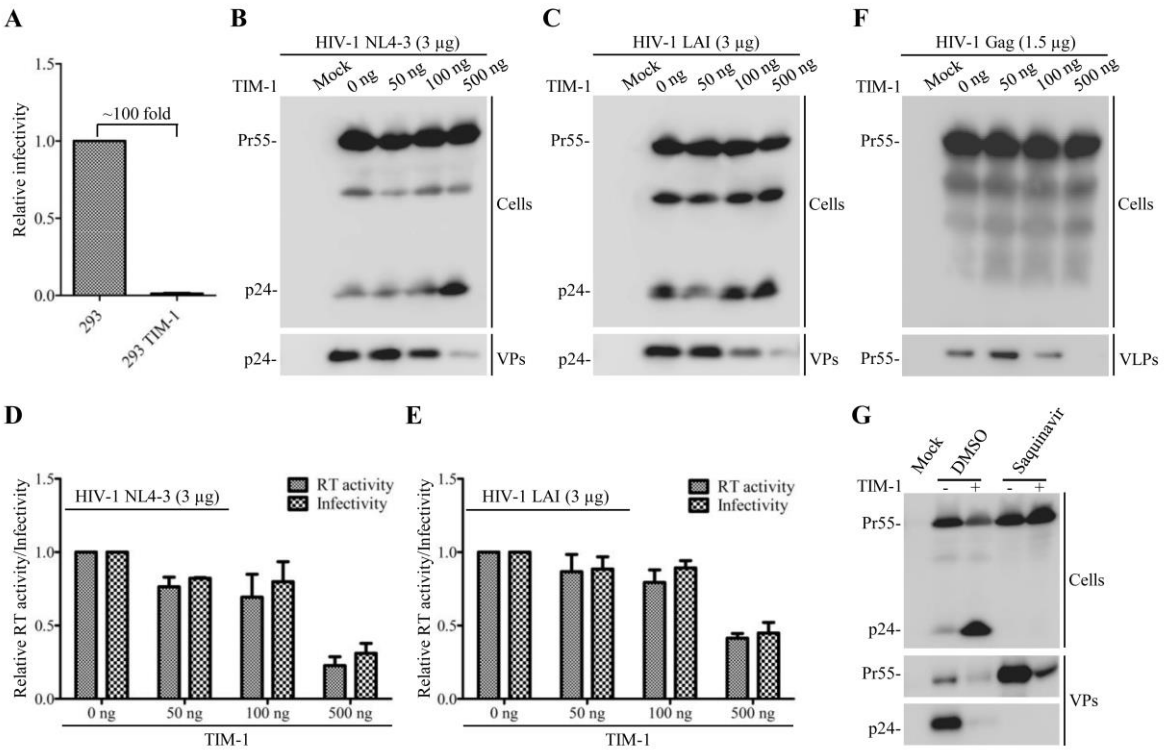

Figure 2.1: TIM-1 inhibits HIV-1 production and Gag release. *(A)* HEK293 or 293 cells stably expressing TIM-1 were transfected with pNL4-3 DNA, and viral infectivity was measured in HeLa-TZM cells. *(B-E)* HEK293T cells were transfected with NL4-3 or LAI HIV-1 proviral clones plus indicated amounts of plasmids encoding TIM-1. Western blotting was performed to examine cell-associated Gag (Cells) and cell-free viral particles (VPs) or VLPs by using an anti-p24 antibody (*B* and *C*). "Mock" indicates untransfected 293T cells. The infectivity of cell-free virus was measured in HeLa-TZM indicator cells (*D* and *E*). The data shown in (*D*) and (*E*) represent three independent experiments. Error bars represent standard deviations. *(F)* HEK293T cells were transfected with plasmids expressing codon-optimized HIV-1 Gag and increasing amounts of TIM-1. Western blotting was performed to determine cell-associated Gag and VLPs. *(G)* HEK293T cells were transfected with HIV-1 NL4-3, and cells were treated either with DMSO or 5 µM Saquinavir. Western blotting was performed using an anti-p24 antibody. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated.

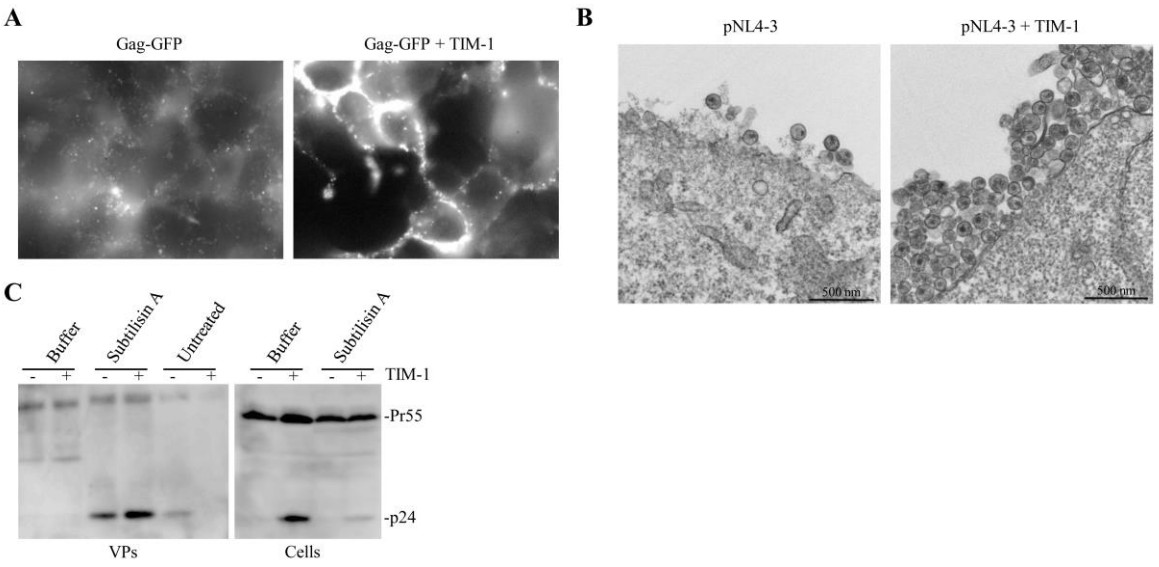

Figure 2.2: TIM-1 retains HIV-1 particles on the cell surface. *(A)* HEK293T cells were co-transfected with plasmids encoding HIV-1 Gag-GFP, with or without TIM-1 expression vector; cells were reseeded onto bottom-top dishes, and live cell images were acquired using a fluorescent microscope (Olympus, 100×). Note that numerous VLPs diffusely are present in "GFP-Gag" control cells as compared to cells expressing "GFP-Gag + TIM-1" where Gag-GFP accumulated at cell-cell contacts. *(B)* HEK293T cells were transfected pNL4-3 or pNL4-3 plus TIM-1 expression vector. Transfected cells were harvested at 24 h post-transfection, fixed in glutaraldehyde, and subjected to TEM. Representative images of thin-sectioned cells are shown. *(C)* HEK293T cells were transfected with pNL4-3 DNA with or without TIM-1 expression plasmid; viral particles released into supernatants were harvested 24 h after transfection ("untreated"). The cells were then treated with stripping buffer alone ("buffer") or buffer containing 1 mg/ml subtilisin A for 15 min at 37°C, followed by adding PMSF to stop the reaction. Cells were washed with PBS, lysed and subjected to Western blotting ("Cell"). The stripped supernatants ("VPs") were concentrated and analyzed by Western blotting. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated.

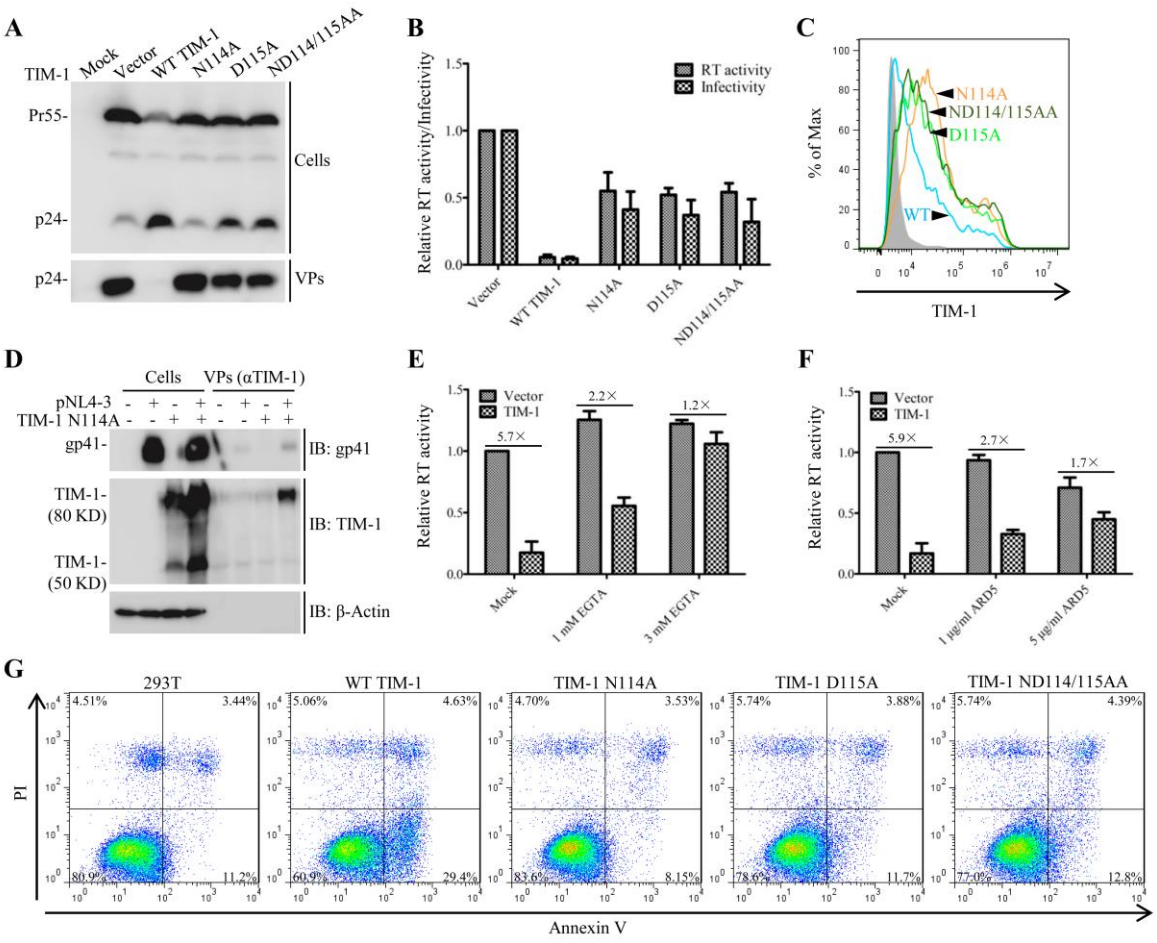

Figure 2.3: Mutation of the PS-binding sites of TIM-1 diminishes its ability to block HIV-1 release. *(A)* HEK293T cells were transfected with HIV-1 NL4-3 proviral DNA, along with plasmids encoding WT TIM-1 or its PS-binding mutants. Western blotting was performed to determine HIV-1 Gag expression in transfected 293T cells and purified viral particles. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated. *(B)* The RT activity and infectivity of HIV-1 harvested in (A) was determined by infection of HeLa-TZM cells. *(C)* Flow cytometric analysis of TIM-1 expression on the surface of transfected 293T cells using an anti-hTIM-1 antibody. (*D*) Incorporation of TIM-1 into HIV-1 virions. 293T cells were transfected with pNL4-3 proviral DNA in the presence or absence of the TIM-1 N114A plasmid. Released virions were purified and co-immunoprecipitated with an anti-TIM-1 antibody at 4°C overnight. The bound virions, along with cell lysates were resolved by SDS-PAGE, followed by Western blotting using anti-HIV-1 gp41 or anti-TIM-1 antibodies. *(E)* Effect of EGTA on the TIM-1-

mediated inhibition of HIV-1 release. The fold inhibitions between mock ("untreated") and EGTA-treated cells were indicated. We set cells not expressing TIM-1 and untreated with EGTA to 1.0 for easy comparison. *(F)* Effect of anti-TIM-1 antibody, ARD5 (against IgV domain) on HIV-1 release. The fold differences in HIV-1 RT activity between mock ("untreated") and antibody-treated cells are indicated. We set cells not expressing TIM-1 and untreated with anti-TIM-1 to 1.0 for easy comparison. *(G)* HEK293T cells were transfected with plasmids encoding WT TIM-1 or its PS-binding mutants, and their ability to induce PS flipping to the outer leaflets of the plasma membrane was assessed by flow cytometry using Annexin V and Propidium iodide (PI) binding kit (Roche). Data from (*B*), (*E*) and (*F*) are averages of at least three independent experiments. Error bars represent standard deviations.

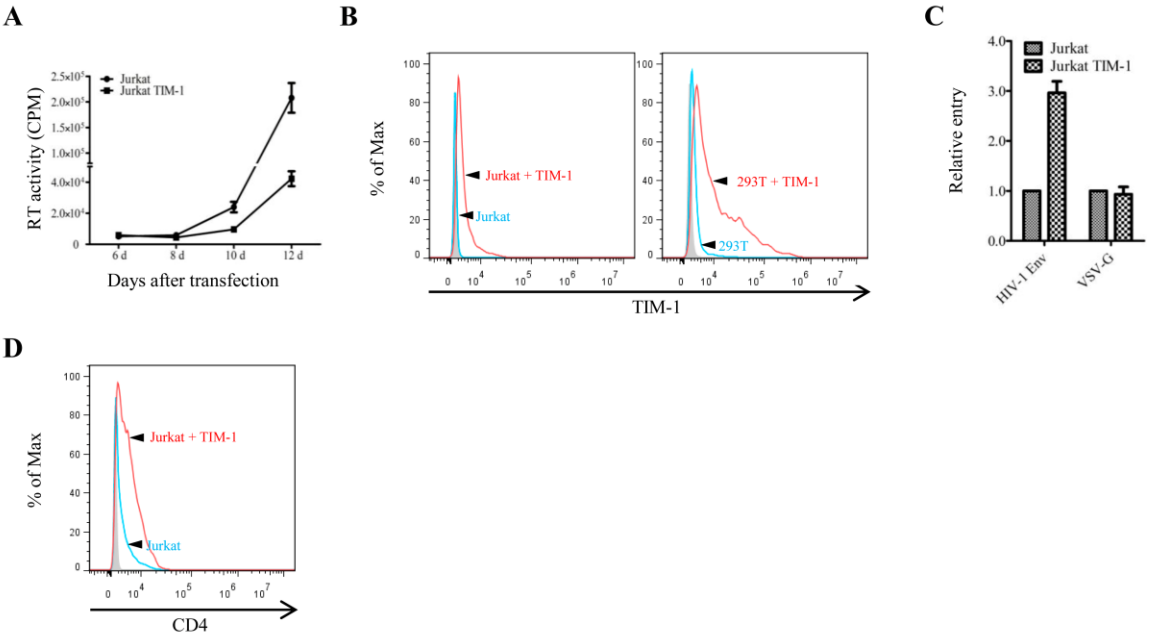

Figure 2.4: Effect of TIM-1 on HIV-1 replication and entry. *(A)* Jurkat or Jurkat cells expressing TIM-1 were transfected with pNL4-3 provial DNA, and viral replication kinetics were determined by measuring RT activities. *(B)* Comparison of TIM-1 expression in Jurkat/TIM-1 and 293T/TIM-1 cells by flow cytometry. *(C)* Expression of TIM-1 increases HIV-1 Env (NL4-3)-mediated but not VSV-G-mediated entry into Jurkat cells. Jurkat or Jurkat/TIM-1 cells were transduced by lentiviral vector (pLenti-GFP-puro) bearing NL4-3 Env and GFP-positive cells were scored by flow cytometry. *(D)* TIM-1 up-regulates CD4 expression in Jurkat cells. The levels of CD4 expression on the cell surface were determined by flow cytometry using an anti-CD4 antibody. The data shown in (*A*) and (*C*) are means and standard deviations of three independent experiments.

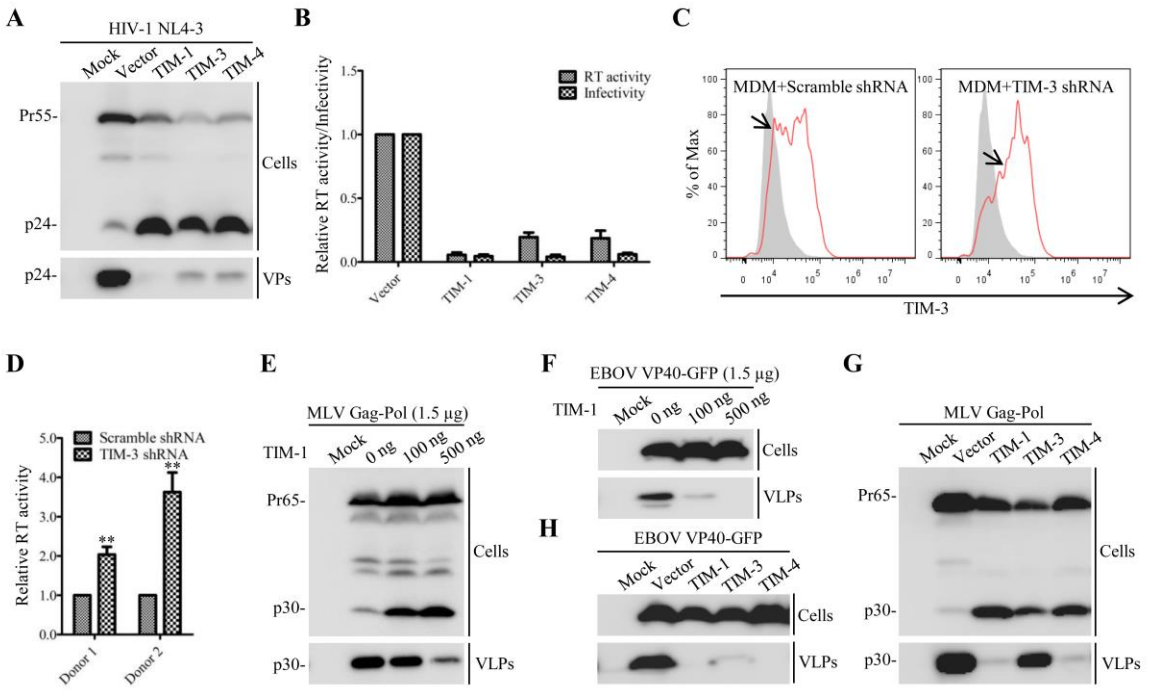

Figure 2.5: Effects of TIM-3, TIM-4, Axl, and RAGE on release of HIV-1, MLV and EBOV. *(A and B)* HEK293T cells were co-transfected with HIV-1 NL4-3 proviral DNA, along with plasmids expressing TIM-1, TIM-3 or TIM-4. Western blotting was performed to monitor cell-associated Gag expression and cell-free virion release by anti-HIV-1 p24 antibody. HIV-1 production was measured by RT activity, and viral infectivity was determined by infecting HeLa-TZM cells. The data shown here are averages of three independent experiments. Error bars represent standard deviations. (*C*) Expression of TIM-3 in MDMs. MDMs were transiently transduced by lentiviral vectors expressing TIM-1 shRNA or scrambled shRNA, and TIM-3 expression was examined by flow cytometry using an anti-TIM-3 antibody. Arrows indicate TIM-3 positive signals. (*D*) Effect of TIM-3 knockdown on HIV-1 release. MDMs transiently transduced by lentiviral vectors expressing TIM-3 shRNA or scrambled shRNA were infected with NL4-3/KFS pseudovirions bearing VSV-G for 6 h. After 3 washes, cells were maintained for an additional 18 h, and the RT activity of the produced virions determined. Results were averages of three independent experiments from two healthy donors. Error bars represent standard deviations; the RT activity of MDMs transduced by scramble shRNA was set to 1. $^{**}p < 0.01$. *(E and F)* HEK293T cells were transfected with plasmids expressing Moloney murine leukemia virus (MLV) Gag-Pol or Ebolavirus (EBOV) VP40-GFP, along with

different amounts of plasmids encoding TIM-1. Forty-eight hours post-transfection, Western blotting was performed to examine MLV Gag and EBOV VP40-GFP expression in the transfected cells and purified virus-like particles (VLPs). *(G and H)* Similar experimental procedures were performed as described for (C and D) except that TIM-3 and TIM-4 were tested. Positions of the HIV-1 Gag precursor Pr55Gag (Pr55), the MLV Gag precursor Pr65Gag (Pr65), the mature HIV-1 CA protein (p24) and mature MLV CA protein (p30) are indicated.

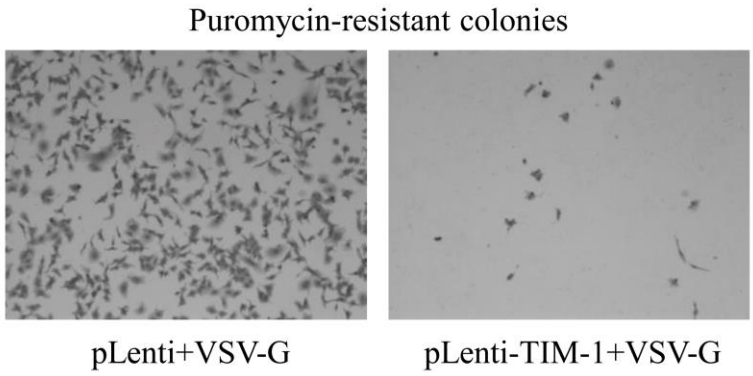

Figure 2.6: HIV-1 lentiviral vectors expressing T-cell immunoglobulin (Ig) and mucin domain 1 (TIM-1) exhibit markedly reduced transduction efficiency. Images show the density of puromycin-resistant colonies in HTX cells transduced by VSV-G pseudotyped HIV-1 vector encoding TIM-1 (pLenti-TIM-1) or GFP (pLenti-puro-GFP). Cells were fixed and stained with Coomassie Brilliant Blue.

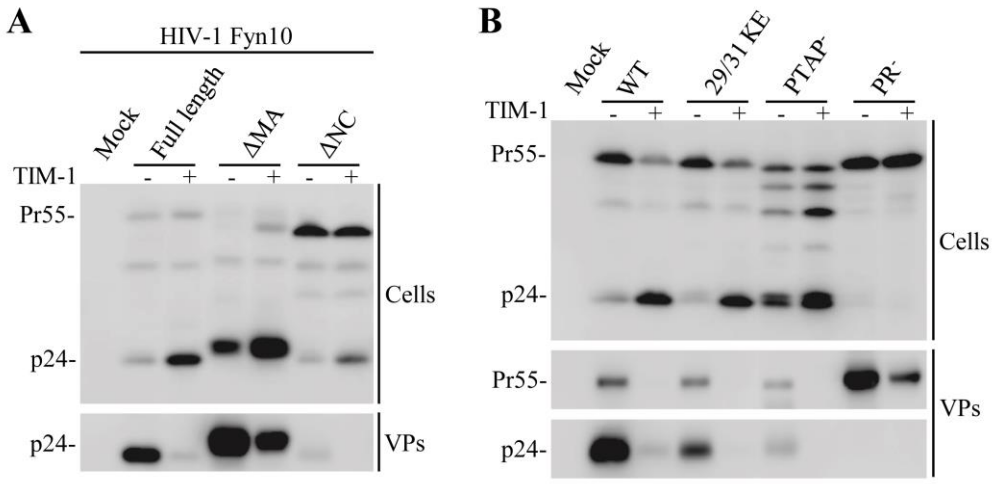

Figure 2.7: TIM-1 blocks HIV-1 release regardless of Gag or protease defects. (A) HEK293T cells were transfected with HIV-1 Fyn10 mutants with matrix (MA), nucleocapsid (NC), or both deleted, along with TIM-1 expression plasmid or empty vector. Western blotting was performed to assess cell-associated Gag (cells) and cell-free viral particle release (VPs). (B) Experimental procedures were the same as described in A, except that 293T cells were transfected with plasmids encoding the wild-type pNL4-3 (WT), 29/31KE, PTAP^{-}, or PR^{-}. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated.

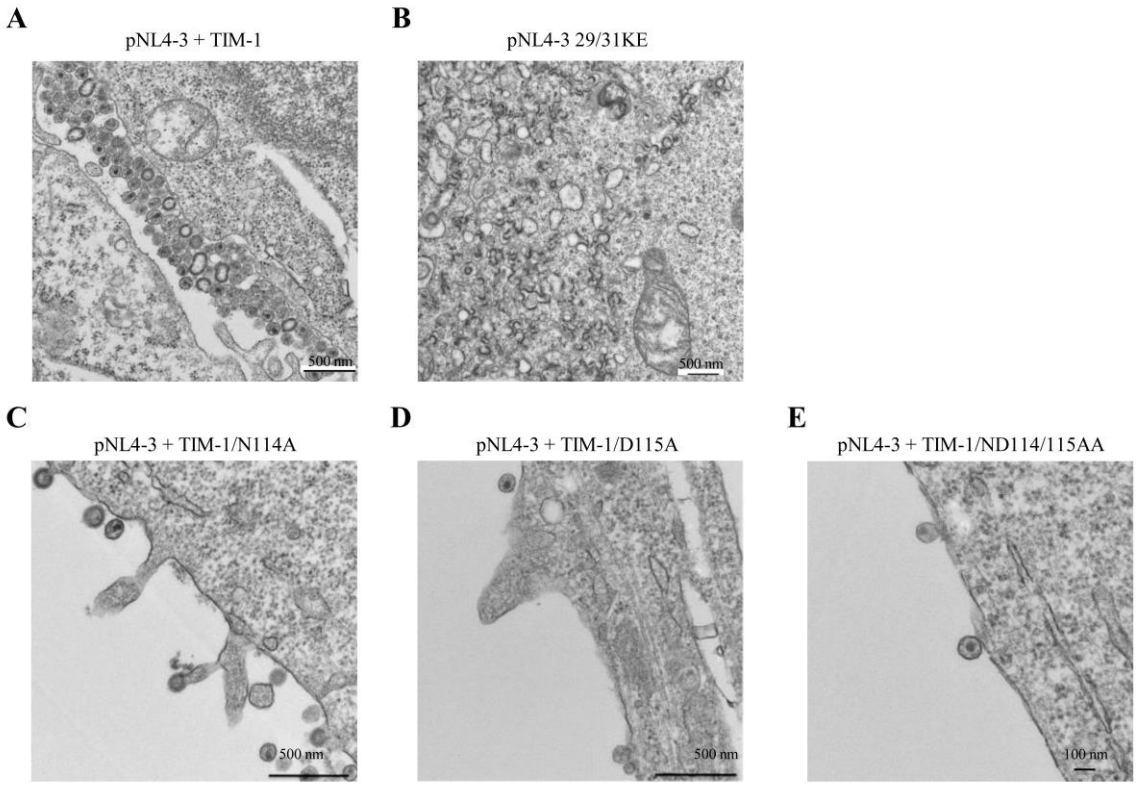

Figure 2.8: Expression of WT TIM-1 does not lead to the accumulation of HIV-1 in intracellular vesicles; TIM-1 mutants deficient for phosphatidylserine (PS) binding do not inhibit HIV-1 release. (A and B) Transmission electron microscopy (TEM) analysis of intracellular HIV-1 virions in 293T cells expressing TIM-1. (C–E) TEM analysis of 293T cells expressing TIM-1 mutants deficient for PS binding and NL4-3. The experimental procedures were the same as described in Figure 2.2B, except that TIM-1 mutants or TIM-1 were examined along with the HIV-1 NL4-3 29/31KE mutant, which is known to assemble in multivesicular bodies (MVBs).

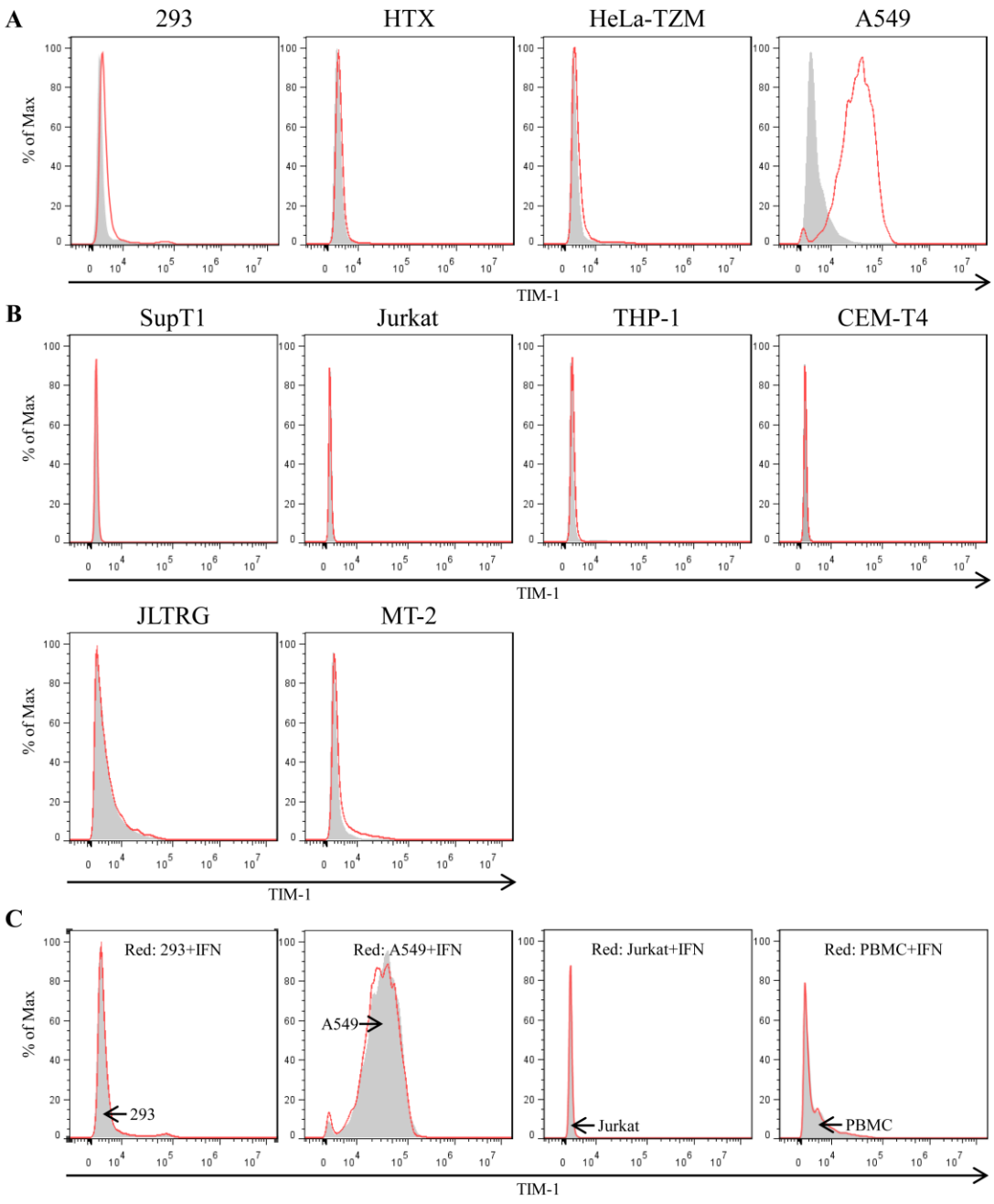

Figure 2.9: Examination of the endogenous TIM-1 expression in different cell lines and the effect of IFN treatment on TIM-1 expression. The endogenous TIM-1 expression on the cell surface was determined by flow cytometry in a panel of epithelial cell lines (293, HTX, HeLa-TZM, A549) (A), T-cell lines (SupT1, Jurkat, CEM-T4, JLTRG, MT-2), and monocytes (THP-1) (B). Gray areas represent the secondary antibody alone controls. Note that a low level of TIM-1 was consistently detected in 293 cells

and that A549 cells express a high level of TIM-1. (C) HEK293, A549, Jurkat, and peripheral blood mononuclear cells (PBMCs) were treated with 1,000 units of IFN-α2b for 18 h, and TIM-1 expression on the cell surface was determined by flow cytometry. Arrows indicate cells without treatment. In all cases, an anti-hTIM-1 antibody was used as primary antibody for binding.

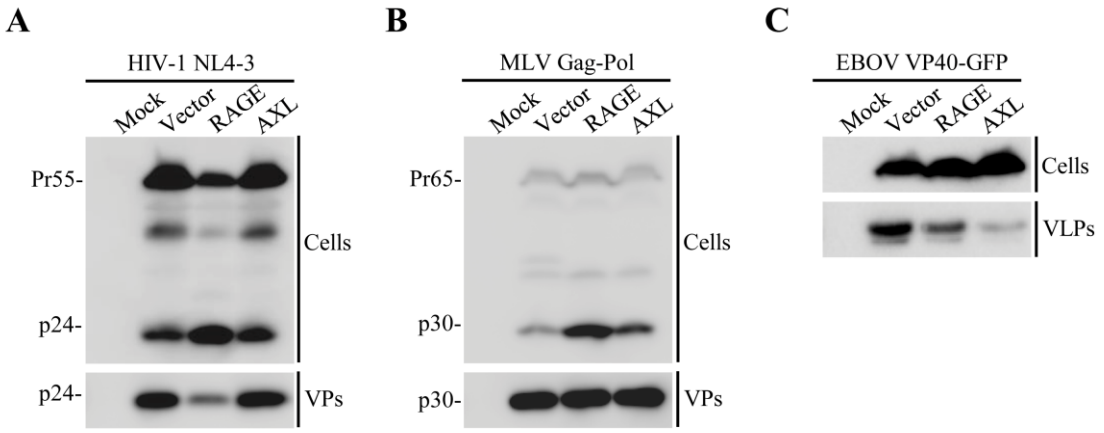

Figure 2.10: Effects of additional PS receptors, Axl and RAGE, on HIV-1, murine leukemia virus (MLV), and Ebola virus (EBOV) release. The experimental procedures were as described as in Figure 2.5, except that Axl and RAGE were tested. Mock, untransfected 293T cells; Vector, cells were transfected with plasmids encoding HIV-1 NL4-3 provirus (A), MLV Gag-Pol (B), or EBOV VP40-GFP (C) plus an empty expression vector. Positions of the HIV-1 Gag precursor Pr55Gag (Pr55), the MLV Gag precursor Pr65Gag (Pr65), the mature HIV-1 CA protein (p24), and mature MLV CA protein (p30) are indicated.

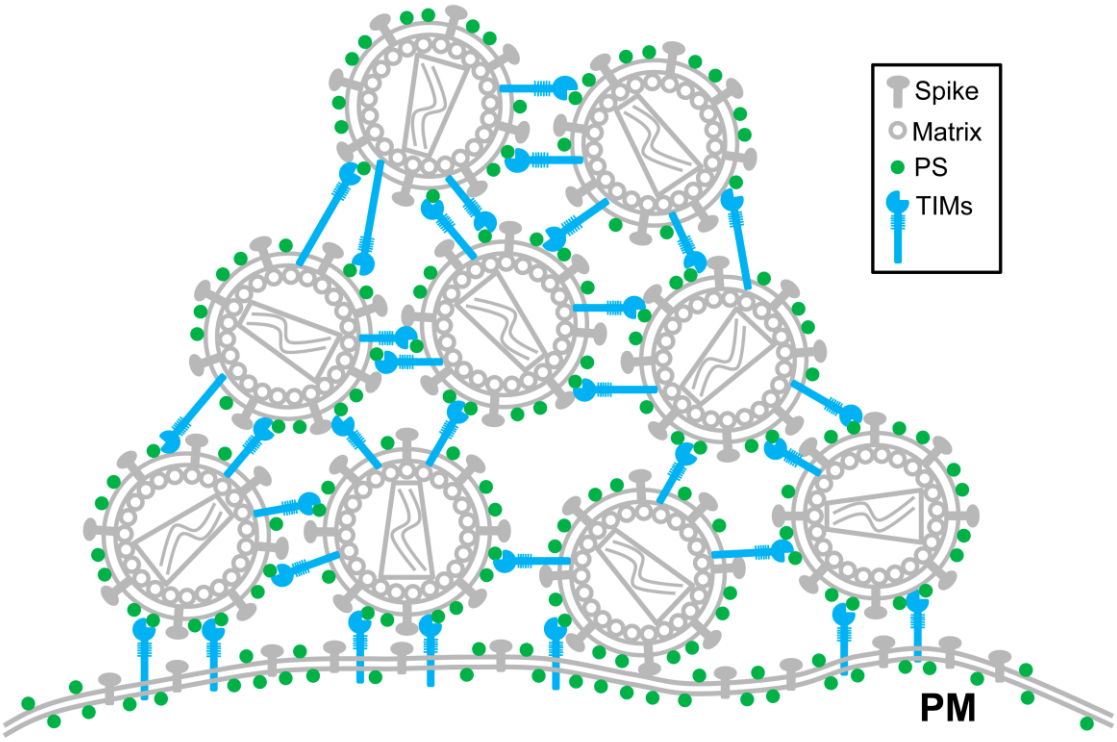

Figure 2.11: A proposed model for TIM-mediated inhibition of HIV-1 release. Expression of TIMs in HIV-1 producer cells results in PS flipping onto the outer leaflet of the plasma membrane. HIV-1 acquires PS and TIMs from viral producer cells upon budding. The intimate interaction between TIMs and PS among HIV-1 virions, as well as that between viral producer cells and virions, collectively leads to accumulation of HIV-1 virions on the plasma membrane.

CHAPTER 3 – LENTIVIRAL NEF PROTEINS ANTAGONIZE TIM-MEDIATED INHIBITION OF VIRAL RELEASE

Minghua Li[a], Eric O. Freed[b], and Shan-Lu Liu[a]

[a]Department of Molecular Microbiology and Immunology, Bond Life Sciences Center, University of Missouri, Columbia, MO 65211; [b]Virus-Cell Interaction Section, HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, MD 21702

(Manuscript in preparation)

Author contributions: Minghua Li, Eric O. Freed, and Shan-Lu Liu designed research; Minghua Li performed research; Minghua Li, Eric O. Freed, and Shan-Lu Liu analyzed data; and Minghua Li, Eric O. Freed, and Shan-Lu Liu wrote the paper.

3.1 Abstract

We recently reported that the T cell immunoglobulin and mucin domain (TIM) proteins inhibit release of HIV-1 and other enveloped viruses by interacting with virion- and cell-associated phosphatidylserine (PS). In this study, we demonstrate that the Nef proteins of HIV-1 and other lentiviruses antagonize TIM-mediated restriction. We show that TIM-1 exhibits stronger inhibition of the release of Nef-deficient relative to Nef-expressing HIV-1 particles and that ectopic expression of Nef relieves this restriction. Consistent with this finding, knockdown of endogenous TIM-3 in human PBMCs effectively enhances the production of Nef-deficient HIV-1 particles. HIV-1 Nef does not appear to downregulate TIM-1 expression on the cell surface, nor does it disrupt TIM-1 incorporation into HIV-1 virions. Interestingly, we observed that coexpression of SERINC3 and SERINC5 potentiates TIM-1 inhibition of HIV-1 release, and that depletion of SERINC proteins in viral-producer cells partially relieves TIM-mediated inhibition of HIV-1 release. These results suggest that SERINCs are involved in TIM-mediated restriction of HIV-1 release. In addition to HIV-1 Nef, the Nef proteins of simian immunodeficiency virus (SIV) strains and HIV-2 also antagonize the antiviral activity of TIM-1, suggesting an evolutionarily conserved role of the lentiviral *nef* gene in antagonizing TIMs. Collectively, our work reveals a new role for lentiviral Nef in antagonizing TIM, and highlights a complex interplay between lentiviral Nef and cellular restriction by TIMs and SERINCs.

3.2 Introduction

The host innate immune system functions as the first line of defense to protect against pathogen invasion. Upon viral infection, cells produce type I interferon (IFN) that

upregulates expression of hundreds of IFN-stimulated genes (ISGs) and executes antiviral activities. These ISGs are collectively referred to as host "restriction factors". As a countermeasure, many viruses, including HIV, have evolved effective countermeasures to overcome these restrictions. For example, HIV-1 Vif protein binds to apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3 (APOBEC3), leading to the proteasomal degradation of APOBEC3 and enhanced viral infectivity (208). HIV-1 Vpu protein counteracts with Tetherin (also known as Bst2), either by preventing its trafficking to plasma membrane or targeting tetherin to endolysosomal compartments for degradation, therefore promoting viral release (307, 308). The strategies, as well as the patterns, by which HIV-1 accessory proteins antagonize cellular restriction have offered critical molecular and genetic insights into our understanding of virus-host interactions.

Human TIM-1, TIM-3 and TIM-4 belong to T-cell immunoglobulin (Ig) and mucin domain (TIM) protein family, which specifically binds to phosphatidylserine (PS) via a conserved IgV domain and regulate host immune response (222). Depending on the patterns of expression in different cell types, TIM-family proteins play distinct roles in cell proliferation, apoptosis, immune tolerance and T cell activation. Polymorphisms of TIM-1 have been associated with some allergic human diseases. Expression of TIM proteins in target cells has been shown to promote entry by a wide range of enveloped viruses (246, 264, 309). Our group has recently shown that TIM-family proteins also potently inhibit release of HIV-1 and other enveloped viruses, including murine leukemia virus (MLV) and Ebola virus (EBOV), and this is achieved by binding to PS present in the viral producer cells and mature virions (310).

In addition TIM-1, TIM-3 and TIM-4 also inhibit HIV release. TIM-3 is abundantly expressed in activated human $CD4^+$ T cells and monocyte-derived macrophages (MDMs) (310, 311), which are the primary targets of HIV-1 infection. We recently demonstrated that knockdown of human TIM-3 in MDMs promotes HIV-1 production, suggesting that endogenous TIM-3, and likely other TIM proteins expressed at basal levels, functions as intrinsic inhibitory factors to block HIV-1 and other virus release (310). However, it is currently unknown if and how HIV-1 might counteract TIM-mediated restriction that would facilitate viral replication. Here we provide evidence that HIV-1 Nef protein is capable of antagonizing the function of TIM-1, possibly by modulating PS of produced HIV-1 virions. Intriguingly, we find that knockdown of SERINC3 or SERINC5, the newly identified restriction factors shown to impair HIV-1 infectivity and antagonized by Nef, relieves the inhibitory effect of TIM-1 on HIV-1 release. Our work unveils a novel antagonism by which HIV-1 Nef counteracts cellular factors TIM-1, possibly through SERINCs and PS, in order to facilitate HIV-1 release.

3.3 Materials and Methods

Plasmids and constructs. The plasmid encoding human TIM-1 gene was cloned to pCIneo vector with an N-terminal FLAG tag as previously reported (310). The molecular clones of HIV-1 NL4-3, SIVmac239, and SIVagmTan were obtained from the National Institutes of Health (NIH) AIDS Reagent Program. The HIV-1 proviral constructs NL4-3 ΔNef, ΔVpu, ΔVif, ΔVpr and ΔEnv were generated by using PCR-based mutagenesis based on NL4-3 backbone. The HIV-1 LAI ΔEnv, LAI ΔEnvΔNef, SIVmac239 ΔEnvΔNef, SIVagmTan ΔEnvΔNef, HIV-2 Rod9 ΔEnvΔNef plasmids were gifts from Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle, WA). The HIV-2

Rod9 plasmid was kindly provided by Chen Liang (McGill University, Canada). The HIV-1 NL4-3 Nef expression plasmids was obtained from Yong-Hui Zheng (Michigan State University, East Lansing, MI). The plasmids encoding HIV-1 NL4-3 IRES-eGFP wild type, ΔNef, ΔVpu, ΔVpuΔNef and Nef alleles of HIV-1 groups M, N, O, P, as well as SIVs were obtained from Frank Kirchhoff (Ulm University Medical Center, Germany). The human SERINC3, SERINC5, and murine leukemia virus GlycoGag expression plasmids were kindly provided by Heinrich Gottlinger (University of Massachusetts, Worcester, MA) and Massimo Pizzato (University of Trento, Italy). The plasmids encoding TIM-3 shRNA, SERINC3 shRNA, SERINC5 shRNA and scramble shRNA were purchased from Sigma (St. Louis, MO).

Cells and Reagents. HEK293T cells and HeLa-TZM-bl indicator cells were grown in Dulbecco's modified Eagle's (DMEM) medium, supplemented with 0.5% penicillin/streptomycin and 10% fetal bovine serum (FBS). Human monocytic THP-1 cells were obtained from NIH AIDS Reagent Program and maintained in RPMI medium and 10% FBS. Jurkat TAg (JTAg) parental cells, SERINC3 and SERINC5 knockout cells ($S3^{-/-}$, $S5^{-/-}$, $S3^{-/-}$ $S5^{-/-}$) were gifts from Heinrich Gottlinger (University of Massachusetts, Worcester, MA). PBMCs were isolated from the blood of healthy donors, and maintained in RPMI medium containing 10% FBS and supplemented with phytohemagglutinin (PHA-P) and interleukin-2 (IL-2). The antibody against HIV-1 Gag and HIV-1 Nef protein were obtained from NIH AIDS Reagent Program. ARD5 antibody against TIM-1 IgV domain and soluble TIM-1 with Fc tag were kindly provided by Wendy Maury (University of Iowa, Iowa City, IA). The anti-TIM-1 and anti-TIM-3 antibodies were purchased from R&D Systems (Minneapolis, MN). The secondary anti-mouse IgG

conjugated to FITC or HRP, phorbol 12-myristate 13-acetate (PMA) and inhibitor GW4869 were purchased from Sigma (St. Louis, MO). Annexin V apoptosis detection kit was purchased from Santa Cruz Biotechnology (Dallas, TX). ELISA plates pre-coated with phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Alabaster, AL). TIM-3 siRNA, negative control siRNA and Aldehyde/Sulfate latex beads were purchased from Life Technologies (Carlsbad, CA).

Virus production and infection. HEK293T cells were transfected with proviral plasmids encoding HIV-1, SIV or HIV-2, along with TIM-1 expression plasmid in the absence or presence of Nef DNA by using calcium-phosphate. Twenty-four hours posttransfection, the supernatants were harvested and clarified through 0.2-μm filter. The virus production was quantified by measuring RT activity as previously described (310). In some cases, the virus infectivity was examined by infecting HeLa-TZM-bl cells. Forty-eight hours later, the firefly luciferase activity was measured according to the manufacturer's instructions. For virus production in PMA- treated THP-1 cells, Jurkat TAg cells and PBMCs, these cells were infected with HIV-1 NL4-3 WT or ΔNef bearing VSV-G for 6 h, cells were washed 3 times with PBS and maintained for additional 18 h. HIV-1 release was monitored by measuring RT activity.

Knockdown of endogenous TIM-3 in human macrophages and PBMCs. THP-1 cells were transduced by lentiviral vectors bearing VSV-G and encoding shRNA targeting TIM-3, followed by selection with 1 μg/ml puromycin. THP-1 stable cell lines were then treated with 12.5 ng/ml PMA for 16 h to allow them to differentiate into macrophages. Knockdown of TIM-3 in PBMCs were achieved by siRNA transfection. Briefly, PBMCs were seeded in a 12-well plate and transfected with 20 pmol TIM-3 siRNA or negative

control siRNA using Lipofectamine RNAiMAX Reagent (Life Technologies). To increase the knockdown efficiency, two rounds of siRNA transfection were applied. The knockdown efficiency of macrophages and PBMCs were evaluated by flow cytometry using an anti-TIM-3 antibody.

***In vitro* TIM-1 and PS binding assay.** ELISA plates pre-coated with phosphatidylserine (PS) were blocked with 2% bovine serum albumin (BSA) for 2 h at room temperature. Plates were then washed 3 times with PBS plus 0.05% Tween 20 (PBST). Soluble TIM-1 alone, or TIM-1 plus increasing amounts of Nef protein or ARD5 were pre-incubated for 1 h at room temperature. The mixture were then applied onto ELISA plates and incubated for additional 2 h at room temperature. After 3 times of wash with PBST, the bound TIM-1 was detected by incubation of HRP conjugated anti-human antibody (Sigma) for 1 h using TMB substrate (Thermo Fisher Scientific). The absorbance at 450 nm was measured.

Quantification of phosphatidylserine (PS) in HIV-1 virions. The protocol was adapted from characterization of exosomes described in (312). Briefly, purified HIV-1 particles were incubated with 4 μm Aldehyde/Sulfate latex beads at room temperature for 1 h. Subsequently, 450 μl of PBS was added into the mixture and incubated for an additional 2 h. The reaction was stopped by adding 200 μl 100 mM glycine. The beads were then washed 3 times with PBS and stained with FITC-conjugated Annexin V (Santa Cruz Biotechnology) at 4 °C for 1 h. After 3 times of wash with PBS, stained beads were resuspended in 100 μl PBS and exposed PS in HIV-1 virions was examined by flow cytometry.

Western blotting. HEK293T cells were transfected with plasmids encoding HIV-1, SIV or HIV-2 proviral DNAs, along with TIM-1, Nef, or GlycoGag expression plasmids. Forty-eight hours later, cells were collected and lysed in the RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS). The supernatants containing HIV-1 were harvested and the viral particles were concentrated by ultracentrifugation at 32,000 rpm (Sorvall, Discovery 100SE) for 2 h at 4 °C. Cell lysates and purified virions were dissolved in 5 x sample buffer, separated on 10% SDS-PAGE gel and probed by anti-HIV-1 p24 (also for SIV and HIV-2 Gag), anti-TIM-1, or anti-Nef antibodies.

Flow cytometry. Twenty-four hours following transient transfection (HEK293T cells), shRNA lentiviral transduction (PMA-treated THP-1 cells), or siRNA treatment (PBMCs), cells were washed twice with cold PBS plus 2% FBS, and then incubated with anti-TIM-1 or anti-TIM-3 antibodies for 1 h at 4 °C. After 3 times of wash with cold PBS, cells were incubated with FITC-conjugated secondary antibody for 45 min at 4 °C. Followed by 3 times of wash with cold PBS and fixation with 3.7% formaldehyde, cells were detected by flow cytometry. For Annexin V staining, the transfected HEK293T cells were resuspended in 1x Assay Buffer (Annexin V apoptosis detection kit) and incubated with FITC-conjugated Annexin V for 15 min at room temperature. Cells were then analyzed directly by flow cytometry.

Quantitative reverse transcription PCR (qRT-PCR). The total cellular RNA was extracted from HEK293T or JTAg cell lines by using RNeasy mini kit (Qiagen). To eliminate genomic DNA, on-column DNase digestion with RNase-Free DNase (Qiagen) was performed. qRT-PCR was conducted by using Power SYBR® Green RNA-to-CT™

1-Step Kit (Applied Biosystems) and 7500 Real-Time PCR System (Applied Biosystems). The threshold-cycle values were normalized by that of β-actin and relative expression of genes of interest was calculated by using the $2^{-\Delta\Delta Ct}$ method. The primers used for qRT-PCR were as follows: SERINC3, 5'-AATTCAGGAACACCAGCCTC-3' and 5'-GGTTGGGATTGCAGGAACGA-3'; SERINC5, 5'-ATCGAGTTCTGACGCTCTGC-3' and 5'-GCTCTTCAGTGTCCTCTCCAC-3'; TIM-1, 5'-CTGCAGGGAGCAATAAGGAG-3' and 5'-TCCAAAGGCCATCTGAAGAC-3'; TIM-3, 5'-TCCAAGGATGCTTACCAC CAG-3' and 5'-GCCAATGTGGATATTTGTGTTAGAT-3'; β-actin, 5'-GGACTTCGAGCAAGAGATGG-3' and 5'-AGCACTGTGTTGGCGTACAG-3'.

Statistical Analysis. Statistical analysis was performed in GraphPad Prism 5 software and the significance of difference was evaluated with ANOVA. Unless specified in the figure legends, all experiments were performed at least three times, the obtained data used for statistical analyses.

3.4 Results

Knockdown of TIM-3 restores the release of HIV-1 ΔNef virus more efficiently than it does the WT. HIV-1 Nef possesses numerous cellular functions, notably downregulation of cell surface molecules including CD4, MHC-1 and SERINCs, thus enhancing viral infectivity and pathogenesis. To explore a possible role of HIV-1 Nef in antagonizing TIM, which is also a cell surface protein, we knocked down TIM-3 in human macrophages and PBMCs, and compared their effect on viral release between HIV-1 NL4-3 WT and Nef-deleted mutant. We first generated THP-1 cell lines stably expressing scramble or TIM-3 shRNA and treated cells with PMA, which allows the cells

to differentiate into macrophages. Subsequently, the PMA-treated THP-1 cells were infected with HIV-1 WT or ΔNef bearing VSV-G; viral release was measured by RT assay. The TIM-3 knockdown efficiency in PMA-treated THP-1 cells (~2-fold) was examined by flow cytometry, as shown in Figure 3.1A and B. We observed a significantly enhanced release for NL4-3 ΔNef in TIM-3 knock down cells, as compared to WT (Figure 3.1C). We also knocked down TIM-3 in PBMCs of three healthy donors (Figure 3.1D) and infected thee cells with VSV-G-pseudotyped HIV-1 LAI virus lacking Env or lacking both Env and Nef. While the knockdown efficiency of TIM-3 in primary PBMCs was rather low (around 20%; Figures 3.1D and E), as would be expected, in all three donors we observed more robust restoration of HIV-1 release for HIV-1 ΔNef as compared to WT (Figure 3.1F). Taken together, these results suggest that HIV-1 Nef present in the WT provirus impair the inhibitory effect of TIM-3 in PMA-treated THP-1 cells and primary human PBMCs.

Nef, but not the other accessory proteins, counteracts the inhibitory effect of TIM-1 on HIV-1 release. The accessory proteins of HIV-1 are known to antagonize many cellular restriction factors, including APOBEC3G and Tetherin (208) (204, 313); HIV-2 envelope glycoprotein (Env) has also been shown to counteract Tetherin (314, 315). To further confirm the function of Nef in TIM-mediated inhibition of HIV-1 release and also explore if other HIV-1 accessory proteins could have a similar function, we co-transfected 293T cells with a series of proviral DNAs encoding either HIV-1 wildtype (WT) NL4-3 or NL4-3 lacking Nef, Vpu, Vif, Vpr and Env. Consistent with our previous report (310), expression of TIM-1 did not change HIV-1 Gag level in viral producer cells, but strongly inhibited release of cell-free virions in a TIM-1 dose-dependent manner

(Figure 3.2A). Notably, TIM-1 was more potent to block the release of ΔNef virions than that of WT and all other accessory gene-deleted HIV-1 proviral counterparts, especially at low levels of TIM-1(Figure 3.2A and B). Consequently, the infectivity of ΔNef HIV-1 was more severely impaired by TIM-1 compared to WT and other proviruses (Figure 3.2C). Similar observations were made for another independent panel of NL4-3 constructs carrying IRES-eGFP (Figure 3.7A and B). Collectively, these results indicate that HIV-1 Nef, but not its Vpu, Vif, Vpr or Env, antagonizes the function of TIM-1 in blocking viral release.

The antagonizing effects of HIV-1 accessory proteins are often achieved by downregulating or sequestering specific ISGs (316). We next asked if Nef is capable of downregulating TIM-1 expression in transfected cells, especially on the cell surface. However, no significant differences were observed between WT and ΔNef HIV-1 in TIM-1 level (Figures 3.2A, D and E), indicating that HIV-1 Nef does not downmodulate nor sequester TIM-1 expression to overcome its restriction on HIV-1 release.

Provision of HIV-1 Nef *in trans* overcomes the stronger inhibition of TIM-1 on HIV-1 ΔNef virus. We next assessed if ectopic expression of HIV-1 Nef protein could overcome the inhibition of TIM-1 on HIV-1 release, especially the ΔNef virus. To this end, we transiently transfected 293T cells with WT NL4-3 or NL4-3 ΔNef, along with a constant amount of TIM-1 yet increasing doses of Nef plasmids. We observed that when NL4-3 Nef protein was overexpressed *in trans*, TIM-1-mediated inhibition of ΔNef HIV-1 release was abolished, as evidenced by increased signals in viral p24 in a dose-dependent manner (Figure 3.3A). Noticeably, we found that release of HIV-1 WT was also enhanced by Nef overexpression (Figure 3.3A). Quantification of viral production

using RT assay showed that release of HIV-1 ΔNef virus was almost completely restored at higher doses of Nef, but this was not so apparent for the WT (Figure 3.3B). These data directly show that HIV-1 Nef protein counteracts TIM-mediated inhibition on HIV-1 release.

We found interestingly that overexpression of HIV-1 Nef in fact upregulates TIM-1 expression in transfected cells (Figure 3.3A), which is apparently at odd with our observed phenotype of Nef that abolishes TIM-1-mediated inhibition of HIV-1 release. Because localization of TIM-1 on the cell surface determines its inhibition of HIV-1 release, we next performed flow cytometry and assessed the TIM-1 expression on the plasma membrane, in the presence and absence of Nef. We found that, regardless of Nef overexpression or not, TIM-1 expression levels on the cell surface were comparable between WT and ΔNef (Figures 3.3C and D). While additional experiments are required to dissect how Nef overexpression upregulates total TIM-1 expression yet has no effect on its expression on the plasma membrane, the antagonistic effect of Nef on TIM-1 is clearly not due to a downregulation of TIM-1 expression.

The antagonizing function of Nef against TIM is conserved among primate lentiviruses. HIV-1 primary isolates are generally classified into M, N, O, and P groups sharing distinct sequence homology and geographical distributions (317). We examined if Nef proteins of these primary HIV-1 isolates, as well as those of HIV-2 and SIV, could have similar functions as the HIV-1 NL4-3 Nef in antagonizing TIM-1. We cotransfected 293T cells with plasmids encoding Nef of these HIV-1 primary isolates, along with HIV-1 NL4-3 ΔNef and TIM-1; release of cell-free HIV-1 virions was monitored by Western blotting and RT activity. Similar to HIV-1 NL4-3 Nef, we found that Nef proteins of all

M, N, O, and P primary isolates readily relieved the inhibitory effect of TIM-1 on HIV-1 ΔNef release, with efficiency comparable to that of NL4-3 (Figure 3.4A and B). Nef proteins of two SIV isolates, SIVcpz (ER505 and MB897) and SIVgor (CP2139 and CR8757), which are the close relatives of HIV-1, also abolished TIM-1 restriction of ΔNef release (Figures 3.4B and C). In agreement with our above finding on NL4-3 Nef, the Nef proteins of all primary HIV-1 and SIV isolates tested upregulated the TIM-1 total expression (Figures 3.4A and C) but not its expression on the cell surface (data not shown).

We next sought to determine if Nef proteins of other primate lentiviruses could also antagonize TIM-1-mediated inhibition of its own viruses. We found that, indeed, TIM-1 strongly inhibited release of Nef-deleted SIVmac239 and HIV-2 virions (note in both cases we used ΔEnvΔNef) compared that of respective WTs, as shown by dramatic reductions of virion-associated p24 levels with increasing dose of TIM-1 (Figures 3.4D and E). Consistently, the RT activities of the Nef-deficient SIVmac239 and HIV-2 were much lower than those of WTs in the presence of TIM-1 (Figure 3.4F); similar observation was made for SIVagmTan (Figure 3.4F). Collectively, these data indicate that the antagonistic effect of Nef on TIM-1 is well conserved among primate lentiviruses.

HIV-1 Nef expressed in viral producer cells alters the levels of PS in produced virions. PS is a phospholipid that is typically located in the inner leaflet of the plasma membrane; upon apoptosis or other stimuli, PS is flipped to the outer leaflet of the cell plasma membrane and mediates cellular processes (224, 225). We and others recently showed that PS-TIM interaction is required for TIM-1-mediated inhibition of virus release and uptake (246, 310). Our group also demonstrated that expression of TIM-1

induces PS flipping. Here we asked if the stronger inhibition of TIM-1 on HIV-1 ΔNef release compared to WT is due to an increase in interaction between TIM-1 and PS, and whether or not HIV-1 Nef impairs TIM-PS interaction. To this end, we first compared the PS levels in cells expressing HIV-1 NL4-3 WT or ΔNef, in the presence or absence of TIM-1, by flow cytometry using FITC-labeled Annexin V. While transfection of TIM-1 or HIV-1 proviral DNAs in 293T cells induced PS flipping, as we had shown before, we observed no difference between HIV-1 WT and ΔNef in PS levels on the plasma membrane, either in the presence or absence of TIM-1 (Figures 3.5A and B). These results indicate that HIV-1 Nef does not appear to affect PS flipping in virus-producing cells, at least in 293T cells we tested.

We next determined the PS levels in produced HIV-1 virions. We cotransfected 293T cells with NL4-3 proviral DNA, along with increasing amounts of TIM-1 or Nef plasmids. Following transfection, the supernatants of cultured cells were harvested and viral particles were purified by ultracentrifugation. We incubated the purified HIV-1 virions with latex beads, followed by flow cytometry using FITC-labeled Annexin V; the mean fluorescence intensities of PS were normalized by virus amounts as measured by RT activity. As would be expected, expression of TIM-1 induced PS flipping on the cell surface and also increased PS in the HIV-1 virions (Figure 3.5C). Interestingly, we found that expression of Nef protein decreased PS levels in incorporated HIV-1 particles in a dose-dependent manner (Figure 3.5C), suggesting that Nef may counteract TIM-1 restriction by decreasing PS incorporation into HIV-1 virions.

Another possibility that Nef antagonizes the inhibitory effect of TIM-1 on HIV-1 release is to directly disrupt the TIM-1 and PS interaction. To test this hypothesis, we

performed ELISA assay by incubating a soluble form of Fc-tagged human TIM-1 protein with PS, which was pre-coated on ELISA plates, in the absence or presence of soluble Nef proteins (NIH AIDS reagent program). After intensive wash and addition of substrate, the PS-associated TIM-1 was quantified by measuring the absorbance at 450 nm. As a positive control, we also included ARD5, an antibody that specifically binds the IgV domain of TIM-1 and known to block the TIM-1 and PS interaction. As shown in Figure 3.5D, soluble Nef proteins did not decrease, but rather slightly increased, the TIM-1 and PS interaction; ARD5 decreased the TIM-1 and PS interaction to a level that was close to the background control as expected. Taken together, these results show that HIV-1 Nef does not appear to directly impede the TIM-1 and Nef interaction, at least *in vitro* based on our ELISA assay.

SERINC3 and SERINC5 proteins participate in the inhibition of TIM-1 on HIV-1 release. SERINC3 and SERINC5 are members of a unique family of transmembrane protein, which has been shown to facilitate the biosynthesis and transportation of phospholipid molecules including PS (190). Notably, two recent studies revealed that SERINC3 and SERINC5 function as intrinsic restriction factors that impair HIV-1 infectivity and their activity can be overcome by HIV-1 Nef and MLV GlycoGag proteins (191, 192). Here we explored the possible role of SERINC3 and SERINC5 in TIM-mediated inhibition of HIV-1 release. We first knocked down SERINC3, which is normally expressed in 293T cells (Figure 3.6A), and we found that inhibition of TIM-1 on HIV-1 ΔNef release was greatly relieved. Release of HIV-1 WT was also increased by knocking down SERINC3, but to a much lesser extent (Figure 3.6B). As might be expected, knockdown of SERINC5 (low expression in 293T cells) only modestly relieved

TIM-1-mediated inhibition of ΔNef HIV-1, but had no effect on the HIV-1 WT (Figure 3.6A and B). These results strongly implied that endogenous SERINCs in 293T cells are involved in TIM-1-mediated inhibition of HIV-1 release, especially the ΔNef virus.

Given that MLV GlycoGag protein antagonizes the function of SERINCs to inhibit viral infectivity, similar to that of HIV-1 Nef, we next examined if expression of MLV GlycoGag also overcomes the likely cooperative effect of SERINCs on TIM-mediated inhibition of HIV-1 release. To this end, we cotransfected 293T cells with a constant amount of TIM-1 yet increasing doses of MLV GlycoGag plasmids, along with HIV-1 NL4-3 WT or its ΔNef mutant. We observed that, similar to HIV-1 Nef, expression of MLV GlycoGag restored HIV-1 release that was inhibited by TIM-1 (Figures 3.6C and D); this was especially the case for the ΔNef virus and despite the fact that MLV GlycoGag somewhat upregulated total TIM-1 expression in the cell lysates as that of HIV-1 Nef (Figures 3.6C and D).

We next determined how SERINC proteins potentiated TIM-1 to inhibit HIV-1 release. We first assessed the possible effect of SERINCs on TIM-1 expression and PS levels in the cells by flow cytometry. As shown in Figure 3.6E and 6F, we found that transient expression of SERINC3 and SERINC5 in 293T cells increased TIM-1 expression as well as PS levels on the plasma membrane, particularly in cells expressing the HIV-1 ΔNef provirus. Consistent with this finding, we noted that co-expression of SERINC3 or SERINC5 together with TIM-1 enhanced TIM-1 restriction of HIV-1 release, although expression of SERINC protein alone had no effect (data not shown). Altogether, these results indicate that SERINC3 and SERINC5 promote TIM-1-mediated inhibition for HIV-1 release, in part by modulation of TIM-1 expression and increasing PS flipping.

3.5 Discussion

HIV-1 accessory protein Nef is a small multifunctional protein that plays critical roles in virulence and AIDS pathogenesis. One of the most documented functions of Nef is its ability to modulate expression of cell surface molecules, including CD4, MHC-I, CD8, CD28, and CD80 (195-199). Nef is reported to interact with these molecules and promote their uptake via clathrin-mediated endocytosis, resulting in removal of these molecules from the plasma membrane. Consistent with its direct role in AIDS pathogenesis, SIV harboring deleted-Nef is less pathogenic in rhesus macaques (202), and slow progression to AIDS has been also associated with Nef deletions in HIV-1 patients (318) (203). Another important function of Nef is to antagonize cellular factors that intrinsically inhibit lentivirus infection. For example, the Nef proteins of HIV-1 group O and most SIVs are capable of counteracting Tetherin by downregulation or intracellular sequestration (204-206). Here, we have demonstrated that the Nef proteins of primate lentiviruses are able to overcome the inhibition of TIMs on HIV-1, HIV-2 and SIV release. Notably, Nef does this not by directly interfering with TIM-1 expression on the cell surface, but appears to decrease virion-associated PS. Notably, we discovered that SERINC proteins, the newly identified restriction factors impairing HIV-1 infectivity that are antagonized by HIV-1 Nef and MLV GlycoGag, potentiate the inhibitory effect of TIM-1 on HIV-1 release, and that this activity is likely achieved by upregulating TIM-1 and/or PS flipping.

How does Nef antagonize the function of TIM on HIV-1 release? Given that TIM and PS interaction is essential for the inhibition of TIM on HIV-1 release, we considered three possibilities, (1) Nef may downregulate TIM expression, (2) Nef could inhibit PS

flipping and virion incorporation, and (3) Nef directly disrupts TIM-PS interaction. By using anti-TIM antibody and FITC-labeled Annexin V, we showed that Nef does not alter the TIM-1 expression on the cell surface, nor does it affect PS flipping onto the outer leaflet of the plasma membrane. We also excluded the possibility that HIV-1 Nef may directly disrupt TIM-1 and PS interaction by performing an *in vitro* binding assay. Ironically, we observed that overexpression of HIV-1 Nef upregulated TIM-1 expression in the total cell lysate, though it had no apparent effect on its expression on the plasma membrane. Further analysis showed that the internalization rate of TIM-1 from the plasma membrane was increased by Nef expression, thus underscoring the possibility that Nef may kinetically modulate TIM-1 and also PS expression on the cell surface, thus somehow counteracting the inhibition of TIM-1 on HIV-1 release. Consistent with this notion, we found that the incorporation of PS into HIV-1 virions was reduced by the presence of HIV-1 Nef in viral producer cells, as quantified by flow cytometry using latex beads. However, these results might need to be treated with caution, because the PS levels in purified HIV-1 virions were normalized by RT activity, which was essentially lower for the TIM-1-expressing cells. Apparently, more sensitive and robust methods are needed to accurately measure the kinetics and content of PS in the cells and in produced virions, as well as to interrogate how they might be modulated by TIM-1 and HIV-1 Nef. Regardless what the detailed molecular mechanisms might be, given that Nef proteins of primary HIV-1, HIV-2 and SIVs isolates possess the same function as that of HIV-1 NL4-3 in counteracting human TIM-1, it will be interesting and necessary to determine if and how TIMs of other nonhuman primate species block release of SIVs and other primate lentiviruses.

One intriguing finding of this study is that SERINC proteins participate in, and specifically potentiate, the inhibitory effect of TIM-1 on HIV-1 release. This may not be so surprising, given that SERINCs are multi-transmembrane proteins that are normally expressed on the plasma membrane, and that the restrictive activity of SERINCs on HIV-1 infectivity is antagonized by HIV-1 Nef. It is perhaps more appealing that SERINC3 and SERINC5 belong to a protein family that has been shown to be important for the biosynthesis of Serine-derived lipids, including PS (190), which is absolutely required for the inhibitory effect of TIM-1 on HIV-1 release (319). Hence, it is conceivable that Nef could antagonize TIM-1 through SERINC's intrinsic capability in modulating PS. Indeed, we found that overexpression of SERINC3 or SERINC5 increases the PS level on the plasma membrane. Also of surprise, we observed that SERINC3 and SERINC5 upregulate TIM-1 expression on the plasma membrane, therefore contributing to the observed cooperative phenotype between SERINCs and TIM in inhibiting HIV-1 release. Perhaps the most convincing piece of evidence that supports for a role of SERINCs is that knockdown of endogenous SERINCs substantially relieves TIM-1 restriction of HIV-1 release, and that similar to HIV-1 Nef, expression of MLV GlycoGag, also significantly restores HIV-1 production that was blocked by TIM-1. Future study will determine how SERINC3 and SERINC5 elevate TIM-1 expression and PS levels in transfected cells and produced virions.

In summary, we have uncovered that lentivirus Nef is an antagonist of TIM-family proteins, and that Nef appears to accomplish this function by influencing the PS incorporation into virions, which is likely influenced by SERINC proteins. The capability of Nef to modulate SERINCs and virion-associated PS broadens our knowledge in

understanding the mechanism by which HIV-1 accessory proteins counteract host restrictions thus facilitating AIDS pathogenesis.

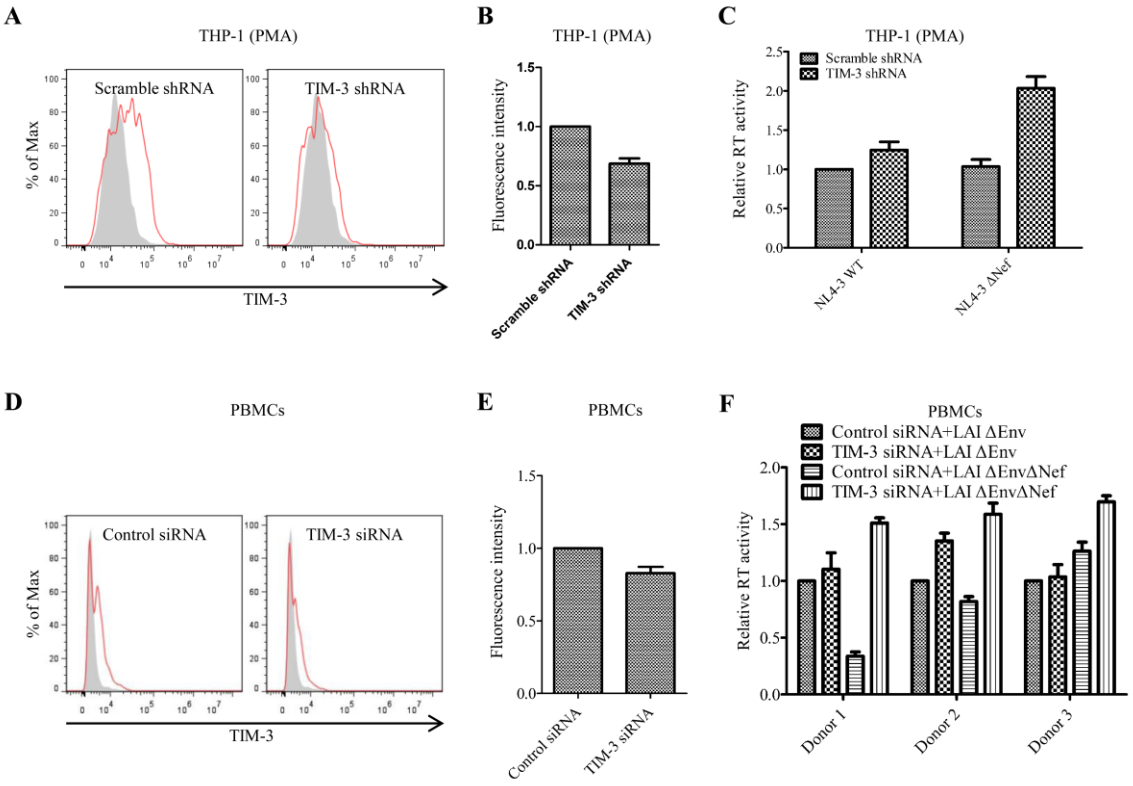

Figure 3.1: Knockdown of TIM-3 in human macrophages and PBMCs is more efficient to restore HIV-1 ΔNef production compared to WT. (A-C) THP-1 cells stably expressing scramble or TIM-3 shRNA were treated with 12.5 ng/ml PMA. Twenty-four hours after treatment, expression of TIM-3 was examined by flow cytometry with an anti-TIM-3 antibody (A) and was quantified by fluorescence intensity (B). The PMA-treated THP-1 cells were infected with NL4-3 or NL4-3 ΔNef bearing VSV-G. Six hours postinfection, cells were washed 3 times with PBS and maintained in RPMI medium for additional 18 h. The release of HIV-1 was determined by measuring RT activity; we set the value of WT scramble RNA to 1.0 and relative RT activities of others are shown (C). (D-F) PBMCs isolated from three healthy donors were treated with control or TIM-3 siRNA. After two rounds of siRNA treatment, the TIM-3 expression level was examined by flow cytometry (D). The knockdown efficiency of TIM-3 was quantified by using geometric means of fluorescence intensity (E). The siRNA-treated PBMCs were infected with HIV LAI ΔEnv or LAI ΔEnvΔNef bearing VSV-G for 6 h; subsequently, PBMCs were washed and maintained for

18 h, and the RT activity of the produced virions was determined (F). The results shown in (B), (C), (E) and (F) are from three independent experiments. Error bars represent standard deviations.

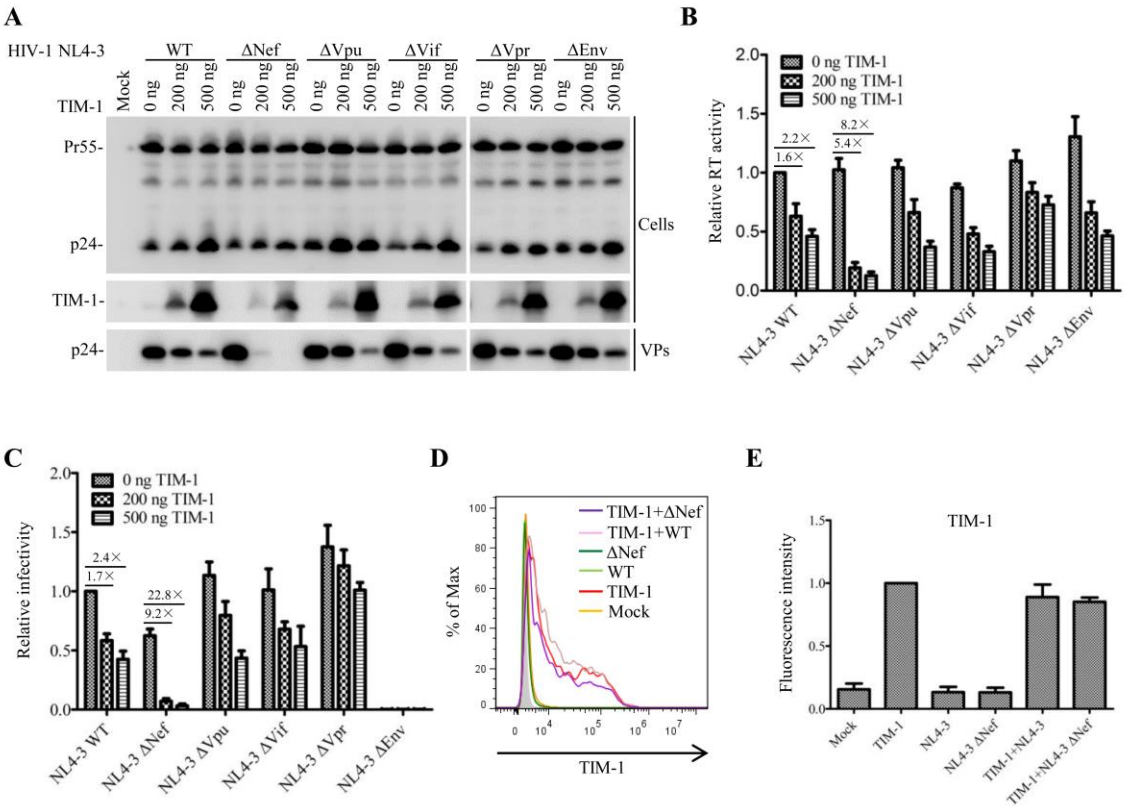

Figure 3.2: TIM-1 exhibits stronger inhibition of NL4-3 ΔNef release compared to WT and other variants. HEK293T cells were transfected with HIV-1 proviral plasmids encoding WT NL4-3 or NL4-3 lacking Nef, Vpu, Vif, Vpr and Env, plus indicated amounts of TIM-1 plasmid. (A) Western blotting was performed to examine cell-associated Gag (Cells) and cell-free viral particles (VPs) release by using an anti-p24 antibody. TIM-1 expression in the total cell lysates was confirmed by an anti-TIM-1 antibody. (B) Viral production was quantified by measuring RT activity. (C) The infectivity of cell-free HIV-1 was tested by infecting HeLa-TZM indicator cells. (D and E) The expression of TIM-1 on cell surface was determined by flow cytometry with an anti-TIM-1 antibody (D) and the results were summarized in (E). The data from (B), (C) and (E) are representative of three independent experiments. Error bars represent standard deviations.

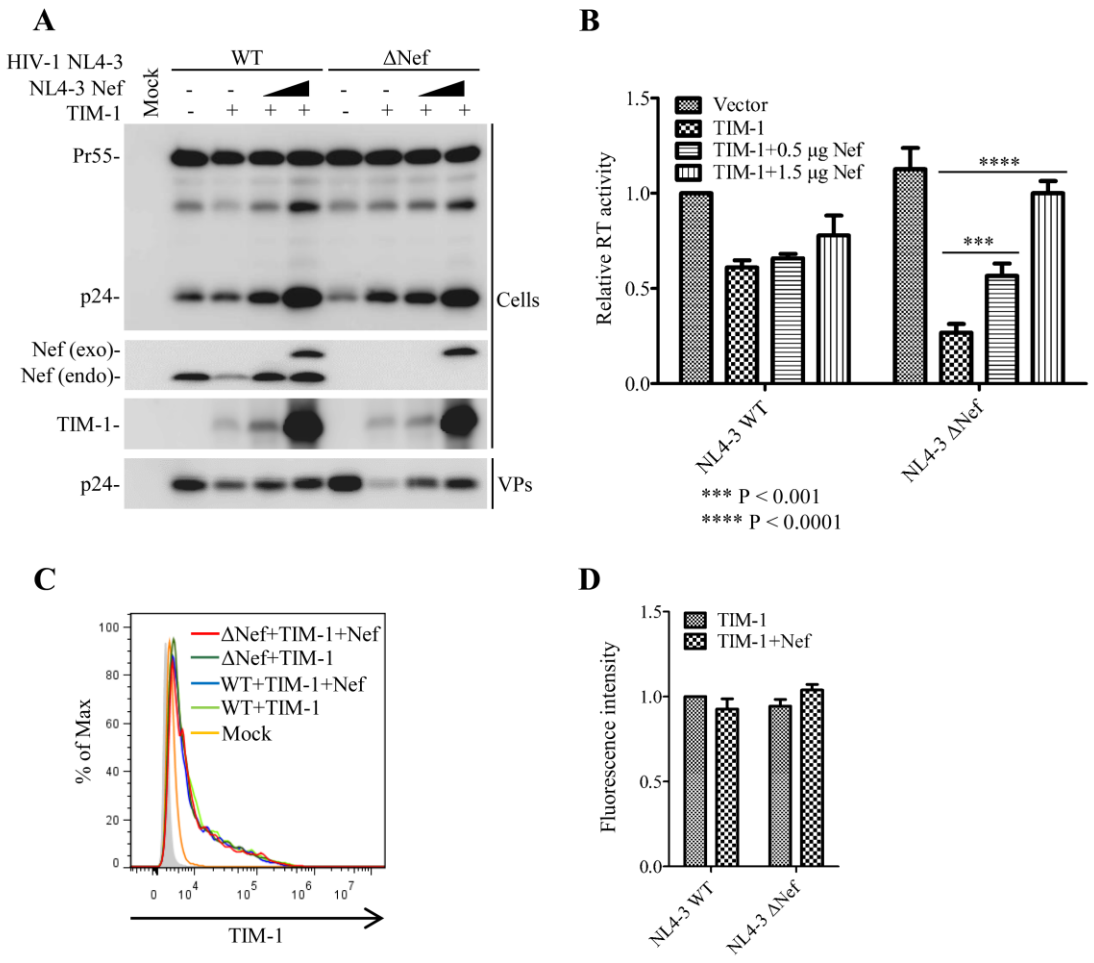

Figure 3.3: Ectopic expression of HIV-1 Nef *in trans* abolishes inhibition of HIV-1 release by TIM-1. HEK293T cells were co-transfected with plasmids expressing NL4-3 WT or ΔNef plus TIM-1, with increasing amounts of NL4-3 Nef expression plasmid (V5-tagged). (A) Expression of HIV-1 Gag, Nef and TIM-1 in transfected cells and present in HIV-1 virions were monitored by western blotting. Note that an anti-Nef antibody was able to detect both endogenous Nef (endo) in the provirus and exogenous Nef (exo) produced by transfection. (B) Released HIV-1 virions in the supernatant were examined by measuring RT activity. (C and D) TIM-1 expression on the cell surface was determined by flow cytometry (C) and quantified by plotting relative fluorescence intensities (D). The results of (B) and (D) are means and standard deviations of four independent experiments.

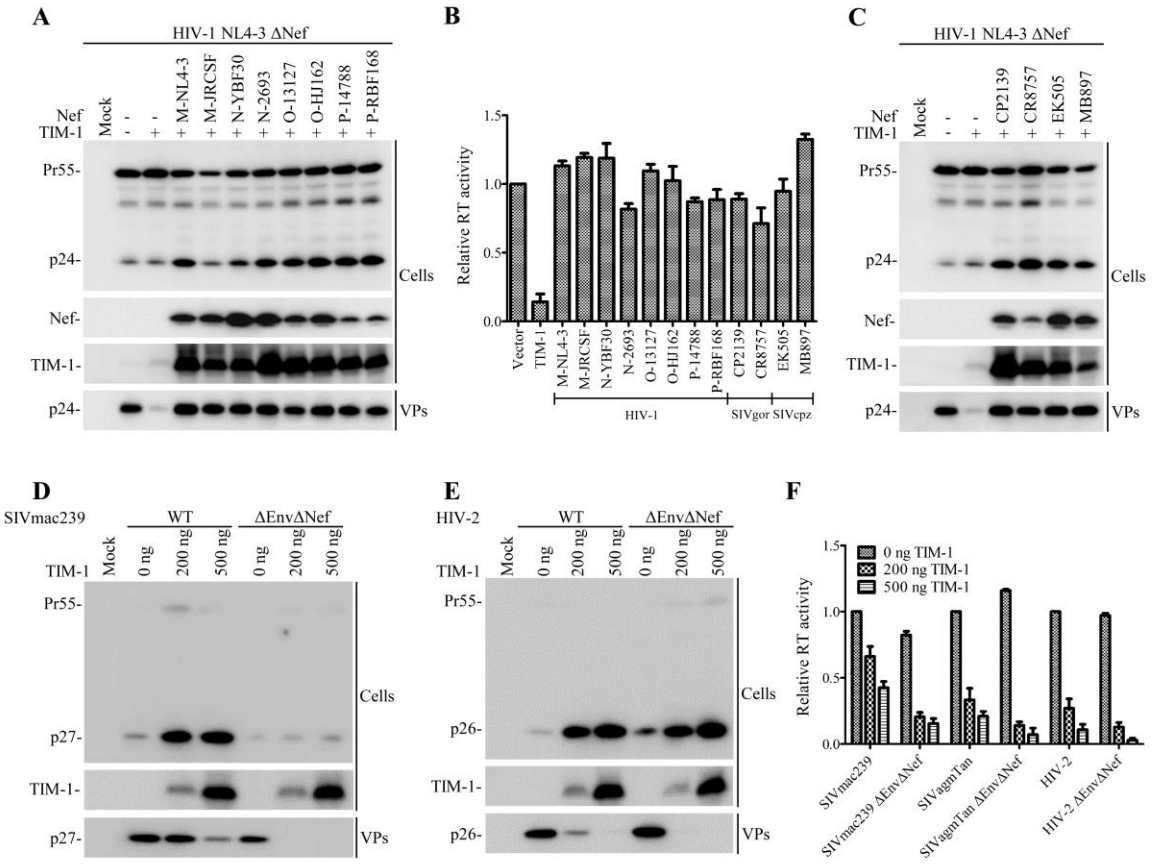

Figure 3.4: Nef proteins derived from primate lentiviruses overcome the potent inhibition of NL4-3 ΔNef release by TIM-1. (A-C) HEK293T cells were transiently transfected with pNL4-3 ΔNef and TIM-1 plasmids along with different Nef-coding constructs of HIV-1 group M (NL4-3, JRCSF), N (YBF30, N-2693), O (O-13127, O-HJ162), P (P-14788, P-RBF168), SIVgor (CP2139, CR8757) and SIVcpz (EK505, MB897). Forty-eight hours posttransfection, western blotting was performed to examine HIV-1 Gag, Nef and TIM-1 expression in the cells; P24 was detected in viral particles (A and C). The production of viral particles was quantified by measuring RT activity (B). (D-F) HEK293T cells were transfected with proviral plasmids encoding SIVmac239, SIVagmTan, HIV-2 WT or HIV-2 ΔEnvΔNef along with increasing amounts of TIM-1 expression plasmids. Cell- and virion-associated Gag proteins in SIVmac239 and HIV-2 were determined by western blotting with an anti-HIV-1 p24 antibody (D and E). The viral particles in the supernatants were examined by RT activity (F). The data shown in (B) and (F) represent three independent experiments. Error bars represent standard deviations.

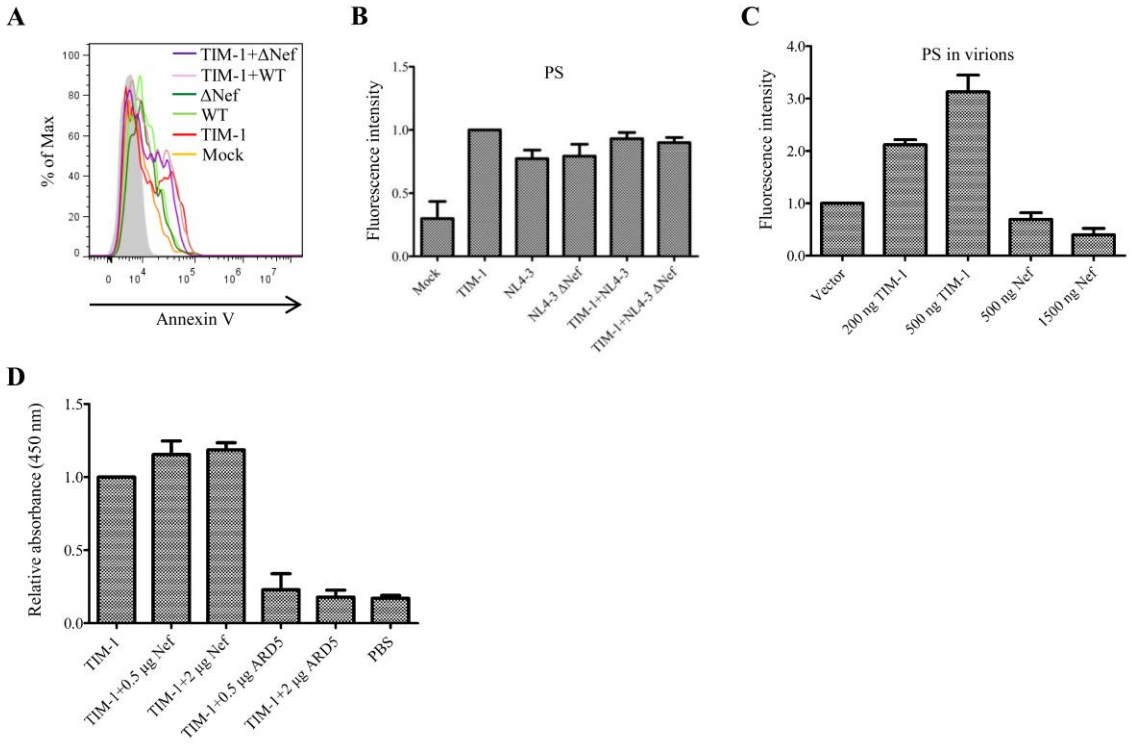

Figure 3.5: Nef expression decreases the incorporation of PS in HIV-1 virions. (A and B) HEK293T cells were transfected with plasmids encoding TIM-1, NL4-3 or NL4-3 ΔNef. Twenty-four hours later, PS exposure on the cell surface was examined by using FITC-labeled Annexin V (A) and quantified (B). (C) The protocol of quantification of virions-associated PS was adapted from (312). Briefly, HEK293T cells were transfected with NL4-3 proviral DNA and increasing amounts of TIM-1 or Nef plasmids. Twenty-four hours posttransfection, the supernatants were collected for viral particles purification. The purified HIV-1 virions were incubated with latex beads at room temperature. After wash with PBS, the exposed PS in HIV-1 particles were examined by flow cytometry using FITC-labeled Annexin V. (D) Soluble TIM-1 proteins were incubated with ELISA plate pre-coated with PS in the absence or presence of soluble Nef proteins. After intensive wash with PBS, the PS-associated TIM-1 was determined by measuring the absorbance at 450 nm. A monoclonal antibody ARD5, known to disrupt TIM-1 and PS binding by blocking IgV domain, serves as a positive control. Data from (B), (C) and (D) are averages of at least three independent experiments. Error bars represent standard deviations.

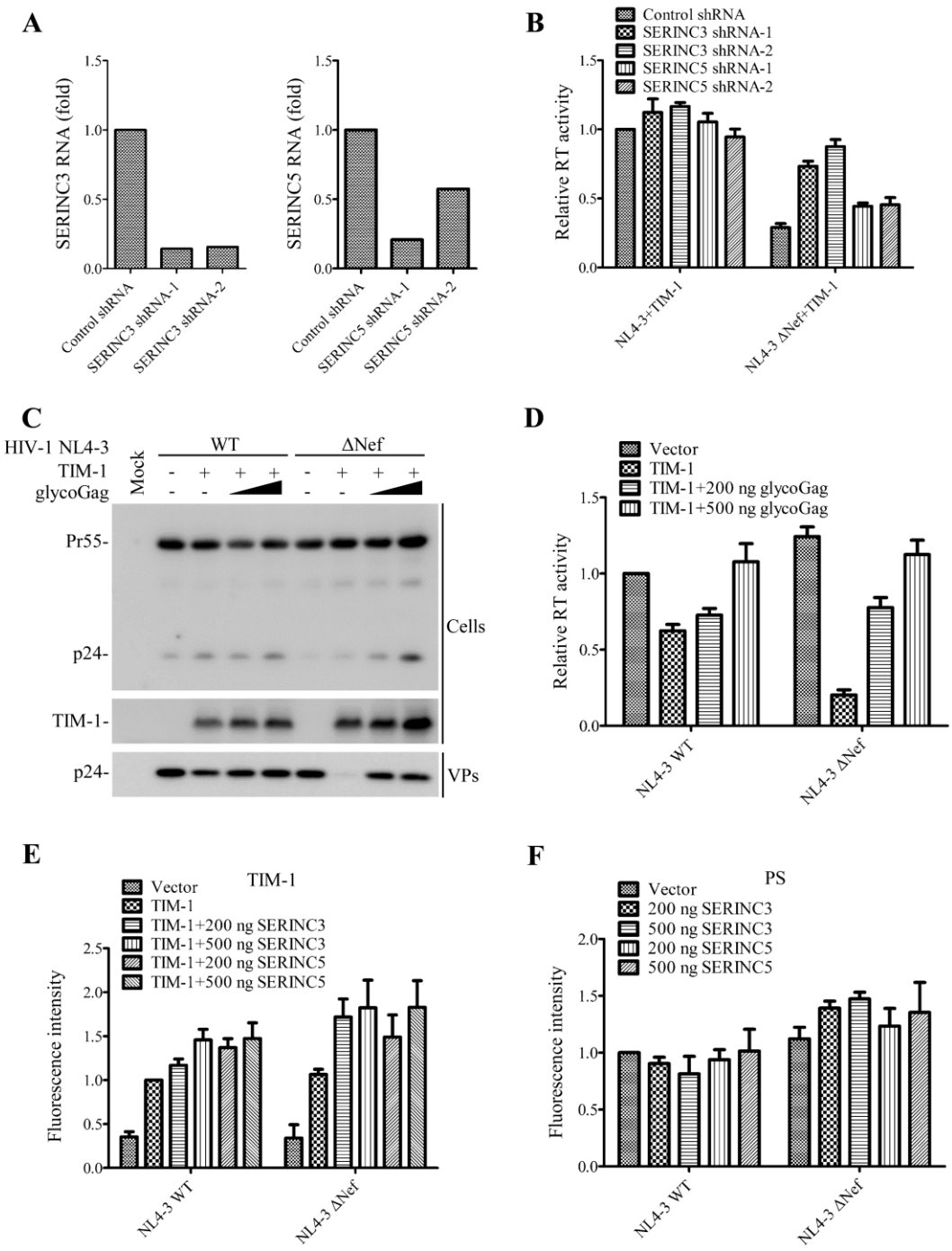

Figure 3.6: SERINC3 and SERINC5 are involved in TIM-1-mediated inhibition of HIV-1 release. (A) Endogenous SERINC3 and SERINC5 in HEK293T cells were depleted by lentiviral shRNA transduction. The knockdown efficiency was evaluated by qRT-PCR. (B) HEK293T cells stably expressing control or SERINC3 or SERINC5 shRNA were transfected with proviral plasmids encoding NL4-3 WT or ΔNef

together with TIM-1 plasmid. Forty-eight hours after transfection, supernatants were harvested and viral production was examined by RT activity. (C and D) HEK293T cells were transiently transfected with plasmids expressing NL4-3 WT or ΔNef together with a TIM-1 plasmid, in the presence or absence of increasing amounts of glycoGag. Western blotting was performed to examine HIV-1 Gag expression in the cells and HIV-1 virions (C). The production of cell-free HIV-1 particles was quantified by RT assay (D). (E) HEK293T cells were transfected with plasmids encoding NL4-3 WT or ΔNef together with TIM-1 and indicated amounts of SERINC3 and SERINC5. Forty-eight hours posttransfection, TIM-1 expression on the cell surface was determined by flow cytometry using an anti-TIM-1 antibody. (F) HEK293T cells were transfected with NL4-3 WT or NL4-3 ΔNef proviral DNAs along with increasing amounts of SERINC3 and SERINC5 plasmids. The PS flipping to the outer leaflet of the plasma membrane was quantified by FITC-labeled Annexin V. The data shown in (B), (D), (E) and (F) are means and standard deviations of at least three independent experiments.

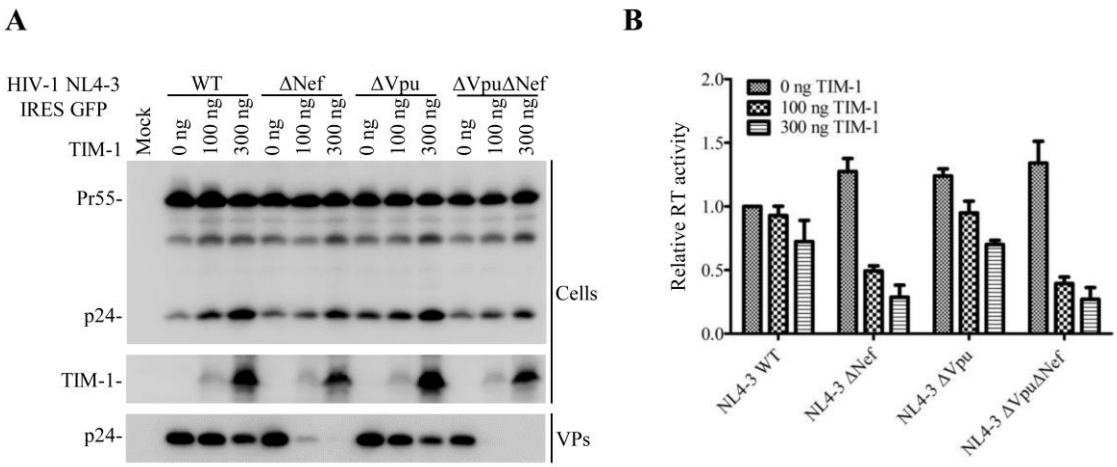

Figure 3.7: Expression of TIM-1 strongly inhibits HIV-1 ΔNef release. (A and B) HEK293T cells were transiently transfected with pBR-NL4-3-IRES-eGFP proviral DNAs expressing NL4-3 WT or ΔNef, ΔVpu, ΔVpuΔNef, along with increasing amounts of TIM-1 plasmid DNA. Cell- and virion-associated Gag proteins were determined by western blotting (A). The production of HIV-1 virions in the supernatants was quantified by measuring RT activity (B). Data are the averages of at least three independent experimental replicates. Error bars represent standard deviations.

CHAPTER 4 – OVERALL SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

In the work described in my Ph.D. thesis, I investigate the functional role of TIM-family proteins in HIV-1 infection and determine how HIV-1 may antagonize TIM-1 restriction. In Chapter 2, I provide evidence that expression of TIM-1 results in the accumulation of mature HIV-1 particles on the plasma membrane and strongly inhibits the release of HIV-1, as well as MLV and EBOV VLPs from viral producer cells. In Chapter 3, I show that TIM-1 is much potent to inhibit HIV-1 ΔNef production compared to WT and other variants. Consistently, ectopic expression of Nef proteins from HIV-1 and other lentiviruses relieve TIM-1 restriction. My results demonstrate that Nef proteins derived from primate lentiviruses can function as TIM-1 antagonists.

The role of TIM-family proteins in HIV-1 infection

TIM-1 has been shown to promote entry of enveloped viruses through the interaction between virions-associated PS and TIM-1 that is present on the surface of target cells (246, 320). Unexpectedly, my data show that expression of TIM-1 in the viral producer cells also significantly blocks HIV-1 release. Again, TIM-1 and PS interaction is critical for TIM-1's inhibitory function in HIV-1 release. This conclusion is supported by the following results: (i) compared to WT TIM-1, expression of TIM-1 mutants deficient for PS binding fail to restrict HIV-1 production; (ii) depletion of divalent calcium, which is required for TIM-1 and PS binding, by EGTA abolishes TIM-1 inhibition of HIV-1 release; (iii) disruption of TIM-1 and PS interaction by ARD5, a monoclonal antibody that is specific against the TIM-1 IgV domain, overcomes TIM-1-mediated blockage of

HIV-1 production. (iv) similar to other restriction factors such as APOBEC3 and tetherin, TIM-1 is incorporated into HIV-1 virions. Hence, TIM-1 and PS present on the cell surface and HIV-1 viral particles form a network to prevent nascent virions from being released at the plasma membrane.

Following viral infection, PS can be flipped to the outer leaflet of the plasma membrane (246, 321). During viral budding, this portion of exposed PS can be also packaged into newly produced progeny virions. Interestingly, my results show that expression of TIM-1 is able to cause PS redistribution to the outer leaflet of the plasma membrane, which apparently contributes to the TIM-1-mediated inhibition of viral release. Given that lipid profiles vary greatly depending on cells and tissues (322), it will be important to examine the effect of TIM-1 on PS flipping in other primary cells, including $CD4^+$ T cells, macrophage and dendritic cells.

Consistent with notion that TIM-1 acts as a cofactor for virus entry, by using pseudotyped virus bearing HIV-1 envelope glycoprotein, I show that expression of TIM-1 in $CD4^+$ Jurkat T cells promotes HIV-1 entry. Intriguingly, I find that CD4 expression level is upregulated when TIM-1 is expressed in Jurkat cells, which may account for the enhanced viral entry by TIM-1. Although TIM-1 in targets cells increases HIV-1 entry, the long-term replication assay using infectious viruses shows that TIM-1 expression in Jurkat cells inhibits HIV-1 replication, suggesting that at least in Jurkat cells, TIM-1's inhibitory function is dominant. However, considering the dual and opposite functions of TIM-1 in viral entry and release shown here, it will be important to determine the role of TIM-1 in HIV-1 infection *in vivo* by using animal models such as humanized mouse or non-human primates.

In addition to cell-free infection, HIV-1 can also spread by cell-to-cell transmission, which is known to be much more efficient than cell-free infection and has been considered as a major mode of HIV-1 spread *in vivo* (323, 324). Importantly, some ISGs have also been shown to block HIV-1 cell-to-cell transmission. For example, tetherin potently not only inhibits cell-free HIV-1 production but also restricts HIV-1 cell-to-cell transmission (325). Similarly, SAMHD1, a potent inhibitor of cell-free HIV-1 infection, also impairs cell-to-cell infection (326). In this sense, it will be interesting and informative to determine if TIM-family proteins also influence HIV-1 cell-to-cell spread.

The mechanisms by which HIV-1 antagonizes TIMs

HIV-1 accessory proteins are known to play critical roles in overcoming host restriction factors, primarily by degradation and/or sequestration of specific ISGs. For example, HIV-1 Vif targets APOBEC3G by recruiting E3 ubiquitin ligase, resulting in polyubiquitination and proteasomal degradation of APOBEC3G (172, 327). In a similar manner, HIV-1 Vpu interacts with tetherin and promotes tetherin ubiquitylation and endolysosomal degradation. Additionally, Vpu inhibits tetherin trafficking to the plasma membrane, thus decreasing tetherin expression at the HIV-1 budding site (328, 329). I provide evidence that Nef proteins from HIV-1 and other primate lentiviruses antagonize TIM-1 restriction. Interestingly, unlike Vif and Vpu, expression of Nef does not cause either downregulation of TIM-1 in the cells or sequestration of TIM-1 from the cell surface. I also show that Nef does not inhibit TIM-1 incorporation into HIV-1 virions.

Given that binding between TIM-1 and PS is essential for TIM-1-mediated inhibition of HIV-1 release, HIV-1 Nef proteins could counteract TIM-1 function at different steps that involve in TIM-1, PS and their interactions. Both HIV-1 infection and TIM-1

expression have been shown to induce PS flipping, I thus asked whether Nef might inhibit PS flipping. However, expression of HIV-1 WT or ΔNef, in the absence or presence of TIM-1, does not appear to change the PS level on the surface of viral producer cells. Although Nef has no apparent effect on the redistribution of PS on the cell surface, it is currently unclear if Nef is able to modulate PS incorporation into HIV-1 virions. In the future study, we should examine the lipid profiles of HIV-1 virions, particularly PS, in the absence or presence of Nef. Additionally, I have considered the possibility that Nef may directly disrupt TIM-1 and PS interaction to counteract TIM-1 restriction. However, incubation of soluble TIM-1 with Nef protein does not interfere PS binding *in vitro*. But this may not automatically exclude the possibility that Nef could affect the TIM-PS interaction *in vivo*. More experiments are needed to resolve this issue in the future.

One exciting finding of my study in Chapter 3 is that SERINC3 and SERINC5 are involved in TIM-1-mediated inhibition of HIV-1 release. I show that expression of SERINC3 and SERINC5 upregulates TIM-1, therefore potentiating TIM-1 inhibitory activity. Consistently, depletion of endogenous SERINC3 and SERINC5 partially relieves TIM-1 restriction, suggesting that SERINCs potentiate TIM-1 function through modulating TIM-1 expression level. SERINC3 and SERINC5 are members of multi-transmembrane proteins that have been to affect biosynthesis and transportation of phospholipid molecules, including PS (190). Therefore, it will be interesting to examine the role of SERINCs in PS synthesis, PS flipping, and PS incorporation into HIV-1 particles as well as the potential roles of Nef during these processes. Furthermore, because SERINC3 and SERINC5 are packaged into HIV-1 virions, it is possible that

SERINCs interact with TIM-1 so as to form a network, thus functioning as a cofactor of TIM-1 to block HIV-1 release. Future study will focus on how SERINCs modulate TIM-1 expression as well as their possible interactions.

In summary, in my Ph.D. thesis work I have discovered a novel function of TIM-family proteins, which potently inhibits the release of HIV-1 and other viruses including MLV and EBOV. Because knockdown of TIM-3 in human macrophages strongly enhances HIV-1 production, my work suggests that TIM-family proteins can act as intrinsic inhibitory factors that block HIV-1 release. Remarkably, Nef proteins derived from HIV-1 and other primate lentiviruses are able to antagonize TIM-1 function, and this involves SERINC3 and SERINC5 that potentiate the TIM-1 activity. Collectively, my work reveals a new role for TIM-family proteins in HIV-1 infection and highlights a complex interplay between lentiviral Nef and cellular restriction by TIMs and SERINCs.

LIST OF PUBLICATIONS

1. **Li, M.**, Freed, E.O., and Liu, S.-L. Lentiviral Nef Proteins Antagonize TIM-mediated Inhibition of Viral Release. (Manuscript in preparation)

2. Miao, C., **Li, M.**, Zheng, Y.-M., Cohen, F.S., and Liu, S.-L. (**2016**). Cell-cell contact promotes Ebola virus GP-mediated infection. ***Virology*** 488, 202-215.

3. Yu, J.*, **Li, M.***, Wilkins, J., Ding, S., Swartz, T.H., Esposito, A.M., Zheng, Y.-M., Freed, E.O., Liang, C., Chen, B.K., and Liu, S.-L. (**2015**). IFITM proteins restrict HIV-1 infection by antagonizing the envelope glycoprotein. ***Cell reports*** 13, 145-156. (***Co-first author**)

4. Li, K., Jia, R., **Li, M.**, Zheng, Y.-M., Miao, C., Yao, Y., Ji, H.-L., Geng, Y., Qiao, W., Albritton, L.M., Liang, C., and Liu, S.-L. (**2015**). A sorting signal suppresses IFITM1 restriction of viral entry. ***Journal of Biological Chemistry*** 290, 4248-4259.

5. **Li, M.**, Ablan, S.D., Miao, C., Zheng, Y.-M., Fuller, M.S., Rennert, P.D., Maury, W., Johnson, M.C., Freed, E.O., and Liu, S.-L. (**2014**). TIM-family proteins inhibit HIV-1 release. ***Proceedings of the National Academy of Sciences USA*** 111, E3699-E3707.

6. Li, K., Markosyan, R.M., Zheng, Y.-M., Golfetto, O., Bungart, B., **Li, M.**, Ding, S., He, Y., Liang, C., Lee, J.C., Gratton, E., Cohen, F.S., and Liu, S.-L. (**2013**). IFITM proteins restrict viral membrane hemifusion. ***PLOS Pathogens*** 9, e1003124.

REFERENCES

1. **Gottlieb MS, Schanker HM, Fan PT, Saxon A, Weisman JD, Pozalski I.** 1981. Pneumocystis pneumonia--Los Angeles. MMWR Morbidity and mortality weekly report **30:**250-252.

2. **Freed EO, Martin MA.** 2001. HIVs and their replication. Fields virology **2:**1971-2041.

3. **Sepkowitz KA.** 2001. AIDS—the first 20 years. New England Journal of Medicine **344:**1764-1772.

4. **Ammann A, Cowan M, Wara D, Goldman H, Perkins H, Lanzerotti R, Gullett J, Duff A, Dritz S, Chin J.** 1982. Possible transfusion-associated acquired immune deficiency syndrome (AIDS)--California. MMWR Morbidity and Mortality Weekly Report **31:**652-654.

5. **Control CfD.** 1983. Immunodeficiency among female sexual partners of males with acquired immune deficiency syndrome (AIDS)-New York. MMWR Morbidity and mortality weekly report **31:**697.

6. **Control CfD.** 1982. Unexplained immunodeficiency and opportunistic infections in infants--New York, New Jersey, California. MMWR Morbidity and mortality weekly report **31:**665.

7. **Ehrenkranz N, Rubini J, Gunn R, Horsburgh C, Collins T, Hasiba U, Hathaway W, Doig W, Hopkins R, Elliott J.** 1982. Pneumocystis carinii pneumonia among persons with hemophilia A. MMWR Morbidity and mortality weekly report **31:**365-367.

8. **Barré-Sinoussi F, Chermann J-C, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet C, Axler-Blin C, Vézinet-Brun F, Rouzioux C.** 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-871.

9. **Montagnier L, Chermann J, Barre-Sinoussi F, Chamaret S, Gruest J, Nugeyre M, Rey F, Dauguet C, Axler-Blin C, Vezinet-Brun F.** 1984. A new human T-lymphotropic retrovirus: characterization and possible role in lymphadenopathy and acquired immune deficiency syndromes. Human T-cell leukemia/lymphoma virus **363**.

10. **Popovic M, Sarngadharan MG, Read E, Gallo RC.** 1984. Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. Science **224:**497-500.

11. **Sarngadharan MG, Popovic M, Bruch L, Schupbach J, Gallo RC.** 1984. Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. Science **224:**506-508.

12. **Coffin J, Haase A, Levy JA, Montagnier L, Oroszlan S, Teich N, Temin H, Toyoshima K, Varmus H, Vogt P.** 1986. Human immunodeficiency viruses [letter]. Science **232:**697.

13. **Clavel F, Guetard D, Brun-Vezinet F, Chamaret S, Rey M-A, Santos-Ferreira M, Laurent AG, Dauguet C, Katlama C, Rouzioux C.** 1986. Isolation of a new human retrovirus from West African patients with AIDS. Science **233:**343-346.

14. **Clavel F.** 1987. HIV-2 the West African AIDS virus. Editorial review. Aids **1:**135-140.

15. **Merson MH, O'Malley J, Serwadda D, Apisuk C.** 2008. The history and challenge of HIV prevention. The Lancet **372:**475-488.

16. **Organization WH.** 2015. Global update on the health sector response to HIV, 2014. 2014.

17. **Hemelaar J.** 2012. The origin and diversity of the HIV-1 pandemic. Trends in molecular medicine **18:**182-192.

18. **Simon F, Mauclère P, Roques P, Loussert-Ajaka I, Müller-Trutwin MC, Saragosti S, Georges-Courbot MC, Barré-Sinoussi F, Brun-Vézinet F.** 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nature medicine **4:**1032-1037.

19. **Roques P, Robertson DL, Souquière S, Apetrei C, Nerrienet E, Barré-Sinoussi F, Müller-Trutwin M, Simon F.** 2004. Phylogenetic characteristics of three new HIV-1 N strains and implications for the origin of group N. Aids **18:**1371-1381.

20. **Vallari A, Holzmayer V, Harris B, Yamaguchi J, Ngansop C, Makamche F, Mbanya D, Kaptué L, Ndembi N, Gürtler L.** 2011. Confirmation of putative HIV-1 group P in Cameroon. Journal of virology **85:**1403-1407.

21. **Maartens G, Celum C, Lewin SR.** 2014. HIV infection: epidemiology, pathogenesis, treatment, and prevention. The Lancet **384:**258-271.

22. **Collaboration ATC.** 2010. Causes of death in HIV-1—infected patients treated with antiretroviral therapy, 1996–2006: collaborative analysis of 13 HIV cohort studies. Clinical Infectious Diseases **50:**1387-1396.

23. **Freiberg MS, Chang C-CH, Kuller LH, Skanderson M, Lowy E, Kraemer KL, Butt AA, Goetz MB, Leaf D, Oursler KA.** 2013. HIV infection and the risk of acute myocardial infarction. JAMA internal medicine **173:**614-622.

24. **Sharp PM, Hahn BH.** 2011. Origins of HIV and the AIDS pandemic. Cold Spring Harbor perspectives in medicine **1:**a006841.

25. **de Silva TI, Cotten M, Rowland-Jones SL.** 2008. HIV-2: the forgotten AIDS virus. Trends in microbiology **16:**588-595.

26. **Rowland-Jones SL, Whittle HC.** 2007. Out of Africa: what can we learn from HIV-2 about protective immunity to HIV-1? Nature immunology **8:**329-331.

27. **Hirsch VM, Olmsted RA, Murphey-Corb M, Purcell RH, Johnson PR.** 1989. An African primate lentivirus (SIVsmclosely related to HIV-2.

28. **King AM, Adams MJ, Lefkowitz EJ.** 2011. Virus taxonomy: classification and nomenclature of viruses: Ninth Report of the International Committee on Taxonomy of Viruses, vol 9. Elsevier.

29. **Rajarapu G.** 2014. Genes and Genome of HIV-1. Journal of Phylogenetics & Evolutionary Biology **2014**.

30. **Robinson HL.** 2002. New hope for an aids vaccine. Nat Rev Immunol **2:**239-250.

31. **Freed EO.** 2015. HIV-1 assembly, release and maturation. Nature Reviews Microbiology **13:**484-496.

32. **Hope T, Trono D.** 2000. Structure, expression, and regulation of the HIV genome. HIV InSite Knowledge Base Chapter.

33. **Arthos J, Cicala C, Martinelli E, Macleod K, Van Ryk D, Wei D, Xiao Z, Veenstra TD, Conrad TP, Lempicki RA.** 2008. HIV-1 envelope protein binds to and signals through integrin α4β7, the gut mucosal homing receptor for peripheral T cells. Nature immunology **9:**301-309.

34. **Geijtenbeek TB, Kwon DS, Torensma R, van Vliet SJ, van Duijnhoven GC, Middel J, Cornelissen IL, Nottet HS, KewalRamani VN, Littman DR.** 2000. DC-SIGN, a dendritic cell–specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100:**587-597.

35. **Saphire AC, Bobardt MD, Zhang Z, David G, Gallay PA.** 2001. Syndecans serve as attachment receptors for human immunodeficiency virus type 1 on macrophages. Journal of virology **75:**9187-9200.

36. **Maddon PJ, Dalgleish AG, McDougal JS, Clapham PR, Weiss RA, Axel R.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333-348.

37. **Wilen CB, Tilton JC, Doms RW.** 2012. HIV: cell binding and entry. Cold Spring Harbor perspectives in medicine **2:**a006866.

38. **Alkhatib G.** 2009. The biology of CCR5 and CXCR4. Current Opinion in HIV and AIDS **4:**96.

39. **Dubay JW, Roberts SJ, Brody B, Hunter E.** 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. Journal of virology **66:**4748-4756.

40. **Freed EO, Myers DJ, Risser R.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proceedings of the National Academy of Sciences **87:**4650-4654.

41. **Bowers K, Pelchen‐Matthews A, Höning S, Vance PJ, Creary L, Haggarty BS, Romano J, Ballensiefen W, Hoxie JA, Marsh M.** 2000. The simian immunodeficiency virus envelope

glycoprotein contains multiple signals that regulate its cell surface expression and endocytosis. Traffic **1:**661-674.

42. **Chan DC, Fass D, Berger JM, Kim PS.** 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell **89:**263-273.

43. **Melikyan GB.** 2008. Common principles and intermediates of viral protein-mediated fusion: the HIV-1 paradigm. Retrovirology **5:**111.

44. **Hu W-S, Hughes SH.** 2012. HIV-1 reverse transcription. Cold Spring Harbor perspectives in medicine **2:**a006882.

45. **Craigie R, Bushman FD.** 2012. Hiv dna integration. Cold Spring Harbor perspectives in medicine **2:**a006890.

46. **Savarino A.** 2007. In-Silico docking of HIV-1 integrase inhibitors reveals a novel drug type acting on an enzyme/DNA reaction intermediate. Retrovirology **4:**1.

47. **Starcich B, Ratner L, Josephs SF, Okamoto T, Gallo RC, Wong-Staal F.** 1985. Characterization of long terminal repeat sequences of HTLV-III. Science **227:**538-540.

48. **Groen JN, Morris KV.** 2013. Chromatin, non-coding RNAs, and the expression of HIV. Viruses **5:**1633-1645.

49. **Zenzie-Gregory B, Sheridan P, Jones K, Smale S.** 1993. HIV-1 core promoter lacks a simple initiator element but contains a bipartite activator at the transcription start site. Journal of Biological Chemistry **268:**15823-15832.

50. **Rittner K, Churcher MJ, Gait MJ, Karn J.** 1995. The human immunodeficiency virus long terminal repeat includes a specialised initiator element which is required for Tat-responsive transcription. Journal of molecular biology **248:**562-580.

51. **Li Y, Flanagan PM, Tschochner H, Kornberg RD.** 1994. RNA polymerase II initiation factor interactions and transcription start site selection. Science **263:**805-807.

52. **Jeang K-T.** 1996. HIV-1 Tat: structure and function. Human Retroviruses and AIDS 1996: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences**:**11-26.

53. **Kao S-Y, Calman AF, Luciw PA, Peterlin BM.** 1986. Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. Nature **330:**489-493.

54. **Zhang Z, Klatt A, Gilmour DS, Henderson AJ.** 2007. Negative elongation factor NELF represses human immunodeficiency virus transcription by pausing the RNA polymerase II complex. Journal of Biological Chemistry **282:**16981-16988.

55. **Tahirov TH, Babayeva ND, Varzavand K, Cooper JJ, Sedore SC, Price DH.** 2010. Crystal structure of HIV-1 Tat complexed with human P-TEFb. Nature **465:**747-751.

56. **Fujinaga K, Irwin D, Huang Y, Taube R, Kurosu T, Peterlin BM.** 2004. Dynamics of human immunodeficiency virus transcription: P-TEFb phosphorylates RD and dissociates negative effectors from the transactivation response element. Molecular and cellular biology **24:**787-795.

57. **Karn J, Stoltzfus CM.** 2012. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. Cold Spring Harbor perspectives in medicine **2:**a006916.

58. **Zapp ML, Green MR.** 1989. Sequence-specific RNA binding by the HIV-1 Rev protein. Nature **342:**714-716.

59. **Fischer U, Huber J, Boelens WC, Mattajt LW, Lührmann R.** 1995. The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. Cell **82:**475-483.

60. **Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO.** 2004. Phosphatidylinositol (4, 5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proceedings of the National Academy of Sciences of the United States of America **101:**14889-14894.

61. **Saad JS, Miller J, Tai J, Kim A, Ghanam RH, Summers MF.** 2006. Structural basis for targeting HIV-1 Gag proteins to the plasma membrane for virus assembly. Proceedings of the National Academy of Sciences **103:**11364-11369.

62. **Lu K, Heng X, Summers MF.** 2011. Structural determinants and mechanism of HIV-1 genome packaging. Journal of molecular biology **410:**609-633.

63. **Levin JG, Guo J, Rouzina I, Musier-Forsyth K.** 2005. Nucleic acid chaperone activity of HIV-1 nucleocapsid protein: critical role in reverse transcription and molecular mechanism. Progress in nucleic acid research and molecular biology **80:**217-286.

64. **Fuller SD, Wilk T, Gowen BE, Kräusslich H-G, Vogt VM.** 1997. Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. Current Biology **7:**729-738.

65. **Sundquist WI, Kräusslich H-G.** 2012. HIV-1 assembly, budding, and maturation. Cold Spring Harbor perspectives in medicine **2:**a006924.

66. **Checkley MA, Luttge BG, Freed EO.** 2011. HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. Journal of molecular biology **410:**582-608.

67. **Peel S, Macheboeuf P, Martinelli N, Weissenhorn W.** 2011. Divergent pathways lead to ESCRT-III-catalyzed membrane fission. Trends in biochemical sciences **36:**199-210.

68. **Carlton JG, Martin-Serrano J.** 2009. The ESCRT machinery: new functions in viral and cellular biology. Biochemical Society Transactions **37:**195-199.

69. **Garrus JE, von Schwedler UK, Pornillos OW, Morham SG, Zavitz KH, Wang HE, Wettstein DA, Stray KM, Côté M, Rich RL.** 2001. Tsg101 and the vacuolar protein sorting pathway are essential for HIV-1 budding. Cell **107:**55-65.

70. **Usami Y, Popov S, Popova E, Inoue M, Weissenhorn W, Göttlinger HG.** 2009. The ESCRT pathway and HIV-1 budding. Biochemical Society Transactions **37:**181-184.

71. **Hanson PI, Roth R, Lin Y, Heuser JE.** 2008. Plasma membrane deformation by circular arrays of ESCRT-III protein filaments. The Journal of cell biology **180:**389-402.

72. **Shestakova A, Hanono A, Drosner S, Curtiss M, Davies BA, Katzmann DJ, Babst M.** 2010. Assembly of the AAA ATPase Vps4 on ESCRT-III. Molecular biology of the cell **21:**1059-1071.

73. **Davies BA, Azmi IF, Payne J, Shestakova A, Horazdovsky BF, Babst M, Katzmann DJ.** 2010. Coordination of substrate binding and ATP hydrolysis in Vps4-mediated ESCRT-III disassembly. Molecular biology of the cell **21:**3396-3408.

74. **Hill M, Tachedjian G, Mak J.** 2005. The packaging and maturation of the HIV-1 Pol proteins. Current HIV research **3:**73-85.

75. **Briggs JA, Grünewald K, Glass B, Förster F, Kräusslich H-G, Fuller SD.** 2006. The mechanism of HIV-1 core assembly: insights from three-dimensional reconstructions of authentic virions. Structure **14:**15-20.

76. **Kuritzkes DR, Walker BD.** 2007. HIV-1: Pathogenesis, clinical manifestations, and treatment. Fields virology **2:**2187-2214.

77. **Hladik F, McElrath MJ.** 2008. Setting the stage: host invasion by HIV. Nature Reviews Immunology **8:**447-457.

78. **UNAIDS W.** 2010. AIDS epidemic update December. The Joint United Nations Programme on HIV and AIDS, Geneva.

79. **Shaw GM, Hunter E.** 2012. HIV transmission. Cold Spring Harbor perspectives in medicine **2:**a006965.

80. **Marlink R, Allan J, McLane M, Essex M, Anderson K, Groopman J.** 1986. Low sensitivity of ELISA testing in early HIV infection. The New England journal of medicine **315:**1549.

81. **Coffin J, Swanstrom R.** 2013. HIV pathogenesis: dynamics and genetics of viral populations and infected cells. Cold Spring Harbor perspectives in medicine **3:**a012526.

82. **Finzi D, Hermankova M, Pierson T, Carruth LM, Buck C, Chaisson RE, Quinn TC, Chadwick K, Margolick J, Brookmeyer R.** 1997. Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. Science **278:**1295-1300.

83. **Finzi D, Blankson J, Siliciano JD, Margolick JB, Chadwick K, Pierson T, Smith K, Lisziewicz J, Lori F, Flexner C.** 1999. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. Nature medicine **5:**512-517.

84. **Chomont N, El-Far M, Ancuta P, Trautmann L, Procopio FA, Yassine-Diab B, Boucher G, Boulassel M-R, Ghattas G, Brenchley JM.** 2009. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. Nature medicine **15:**893-900.

85. **Wightman F, Solomon A, Khoury G, Green JA, Gray L, Gorry PR, Ho YS, Saksena NK, Hoy J, Crowe SM.** 2010. Both CD31+ and CD31-naive CD4+ T cells are persistent HIV type 1-infected reservoirs in individuals receiving antiretroviral therapy. Journal of Infectious Diseases **202:**1738-1748.

86. **Ellery P, Sonza S, Paukovics G.** CD14lo/CD16hi monocytes are more permissive to HIV infection in vitro and may selectively harbour HIV-1 in patients on highly active antiretroviral therapy, p. *In* (ed),

87. **Gorry P, Purcell D, Howard J, McPhee D.** 1998. Restricted HIV-1 infection of human astrocytes: potential role of nef in the regulation of virus replication. Journal of neurovirology **4:**377-386.

88. **Zhou Y, Zhang H, Siliciano JD, Siliciano RF.** 2005. Kinetics of human immunodeficiency virus type 1 decay following entry into resting CD4+ T cells. Journal of virology **79:**2199-2210.

89. **Wei X, Ghosh SK, Taylor ME, Johnson VA, Emini EA, Deutsch P, Lifson JD, Bonhoeffer S, Nowak MA, Hahn BH.** 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117-122.

90. **Siliciano RF, Greene WC.** 2011. HIV latency. Cold Spring Harbor perspectives in medicine **1:**a007096.

91. **Lassen K, Han Y, Zhou Y, Siliciano J, Siliciano RF.** 2004. The multifactorial nature of HIV-1 latency. Trends in molecular medicine **10:**525-531.

92. **Kauder SE, Bosque A, Lindqvist A, Planelles V, Verdin E.** 2009. Epigenetic regulation of HIV-1 latency by cytosine methylation. PLoS Pathog **5:**e1000495.

93. **Lenasi T, Contreras X, Peterlin BM.** 2008. Transcriptional interference antagonizes proviral gene expression to promote HIV latency. Cell host & microbe **4:**123-133.

94. **Han Y, Lin YB, An W, Xu J, Yang H-C, O'Connell K, Dordai D, Boeke JD, Siliciano JD, Siliciano RF.** 2008. Orientation-dependent regulation of integrated HIV-1 expression by host gene transcriptional readthrough. Cell host & microbe **4:**134-146.

95. **Arts EJ, Hazuda DJ.** 2012. HIV-1 antiretroviral drug therapy. Cold Spring Harbor perspectives in medicine **2:**a007161.

96. **Kondru R, Zhang J, Ji C, Mirzadegan T, Rotstein D, Sankuratri S, Dioszegi M.** 2008. Molecular interactions of CCR5 with major classes of small-molecule anti-HIV CCR5 antagonists. Molecular pharmacology **73:**789-800.

97. **Lalezari JP, Henry K, O'Hearn M, Montaner JS, Piliero PJ, Trottier B, Walmsley S, Cohen C, Kuritzkes DR, Eron Jr JJ.** 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. New England Journal of Medicine **348:**2175-2185.

98. **Hart G, Orr D, Penn C, Figueiredo H, Gray N, Boehme R, Cameron J.** 1992. Effects of (-)-2'-deoxy-3'-thiacytidine (3TC) 5'-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrobial agents and chemotherapy **36:**1688-1694.

99. **Richman DD.** 2001. HIV chemotherapy. Nature **410:**995-1001.

100. **Tantillo C, Ding J, Jacobo-Molina A, Nanni RG, Boyer PL, Hughes SH, Pauwels R, Andries K, Janssen PA, Arnold E.** 1994. Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase: implications for mechanisms of drug inhibition and resistance. Journal of molecular biology **243:**369-387.

101. **Spence RA, Kati WM, Anderson KS, Johnson KA.** 1995. Mechanism of inhibition of HIV-1 reverse transcriptase by nonnucleoside inhibitors. Science **267:**988-993.

102. **Grobler JA, Stillmock K, Hu B, Witmer M, Felock P, Espeseth AS, Wolfe A, Egbertson M, Bourgeois M, Melamed J.** 2002. Diketo acid inhibitor mechanism and HIV-1 integrase: implications for metal binding in the active site of phosphotransferase enzymes. Proceedings of the National Academy of Sciences **99:**6661-6666.

103. **Flexner C.** 1998. HIV-protease inhibitors. N Engl J Med **338:**1281-1292.

104. **Lederman MM, Connick E, Landay A, Kuritzkes DR, Spritzler J, Clair MS, Kotzin BL, Fox L, Chiozzi MH, Leonard JM.** 1998. Immunologic responses associated with 12 weeks of combination antiretroviral therapy consisting of zidovudine, lamivudine, and ritonavir: results of AIDS Clinical Trials Group Protocol 315. Journal of Infectious Diseases **178:**70-79.

105. **Tomaras GD, Yates NL, Liu P, Qin L, Fouda GG, Chavez LL, Decamp AC, Parks RJ, Ashley VC, Lucas JT.** 2008. Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. Journal of virology **82:**12449-12463.

106. **Overbaugh J, Morris L.** 2012. The antibody response against HIV-1. Cold Spring Harbor perspectives in medicine **2:**a007039.

107. **Burton DR, Desrosiers RC, Doms RW, Koff WC, Kwong PD, Moore JP, Nabel GJ, Sodroski J, Wilson IA, Wyatt RT.** 2004. HIV vaccine design and the neutralizing antibody problem. Nature immunology **5:**233-236.

108. **Korber B, Muldoon M, Theiler J, Gao F, Gupta R, Lapedes A, Hahn B, Wolinsky S, Bhattacharya T.** 2000. Timing the ancestor of the HIV-1 pandemic strains. Science **288:**1789-1796.

109. **Bunnik EM, Pisas L, Van Nuenen AC, Schuitemaker H.** 2008. Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. Journal of virology **82:**7932-7941.

110. **Rizzardi GP, Harari A, Capiluppi B, Tambussi G, Ellefsen K, Ciuffreda D, Champagne P, Bart P-A, Chave J-P, Lazzarin A.** 2002. Treatment of primary HIV-1 infection with cyclosporin A coupled with highly active antiretroviral therapy. The Journal of clinical investigation **109:**681-688.

111. **Gray E, Moore P, Choge I, Decker J, Bibollet-Ruche F, Li H, Leseka N, Treurnicht F, Mlisana K, Shaw G.** 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. Journal of virology **81:**6187-6196.

112. **Stamatatos L, Morris L, Burton DR, Mascola JR.** 2009. Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? Nature medicine **15:**866-870.

113. **Kwong PD, Mascola JR, Nabel GJ.** 2011. Rational design of vaccines to elicit broadly neutralizing antibodies to HIV-1. Cold Spring Harbor perspectives in medicine **1:**a007278.

114. **Scheid JF, Mouquet H, Feldhahn N, Seaman MS, Velinzon K, Pietzsch J, Ott RG, Anthony RM, Zebroski H, Hurley A.** 2009. Broad diversity of neutralizing antibodies isolated from memory B cells in HIV-infected individuals. Nature **458:**636-640.

115. **Walker LM, Phogat SK, Chan-Hui P-Y, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL.** 2009. Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. Science **326:**285-289.

116. **Mascola JR, Haynes BF.** 2013. HIV‐1 neutralizing antibodies: understanding nature's pathways. Immunological reviews **254:**225-244.

117. **Parren PW, Marx PA, Hessell AJ, Luckay A, Harouse J, Cheng-Mayer C, Moore JP, Burton DR.** 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. Journal of virology **75:**8340-8347.

118. **Mascola JR.** 2003. Defining the protective antibody response for HIV-1. Current molecular medicine **3:**209-216.

119. **O'Connell RJ, Kim JH, Corey L, Michael NL.** 2012. Human immunodeficiency virus vaccine trials. Cold Spring Harbor perspectives in medicine **2:**a007351.

120. **Walker BD, Korber BT.** 2001. Immune control of HIV: the obstacles of HLA and viral diversity. Nature immunology **2**.

121. **Mascola JR, Montefiori DC.** 2009. The role of antibodies in HIV vaccines. Annual review of immunology **28:**413-444.

122. **Hessell AJ, Rakasz EG, Tehrani DM, Huber M, Weisgrau KL, Landucci G, Forthal DN, Koff WC, Poignard P, Watkins DI.** 2010. Broadly neutralizing monoclonal antibodies 2F5 and 4E10 directed against the human immunodeficiency virus type 1 gp41 membrane-proximal external region protect against mucosal challenge by simian-human immunodeficiency virus SHIVBa-L. Journal of virology **84:**1302-1313.

123. **Zhou T, Georgiev I, Wu X, Yang Z-Y, Dai K, Finzi A, Do Kwon Y, Scheid JF, Shi W, Xu L.** 2010. Structural basis for broad and potent neutralization of HIV-1 by antibody VRC01. Science **329:**811-817.

124. **Cocchi F, DeVico AL, Garzino-Demo A, Lusso P, Gallo RC.** 1996. Response: Role of β-Chemokines in Suppressing HIV Replication. Science **274:**1394-1395.

125. **Chun T-W, Engel D, Berrey MM, Shea T, Corey L, Fauci AS.** 1998. Early establishment of a pool of latently infected, resting CD4+ T cells during primary HIV-1 infection. Proceedings of the National Academy of Sciences **95:**8869-8873.

126. **Poon B, Hsu J, Gudeman V, Chen I, Grovit-Ferbas K.** 2005. Formaldehyde-treated, heat-inactivated virions with increased human immunodeficiency virus type 1 env can be used to induce high-titer neutralizing antibody responses. Journal of virology **79:**10210-10217.

127. **Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Premsri N, Namwat C, de Souza M, Adams E.** 2009. Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. New England Journal of Medicine **361:**2209-2220.

128. **Pitisuttithum P, Rerks-Ngarm S, Bussaratid V, Dhitavat J, Maekanantawat W, Pungpak S, Suntharasamai P, Vanijanonta S, Nitayapan S, Kaewkungwal J.** 2011. Safety and reactogenicity of canarypox ALVAC-HIV (vCP1521) and HIV-1 gp120 AIDSVAX B/E vaccination in an efficacy trial in Thailand. PloS one **6:**e27837.

129. **Michael NL.** 2009. Primary and sub-group analyses of the Thai Phase III HIV vaccine trial. AIDS Vaccine **2009**.

130. **Barber GN.** 2011. Cytoplasmic DNA innate immune pathways. Immunological Reviews **243:**99-108.

131. **Wu J, Chen ZJ.** 2014. Innate Immune Sensing and Signaling of Cytosolic Nucleic Acids. Annual Review of Immunology **32:**461-488.

132. **Kawai T, Akira S.** Toll-like Receptors and Their Crosstalk with Other Innate Receptors in Infection and Immunity. Immunity **34:**637-650.

133. **Yoneyama M, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T.** 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol **5:**730-737.

134. **Wu J, Sun L, Chen X, Du F, Shi H, Chen C, Chen ZJ.** 2013. Cyclic GMP-AMP Is an Endogenous Second Messenger in Innate Immune Signaling by Cytosolic DNA. Science **339:**826-830.

135. **Unterholzner L, Keating SE, Baran M, Horan KA, Jensen SB, Sharma S, Sirois CM, Jin T, Latz E, Xiao TS, Fitzgerald KA, Paludan SR, Bowie AG.** 2010. IFI16 is an innate immune sensor for intracellular DNA. Nat Immunol **11:**997-1004.

136. **Zhang Z, Yuan B, Bao M, Lu N, Kim T, Liu Y-J.** 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. Nat Immunol **12:**959-965.

137. **Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, Ban T, Lu Y, Miyagishi M, Kodama T, Honda K, Ohba Y, Taniguchi T.** 2007. DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature **448:**501-505.

138. **Doyle T, Goujon C, Malim MH.** 2015. HIV-1 and interferons: who's interfering with whom? Nature Reviews Microbiology **13:**403-413.

139. **Gao D, Wu J, Wu Y-T, Du F, Aroh C, Yan N, Sun L, Chen ZJ.** 2013. Cyclic GMP-AMP synthase is an innate immune sensor of HIV and other retroviruses. Science **341:**903-906.

140. **Sun L, Wu J, Du F, Chen X, Chen ZJ.** 2013. Cyclic GMP-AMP Synthase Is a Cytosolic DNA Sensor That Activates the Type I Interferon Pathway. Science **339:**786-791.

141. **Huang Y-H, Liu X-Y, Du X-X, Jiang Z-F, Su X-D.** 2012. The structural basis for the sensing and binding of cyclic di-GMP by STING. Nat Struct Mol Biol **19:**728-730.

142. **Shu C, Yi G, Watts T, Kao CC, Li P.** 2012. Structure of STING bound to cyclic di-GMP reveals the mechanism of cyclic dinucleotide recognition by the immune system. Nat Struct Mol Biol **19:**722-724.

143. **Abe T, Harashima A, Xia T, Konno H, Konno K, Morales A, Ahn J, Gutman D, Barber Glen N.** 2013. STING Recognition of Cytoplasmic DNA Instigates Cellular Defense. Molecular Cell **50:**5-15.

144. **Cai X, Chiu Y-H, Chen Zhijian J.** 2014. The cGAS-cGAMP-STING Pathway of Cytosolic DNA Sensing and Signaling. Molecular Cell **54:**289-296.

145. **Hoffmann H-H, Schneider WM, Rice CM.** 2015. Interferons and viruses: an evolutionary arms race of molecular interactions. Trends in Immunology **36:**124-138.

146. **Doitsh G, Galloway NL, Geng X, Yang Z, Monroe KM, Zepeda O, Hunt PW, Hatano H, Sowinski S, Muñoz-Arias I.** 2014. Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature **505:**509-514.

147. **Beignon A-S, McKenna K, Skoberne M, Manches O, DaSilva I, Kavanagh DG, Larsson M, Gorelick RJ, Lifson JD, Bhardwaj N.** 2005. Endocytosis of HIV-1 activates plasmacytoid

dendritic cells via Toll-like receptor–viral RNA interactions. The Journal of clinical investigation **115:**3265-3275.

148. **Kader M, Smith AP, Guiducci C, Wonderlich ER, Normolle D, Watkins SC, Barrat FJ, Barratt-Boyes SM.** 2013. Blocking TLR7-and TLR9-mediated IFN-α production by plasmacytoid dendritic cells does not diminish immune activation in early SIV infection. PLoS Pathog **9:**e1003530.

149. **Manel N, Littman DR.** 2011. Hiding in plain sight: how HIV evades innate immune responses. Cell **147:**271-274.

150. **Yan N, Regalado-Magdos AD, Stiggelbout B, Lee-Kirsch MA, Lieberman J.** 2010. The cytosolic exonuclease TREX1 inhibits the innate immune response to human immunodeficiency virus type 1. Nature immunology **11:**1005-1013.

151. **Levin D, Harari D, Schreiber G.** 2011. Stochastic Receptor Expression Determines Cell Fate upon Interferon Treatment. Molecular and Cellular Biology **31:**3252-3266.

152. **Thomas C, Moraga I, Levin D, Krutzik Peter O, Podoplelova Y, Trejo A, Lee C, Yarden G, Vleck Susan E, Glenn Jeffrey S, Nolan Garry P, Piehler J, Schreiber G, Garcia KC.** 2011. Structural Linkage between Ligand Discrimination and Receptor Activation by Type I Interferons. Cell **146:**621-632.

153. **Schneider WM, Chevillotte MD, Rice CM.** 2014. Interferon-Stimulated Genes: A Complex Web of Host Defenses. Annual Review of Immunology **32:**513-545.

154. **Yan N, Chen ZJ.** 2012. Intrinsic antiviral immunity. Nature immunology **13:**214-222.

155. **Moffatt P, Gaumond MH, Salois P, Sellin K, Bessette MC, Godin É, de Oliveira PT, Atkins GJ, Nanci A, Thomas G.** 2008. Bril: a novel bone‐specific modulator of mineralization. Journal of Bone and Mineral Research **23:**1497-1508.

156. **Siegrist F, Ebeling M, Certa U.** 2011. The small interferon-induced transmembrane genes and proteins. Journal of Interferon & Cytokine Research **31:**183-197.

157. **Bailey CC, Zhong G, Huang I-C, Farzan M.** 2014. IFITM-family proteins: The cell's first line of antiviral defense. Annual review of virology **1:**261.

158. **Lu J, Pan Q, Rong L, Liu S-L, Liang C.** 2011. The IFITM proteins inhibit HIV-1 infection. Journal of virology **85:**2126-2137.

159. **Li K, Markosyan RM, Zheng Y-M, Golfetto O, Bungart B, Li M, Ding S, He Y, Liang C, Lee JC.** 2013. IFITM proteins restrict viral membrane hemifusion. PLoS Pathog **9:**e1003124.

160. **Compton AA, Bruel T, Porrot F, Mallet A, Sachse M, Euvrard M, Liang C, Casartelli N, Schwartz O.** 2014. IFITM proteins incorporated into HIV-1 virions impair viral fusion and spread. Cell host & microbe **16:**736-747.

161. **Yu J, Li M, Wilkins J, Ding S, Swartz TH, Esposito AM, Zheng Y-M, Freed EO, Liang C, Chen BK.** 2015. IFITM proteins restrict HIV-1 infection by antagonizing the envelope glycoprotein. Cell reports **13:**145-156.

162. **Nisole S, Stoye JP, Saïb A.** 2005. TRIM family proteins: retroviral restriction and antiviral defence. Nature Reviews Microbiology **3:**799-808.

163. **Malim MH, Bieniasz PD.** 2012. HIV restriction factors and mechanisms of evasion. Cold Spring Harbor perspectives in medicine **2:**a006940.

164. **Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J.** 2004. The cytoplasmic body component TRIM5α restricts HIV-1 infection in Old World monkeys. Nature **427:**848-853.

165. **Hatziioannou T, Princiotta M, Piatak M, Yuan F, Zhang F, Lifson JD, Bieniasz PD.** 2006. Generation of simian-tropic HIV-1 by restriction factor evasion. Science **314:**95-95.

166. **Hatziioannou T, Perez-Caballero D, Yang A, Cowan S, Bieniasz PD.** 2004. Retrovirus resistance factors Ref1 and Lv1 are species-specific variants of TRIM5α. Proceedings of the National Academy of Sciences of the United States of America **101:**10774-10779.

167. **Laguette N, Sobhian B, Casartelli N, Ringeard M, Chable-Bessia C, Ségéral E, Yatim A, Emiliani S, Schwartz O, Benkirane M.** 2011. SAMHD1 is the dendritic-and myeloid-cell-specific HIV-1 restriction factor counteracted by Vpx. Nature **474:**654-657.

168. **Hrecka K, Hao C, Gierszewska M, Swanson SK, Kesik-Brodacka M, Srivastava S, Florens L, Washburn MP, Skowronski J.** 2011. Vpx relieves inhibition of HIV-1 infection of macrophages mediated by the SAMHD1 protein. Nature **474:**658-661.

169. **Laguette N, Benkirane M.** 2012. How SAMHD1 changes our view of viral restriction. Trends in immunology **33:**26-33.

170. **Goldstone DC, Ennis-Adeniran V, Hedden JJ, Groom HC, Rice GI, Christodoulou E, Walker PA, Kelly G, Haire LF, Yap MW.** 2011. HIV-1 restriction factor SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase. Nature **480:**379-382.

171. **Ryoo J, Choi J, Oh C, Kim S, Seo M, Kim S-Y, Seo D, Kim J, White TE, Brandariz-Nuñez A.** 2014. The ribonuclease activity of SAMHD1 is required for HIV-1 restriction. Nature medicine **20:**936-941.

172. **Sheehy AM, Gaddis NC, Choi JD, Malim MH.** 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. Nature **418:**646-650.

173. **Bogerd HP, Cullen BR.** 2008. Single-stranded RNA facilitates nucleocapsid: APOBEC3G complex formation. Rna **14:**1228-1236.

174. **Yu Q, König R, Pillai S, Chiles K, Kearney M, Palmer S, Richman D, Coffin JM, Landau NR.** 2004. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. Nature structural & molecular biology **11:**435-442.

175. **Bishop KN, Verma M, Kim E-Y, Wolinsky SM, Malim MH.** 2008. APOBEC3G inhibits elongation of HIV-1 reverse transcripts. PLoS Pathog **4:**e1000231.

176. **Kim B-H, Shenoy AR, Kumar P, Bradfield CJ, MacMicking JD.** 2012. IFN-inducible GTPases in host cell defense. Cell host & microbe **12:**432-444.

177. **Haller O, Kochs G.** 2011. Human MxA protein: an interferon-induced dynamin-like GTPase with broad antiviral activity. Journal of Interferon & Cytokine Research **31:**79-87.

178. **Kane M, Yadav SS, Bitzegeio J, Kutluay SB, Zang T, Wilson SJ, Schoggins JW, Rice CM, Yamashita M, Hatziioannou T.** 2013. MX2 is an interferon-induced inhibitor of HIV-1 infection. Nature **502:**563-566.

179. **Goujon C, Moncorgé O, Bauby H, Doyle T, Ward CC, Schaller T, Hué S, Barclay WS, Schulz R, Malim MH.** 2013. Human MX2 is an interferon-induced post-entry inhibitor of HIV-1 infection. Nature **502:**559-562.

180. **Mavrommatis E, Fish EN, Platanias LC.** 2013. The schlafen family of proteins and their regulation by interferons. Journal of Interferon & Cytokine Research **33:**206-210.

181. **Li M, Kao E, Gao X, Sandig H, Limmer K, Pavon-Eternod M, Jones TE, Landry S, Pan T, Weitzman MD.** 2012. Codon-usage-based inhibition of HIV protein synthesis by human schlafen 11. Nature **491:**125-128.

182. **Abdel-Mohsen M, Raposo RA, Deng X, Li M, Liegler T, Sinclair E, Salama MS, Ghanem HE-d, Hoh R, Wong JK.** 2013. Expression profile of host restriction factors in HIV-1 elite controllers. Retrovirology **10:**106.

183. **Altfeld M, Gale Jr M.** 2015. Innate immunity against HIV-1 infection. Nature immunology **16:**554-562.

184. **Kupzig S, Korolchuk V, Rollason R, Sugden A, Wilde A, Banting G.** 2003. Bst‐2/HM1. 24 is a raft‐associated apical membrane protein with an unusual topology. Traffic **4:**694-709.

185. **Evans DT, Serra-Moreno R, Singh RK, Guatelli JC.** 2010. BST-2/tetherin: a new component of the innate immune response to enveloped viruses. Trends in microbiology **18:**388-396.

186. **Habermann A, Krijnse-Locker J, Oberwinkler H, Eckhardt M, Homann S, Andrew A, Strebel K, Kräusslich H-G.** 2010. CD317/tetherin is enriched in the HIV-1 envelope and downregulated from the plasma membrane upon virus infection. Journal of virology **84:**4646-4658.

187. **Perez-Caballero D, Zang T, Ebrahimi A, McNatt MW, Gregory DA, Johnson MC, Bieniasz PD.** 2009. Tetherin inhibits HIV-1 release by directly tethering virions to cells. Cell **139:**499-511.

188. **Giese S, Marsh M.** 2014. Tetherin can restrict cell-free and cell-cell transmission of HIV from primary macrophages to T cells. PLoS Pathog **10:**e1004189.

189. **Fackler OT.** 2015. Spotlight on HIV-1 Nef: SERINC3 and SERINC5 Identified as Restriction Factors Antagonized by the Pathogenesis Factor. Viruses **7:**6730-6738.

190. **Inuzuka M, Hayakawa M, Ingi T.** 2005. Serinc, an Activity-regulated Protein Family, Incorporates Serine into Membrane Lipid Synthesis. Journal of Biological Chemistry **280:**35776-35783.

191. **Rosa A, Chande A, Ziglio S, De Sanctis V, Bertorelli R, Goh SL, McCauley SM, Nowosielska A, Antonarakis SE, Luban J, Santoni FA, Pizzato M.** 2015. HIV-1 Nef promotes infection by excluding SERINC5 from virion incorporation. Nature **526:**212-217.

192. **Usami Y, Wu Y, Gottlinger HG.** 2015. SERINC3 and SERINC5 restrict HIV-1 infectivity and are counteracted by Nef. Nature **526:**218-223.

193. **Malim MH, Emerman M.** 2008. HIV-1 accessory proteins—ensuring viral survival in a hostile environment. Cell host & microbe **3:**388-398.

194. **Neil SJ, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425-430.

195. **Jin Y-J, Cai CY, Zhang X, Zhang H-T, Hirst JA, Burakoff SJ.** 2005. HIV Nef-mediated CD4 down-regulation is adaptor protein complex 2 dependent. The Journal of Immunology **175:**3157-3164.

196. **Stove V, Van de Walle I, Naessens E, Coene E, Stove C, Plum J, Verhasselt B.** 2005. Human immunodeficiency virus Nef induces rapid internalization of the T-cell coreceptor CD8αβ. Journal of virology **79:**11422-11433.

197. **Swigut T, Shohdy N, Skowronski J.** 2001. Mechanism for down‐regulation of CD28 by Nef. The EMBO journal **20:**1593-1604.

198. **Chaudhry A, Das SR, Jameel S, George A, Bal V, Mayor S, Rath S.** 2007. A two-pronged mechanism for HIV-1 Nef-mediated endocytosis of immune costimulatory molecules CD80 and CD86. Cell host & microbe **1:**37-49.

199. **Atkins KM, Thomas L, Youker RT, Harriff MJ, Pissani F, You H, Thomas G.** 2008. HIV-1 Nef Binds PACS-2 to Assemble a Multikinase Cascade That Triggers Major Histocompatibility Complex Class I (MHC-I) Down-regulation ANALYSIS USING SHORT INTERFERING RNA AND KNOCK-OUT MICE. Journal of Biological Chemistry **283:**11772-11784.

200. **Collins KL, Chen BK, Kalams SA, Walker BD, Baltimore D.** 1998. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. Nature **391:**397-401.

201. **Lama J, Mangasarian A, Trono D.** 1999. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef-and Vpu-inhibitable manner. Current Biology **9:**622-631.

202. **Kestier HW, Ringler DJ, Mori K, Panicali DL, Sehgal PK, Daniel MD, Desrosiers RC.** 1991. Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell **65:**651-662.

203. **Gorry PR, McPhee DA, Verity E, Dyer WB, Wesselingh SL, Learmont J, Sullivan JS, Roche M, Zaunders JJ, Gabuzda D.** 2007. Pathogenicity and immunogenicity of attenuated, nef-deleted HIV-1 strains in vivo. Retrovirology **4:**1.

204. **Kluge Silvia F, Mack K, Iyer Shilpa S, Pujol François M, Heigele A, Learn Gerald H, Usmani Shariq M, Sauter D, Joas S, Hotter D, Bibollet-Ruche F, Plenderleith Lindsey J, Peeters M, Geyer M, Sharp Paul M, Fackler Oliver T, Hahn Beatrice H, Kirchhoff F.** 2014. Nef Proteins of Epidemic HIV-1 Group O Strains Antagonize Human Tetherin. Cell Host & Microbe **16:**639-650.

205. **Zhang F, Wilson SJ, Landford WC, Virgen B, Gregory D, Johnson MC, Munch J, Kirchhoff F, Bieniasz PD, Hatziioannou T.** 2009. Nef proteins from simian immunodeficiency viruses are tetherin antagonists. Cell host & microbe **6:**54-67.

206. **Zhang F, Landford WN, Ng M, McNatt MW, Bieniasz PD, Hatziioannou T.** 2011. SIV Nef Proteins Recruit the AP-2 Complex to Antagonize Tetherin and Facilitate Virion Release. PLoS Pathog **7:**e1002039.

207. **Fisher AG, Ensoli B, Ivanoff L, Chamberlain M, Petteway S, Ratner L, Gallo RC, Wong-Staal F.** 1987. The sor gene of HIV-1 is required for efficient virus transmission in vitro. Science **237:**888-893.

208. **Yu X, Yu Y, Liu B, Luo K, Kong W, Mao P, Yu X-F.** 2003. Induction of APOBEC3G Ubiquitination and Degradation by an HIV-1 Vif-Cul5-SCF Complex. Science **302:**1056-1060.

209. **Stopak K, de Noronha C, Yonemoto W, Greene WC.** 2003. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. Molecular cell **12:**591-601.

210. **Frankel AD, Young JA.** 1998. HIV-1: fifteen proteins and an RNA. Annual review of biochemistry **67:**1-25.

211. **Margottin F, Bour SP, Durand H, Selig L, Benichou S, Richard V, Thomas D, Strebel K, Benarous R.** 1998. A novel human WD protein, h-βTrCP, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Molecular cell **1:**565-574.

212. **Kerkau T, Bacik I, Bennink JR, Yewdell JW, Hünig T, Schimpl A, Schubert U.** 1997. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step

in the biosynthesis of major histocompatibility complex (MHC) class I molecules. The Journal of experimental medicine **185:**1295-1306.

213. **Dubé M, Roy BB, Guiot-Guillain P, Binette J, Mercier J, Chiasson A, Cohen ÉA.** 2010. Antagonism of tetherin restriction of HIV-1 release by Vpu involves binding and sequestration of the restriction factor in a perinuclear compartment. PLoS Pathog **6:**e1000856.

214. **Mitchell RS, Katsura C, Skasko MA, Fitzpatrick K, Lau D, Ruiz A, Stephens EB, Margottin-Goguet F, Benarous R, Guatelli JC.** 2009. Vpu antagonizes BST-2–mediated restriction of HIV-1 release via β-TrCP and endo-lysosomal trafficking. PLoS Pathog **5:**e1000450.

215. **Neil SJ.** 2013. The antiviral activities of tetherin, p 67-104, Intrinsic Immunity. Springer.

216. **Cohen EA, Terwilliger EF, Jalinoos Y, Proulx J, Sodroski JG, Haseltine WA.** 1990. Identification of HIV-1 vpr product and function. JAIDS Journal of Acquired Immune Deficiency Syndromes **3:**11-18.

217. **Wen X, Duus KM, Friedrich TD, de Noronha CM.** 2007. The HIV1 protein Vpr acts to promote G2 cell cycle arrest by engaging a DDB1 and Cullin4A-containing ubiquitin ligase complex using VprBP/DCAF1 as an adaptor. Journal of Biological Chemistry **282:**27046-27057.

218. **Le Rouzic E, Benichou S.** 2005. The Vpr protein from HIV-1: distinct roles along the viral life cycle. Retrovirology **2:**11.

219. **Mashiba M, Collins DR, Terry VH, Collins KL.** 2014. Vpr overcomes macrophage-specific restriction of HIV-1 Env expression and virion production. Cell host & microbe **16:**722-735.

220. **McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, Freeman GJ, Umetsu DT, DeKruyff RH.** 2001. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. Nature immunology **2:**1109-1116.

221. **McIntire JJ, Umetsu DT, DeKruyff RH.** TIM-1, a novel allergy and asthma susceptibility gene, p 335-348. *In* (ed), Springer,

222. **Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH.** 2010. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. Immunological reviews **235:**172-189.

223. **Santiago C, Ballesteros A, Martinez-Muñoz L, Mellado M, Kaplan GG, Freeman GJ, Casasnovas JM.** 2007. Structures of T cell immunoglobulin mucin protein 4 show a metal-Ion-dependent ligand binding site where phosphatidylserine binds. Immunity **27:**941-951.

224. **Leventis PA, Grinstein S.** 2010. The Distribution and Function of Phosphatidylserine in Cellular Membranes. Annual Review of Biophysics **39:**407-427.

225. **Marino G, Kroemer G.** 2013. Mechanisms of apoptotic phosphatidylserine exposure. Cell Res **23:**1247-1248.

226. **DeKruyff RH, Bu X, Ballesteros A, Santiago C, Chim Y-LE, Lee H-H, Karisola P, Pichavant M, Kaplan GG, Umetsu DT.** 2010. T cell/transmembrane, Ig, and mucin-3 allelic variants differentially recognize phosphatidylserine and mediate phagocytosis of apoptotic cells. The journal of immunology **184:**1918-1930.

227. **Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S.** 2007. Identification of Tim4 as a phosphatidylserine receptor. Nature **450:**435-439.

228. **Umetsu SE, Lee W-L, McIntire JJ, Downey L, Sanjanwala B, Akbari O, Berry GJ, Nagumo H, Freeman GJ, Umetsu DT.** 2005. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. Nature immunology **6:**447-454.

229. **Ichimura T, Bonventre JV, Bailly V, Wei H, Hession CA, Cate RL, Sanicola M.** 1998. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. Journal of Biological Chemistry **273:**4135-4142.

230. **Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK.** 2005. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nature immunology **6:**1245-1252.

231. **Nakayama M, Akiba H, Takeda K, Kojima Y, Hashiguchi M, Azuma M, Yagita H, Okumura K.** 2009. Tim-3 mediates phagocytosis of apoptotic cells and cross-presentation. Blood **113:**3821-3830.

232. **Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, Zheng XX, Strom TB, Hafler DA, Kuchroo VK.** 2009. TIM‐3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. European journal of immunology **39:**2492-2501.

233. **Soo Hoo W, Jensen E, Saadat A, Nieto D, Moss R, Carlo D, Moll T.** 2006. Vaccination with cell immunoglobulin mucin‐1 antibodies and inactivated influenza enhances vaccine‐specific lymphocyte proliferation, interferon‐γ production and cross‐strain reactivity. Clinical & Experimental Immunology **145:**123-129.

234. **Ahmadi K, Lanchbury J, Reed P, Chiano M, Thompson D, Galley M, Line A, Lank E, Wong H, Strachan D.** 2003. Novel association suggests multiple independent QTLs within chromosome 5q21–33 region control variation in total humans IgE levels. Genes and immunity **4:**289-297.

235. **Sizing ID, Bailly V, McCoon P, Chang W, Rao S, Pablo L, Rennard R, Walsh M, Li Z, Zafari M.** 2007. Epitope-dependent effect of anti-murine TIM-1 monoclonal antibodies on T cell activity and lung immune responses. The Journal of Immunology **178:**2249-2261.

236. **Degauque N, Mariat C, Kenny J, Zhang D, Gao W, Vu MD, Alexopoulos S, Oukka M, Umetsu DT, DeKruyff RH.** 2008. Immunostimulatory Tim-1–specific antibody

deprograms Tregs and prevents transplant tolerance in mice. The Journal of clinical investigation **118:**735-741.

237. **Ueno T, Habicht A, Clarkson MR, Albin MJ, Yamaura K, Boenisch O, Popoola J, Wang Y, Yagita H, Akiba H.** 2008. The emerging role of T cell Ig mucin 1 in alloimmune responses in an experimental mouse transplant model. The Journal of clinical investigation **118:**742-751.

238. **Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV.** 2008. Kidney injury molecule–1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. The Journal of clinical investigation **118:**1657-1668.

239. **Han WK, Alinani A, Wu C-L, Michaelson D, Loda M, McGovern FJ, Thadhani R, Bonventre JV.** 2005. Human kidney injury molecule-1 is a tissue and urinary tumor marker of renal cell carcinoma. Journal of the American Society of Nephrology **16:**1126-1134.

240. **Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone S.** 1996. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. The EMBO journal **15:**4282.

241. **Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA.** 2002. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature **415:**536-541.

242. **Sabatos CA, Chakravarti S, Cha E, Schubart A, Sánchez-Fueyo A, Zheng XX, Coyle AJ, Strom TB, Freeman GJ, Kuchroo VK.** 2003. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. Nature immunology **4:**1102-1110.

243. **Park D, Hochreiter-Hufford A, Ravichandran KS.** 2009. The phosphatidylserine receptor TIM-4 does not mediate direct signaling. Current Biology **19:**346-351.

244. **Feigelstock D, Thompson P, Mattoo P, Zhang Y, Kaplan GG.** 1998. The human homolog of HAVcr-1 codes for a hepatitis A virus cellular receptor. Journal of virology **72:**6621-6628.

245. **Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL, Moller-Tank S, Meyerholz DK, Rennert P, Mullins RF, Brindley M.** 2011. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. Proceedings of the National Academy of Sciences **108:**8426-8431.

246. **Meertens L, Carnec X, Lecoin MP, Ramdasi R, Guivel-Benhassine F, Lew E, Lemke G, Schwartz O, Amara A.** 2012. The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. Cell host & microbe **12:**544-557.

247. **Jemielity S, Wang JJ, Chan YK, Ahmed AA, Li W, Monahan S, Bu X, Farzan M, Freeman GJ, Umetsu DT.** 2013. TIM-family proteins promote infection of multiple enveloped viruses through virion-associated phosphatidylserine. PLoS pathog **9:**e1003232.

248. **McIntire JJ, Umetsu SE, Macaubas C, Hoyte EG, Cinnioglu C, Cavalli-Sforza LL, Barsh GS, Hallmayer JF, Underhill PA, Risch NJ.** 2003. Immunology: hepatitis A virus link to atopic disease. Nature **425:**576-576.

249. **Wichukchinda N, Nakajima T, Saipradit N, Nakayama EE, Ohtani H, Rojanawiwat A, Pathipvanich P, Ariyoshi K, Sawanpanyalert P, Shioda T.** 2010. TIM1 haplotype may control the disease progression to AIDS in a HIV-1-infected female cohort in Thailand. AIDS **24:**1625-1631.

250. **Jin H-T, Anderson AC, Tan WG, West EE, Ha S-J, Araki K, Freeman GJ, Kuchroo VK, Ahmed R.** 2010. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. Proceedings of the National Academy of Sciences **107:**14733-14738.

251. **Jones RB, Ndhlovu LC, Barbour JD, Sheth PM, Jha AR, Long BR, Wong JC, Satkunarajah M, Schweneker M, Chapman JM.** 2008. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. The Journal of experimental medicine **205:**2763-2779.

252. **Elahi S, Niki T, Hirashima M, Horton H.** 2012. Galectin-9 binding to Tim-3 renders activated human CD4+ T cells less susceptible to HIV-1 infection. Blood **119:**4192-4204.

253. **Chen H, Li C, Huang J, Cung T, Seiss K, Beamon J, Carrington MF, Porter LC, Burke PS, Yang Y.** 2011. CD4+ T cells from elite controllers resist HIV-1 infection by selective upregulation of p21. The Journal of clinical investigation **121:**1549-1560.

254. **Kuchroo VK, Umetsu DT, DeKruyff RH, Freeman GJ.** 2003. The TIM gene family: emerging roles in immunity and disease. Nat Rev Immunol **3:**454-462.

255. **Freeman GJ, Casasnovas JM, Umetsu DT, DeKruyff RH.** 2010. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. Immunol Rev **235:**172-189.

256. **Chae SC, Song JH, Heo JC, Lee YC, Kim JW, Chung HT.** 2003. Molecular variations in the promoter and coding regions of human Tim-1 gene and their association in Koreans with asthma. Hum Immunol **64:**1177-1182.

257. **Umetsu DT, Umetsu SE, Freeman GJ, DeKruyff RH.** 2008. TIM gene family and their role in atopic diseases. Curr Top Microbiol Immunol **321:**201-215.

258. **McIntire JJ, Umetsu SE, Macaubas C, Hoyte EG, Cinnioglu C, Cavalli-Sforza LL, Barsh GS, Hallmayer JF, Underhill PA, Risch NJ, Freeman GJ, DeKruyff RH, Umetsu DT.** 2003. Immunology: hepatitis A virus link to atopic disease. Nature **425:**576.

259. **Kaplan G, Totsuka A, Thompson P, Akatsuka T, Moritsugu Y, Feinstone SM.** 1996. Identification of a surface glycoprotein on African green monkey kidney cells as a receptor for hepatitis A virus. EMBO J **15:**4282-4296.

260. **Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL, Moller-Tank S, Meyerholz DK, Rennert P, Mullins RF, Brindley M, Sandersfeld LM, Quinn K, Weller M, McCray PB, Jr., Chiorini J, Maury W.** 2011. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. Proc Natl Acad Sci U S A **108:**8426-8431.

261. **Meertens L, Carnec X, Lecoin MP, Ramdasi R, Guivel-Benhassine F, Lew E, Lemke G, Schwartz O, Amara A.** 2012. The TIM and TAM families of phosphatidylserine receptors mediate dengue virus entry. Cell Host Microbe **12:**544-557.

262. **Kim HY, Eyheramonho MB, Pichavant M, Gonzalez Cambaceres C, Matangkasombut P, Cervio G, Kuperman S, Moreiro R, Konduru K, Manangeeswaran M, Freeman GJ, Kaplan GG, DeKruyff RH, Umetsu DT, Rosenzweig SD.** 2011. A polymorphism in TIM1 is associated with susceptibility to severe hepatitis A virus infection in humans. J Clin Invest **121:**1111-1118.

263. **Moller-Tank S, Kondratowicz AS, Davey RA, Rennert PD, Maury W.** 2013. Role of the phosphatidylserine receptor TIM-1 in enveloped-virus entry. J Virol **87:**8327-8341.

264. **Jemielity S, Wang JJ, Chan YK, Ahmed AA, Li W, Monahan S, Bu X, Farzan M, Freeman GJ, Umetsu DT, Dekruyff RH, Choe H.** 2013. TIM-family proteins promote infection of multiple enveloped viruses through virion-associated phosphatidylserine. PLoS Pathog **9:**e1003232.

265. **Rennert PD.** 2011. Novel roles for TIM-1 in immunity and infection. Immunol Lett **141:**28-35.

266. **Umetsu SE, Lee WL, McIntire JJ, Downey L, Sanjanwala B, Akbari O, Berry GJ, Nagumo H, Freeman GJ, Umetsu DT, DeKruyff RH.** 2005. TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. Nat Immunol **6:**447-454.

267. **Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK.** 2002. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. Nature **415:**536-541.

268. **Kobayashi N, Karisola P, Pena-Cruz V, Dorfman DM, Jinushi M, Umetsu SE, Butte MJ, Nagumo H, Chernova I, Zhu B, Sharpe AH, Ito S, Dranoff G, Kaplan GG, Casasnovas JM, Umetsu DT, Dekruyff RH, Freeman GJ.** 2007. TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. Immunity **27:**927-940.

269. **Miyanishi M, Tada K, Koike M, Uchiyama Y, Kitamura T, Nagata S.** 2007. Identification of Tim4 as a phosphatidylserine receptor. Nature **450:**435-439.

270. **McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, Freeman GJ, Umetsu DT, DeKruyff RH.** 2001. Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. Nat Immunol **2:**1109-1116.

271. **Jost S, Moreno-Nieves UY, Garcia-Beltran WF, Rands K, Reardon J, Toth I, Piechocka-Trocha A, Altfeld M, Addo MM.** 2013. Dysregulated Tim-3 expression on natural killer cells is associated with increased Galectin-9 levels in HIV-1 infection. Retrovirology **10:**74.

272. **Joshi A, Ablan SD, Soheilian F, Nagashima K, Freed EO.** 2009. Evidence that productive human immunodeficiency virus type 1 assembly can occur in an intracellular compartment. J Virol **83:**5375-5387.

273. **Ono A, Ablan SD, Lockett SJ, Nagashima K, Freed EO.** 2004. Phosphatidylinositol (4,5) bisphosphate regulates HIV-1 Gag targeting to the plasma membrane. Proc Natl Acad Sci U S A **101:**14889-14894.

274. **Ono A, Waheed AA, Freed EO.** 2007. Depletion of cellular cholesterol inhibits membrane binding and higher-order multimerization of human immunodeficiency virus type 1 Gag. Virology **360:**27-35.

275. **Côté M, Zheng YM, Albritton LM, Liu S-L.** 2008. Fusogenicity of Jaagsiekte sheep retrovirus envelope protein is dependent on low pH and is enhanced by cytoplasmic tail truncations. J Virol **82:**2543-2554.

276. **Ono A, Freed EO.** 2004. Cell-type-dependent targeting of human immunodeficiency virus type 1 assembly to the plasma membrane and the multivesicular body. J Virol **78:**1552-1563.

277. **Freed EO, Englund G, Martin MA.** 1995. Role of the basic domain of human immunodeficiency virus type 1 matrix in macrophage infection. J Virol **69:**3949-3954.

278. **Waheed AA, Ono A, Freed EO.** 2009. Methods for the study of HIV-1 assembly. Methods Mol Biol **485:**163-184.

279. **Li K, Markosyan RM, Zheng Y-M, Golfetto O, Bungart B, Li M, Liang C, He Y, Lee JC, Gratton E, Cohen FS, Liu S-L.** 2013. IFITM Proteins Restrict Viral Membrane Hemifusion. PLoS Pathog **9:**e1003124.

280. **Côté M, Zheng YM, Li K, Xiang S-H, Albritton LM, Liu S-L.** 2012. Critical Role of a Leucine-Valine Change in the Distinct Low pH Requirements for Membrane Fusion between Two Related Retrovirus Envelope. J Biol Chem **287:**7640-7651.

281. **Joshi A, Garg H, Ablan SD, Freed EO.** 2011. Evidence of a role for soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) machinery in HIV-1 assembly and release. J Biol Chem **286:**29861-29871.

282. **Huang M, Orenstein JM, Martin MA, Freed EO.** 1995. p6Gag is required for particle production from full-length human immunodeficiency virus type 1 molecular clones expressing protease. J Virol **69:**6810-6818.

283. **Neil SJ, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425-430.

284. **Van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R, Johnson MC, Stephens EB, Guatelli J.** 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell Host Microbe **3:**245-252.

285. **Fitzpatrick K, Skasko M, Deerinck TJ, Crum J, Ellisman MH, Guatelli J.** 2010. Direct restriction of virus release and incorporation of the interferon-induced protein BST-2 into HIV-1 particles. PLoS Pathog **6:**e1000701.

286. **Santiago C, Ballesteros A, Martinez-Munoz L, Mellado M, Kaplan GG, Freeman GJ, Casasnovas JM.** 2007. Structures of T cell immunoglobulin mucin protein 4 show a metal-Ion-dependent ligand binding site where phosphatidylserine binds. Immunity **27:**941-951.

287. **Chukkapalli V, Inlora J, Todd GC, Ono A.** 2013. Evidence in support of RNA-mediated inhibition of phosphatidylserine-dependent HIV-1 Gag membrane binding in cells. J Virol **87:**7155-7159.

288. **Vlach J, Saad JS.** 2013. Trio engagement via plasma membrane phospholipids and the myristoyl moiety governs HIV-1 matrix binding to bilayers. Proc Natl Acad Sci U S A **110:**3525-3530.

289. **Alfadhli A, Huseby D, Kapit E, Colman D, Barklis E.** 2007. Human immunodeficiency virus type 1 matrix protein assembles on membranes as a hexamer. J Virol **81:**1472-1478.

290. **Dick RA, Goh SL, Feigenson GW, Vogt VM.** 2012. HIV-1 Gag protein can sense the cholesterol and acyl chain environment in model membranes. Proc Natl Acad Sci U S A **109:**18761-18766.

291. **Binne LL, Scott ML, Rennert PD.** 2007. Human TIM-1 associates with the TCR complex and up-regulates T cell activation signals. J Immunol **178:**4342-4350.

292. **Mercer J, Helenius A.** 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. Science **320:**531-535.

293. **Soares MM, King SW, Thorpe PE.** 2008. Targeting inside-out phosphatidylserine as a therapeutic strategy for viral diseases. Nat Med **14:**1357-1362.

294. **Callahan MK, Popernack PM, Tsutsui S, Truong L, Schlegel RA, Henderson AJ.** 2003. Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. J Immunol **170:**4840-4845.

295. **Freed EO, Delwart EL, Buchschacher GL, Jr., Panganiban AT.** 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. Proc Natl Acad Sci U S A **89:**70-74.

296. **Bhattacharyya S, Zagorska A, Lew ED, Shrestha B, Rothlin CV, Naughton J, Diamond MS, Lemke G, Young JA.** 2013. Enveloped viruses disable innate immune responses in dendritic cells by direct activation of TAM receptors. Cell Host Microbe **14:**136-147.

297. **Morizono K, Xie Y, Olafsen T, Lee B, Dasgupta A, Wu AM, Chen IS.** 2011. The soluble serum protein Gas6 bridges virion envelope phosphatidylserine to the TAM receptor tyrosine kinase Axl to mediate viral entry. Cell Host Microbe **9:**286-298.

298. **Morizono K, Chen IS.** 2014. Role of phosphatidylserine receptors in enveloped virus infection. Journal of virology **88:**4275-4290.

299. **Malim MH, Bieniasz PD.** 2012. HIV Restriction Factors and Mechanisms of Evasion. Cold Spring Harb Perspect Med **2:**a006940.

300. **Lim ES, Wu LI, Malik HS, Emerman M.** 2012. The function and evolution of the restriction factor Viperin in primates was not driven by lentiviruses. Retrovirology **9:**55.

301. **Venkatesh S, Bieniasz PD.** 2013. Mechanism of HIV-1 virion entrapment by tetherin. PLoS Pathog **9:**e1003483.

302. **Santiago C, Ballesteros A, Tami C, Martinez-Munoz L, Kaplan GG, Casasnovas JM.** 2007. Structures of T Cell immunoglobulin mucin receptors 1 and 2 reveal mechanisms for regulation of immune responses by the TIM receptor family. Immunity **26:**299-310.

303. **Cao E, Zang X, Ramagopal UA, Mukhopadhaya A, Fedorov A, Fedorov E, Zencheck WD, Lary JW, Cole JL, Deng H, Xiao H, Dilorenzo TP, Allison JP, Nathenson SG, Almo SC.** 2007. T cell immunoglobulin mucin-3 crystal structure reveals a galectin-9-independent ligand-binding surface. Immunity **26:**311-321.

304. **Ohtani H, Naruse TK, Iwasaki Y, Akari H, Ishida T, Matano T, Kimura A.** 2012. Lineage-specific evolution of T-cell immunoglobulin and mucin domain 1 gene in the primates. Immunogenetics **64:**669-678.

305. **Sharma G, Ohtani H, Kaur G, Naruse TK, Sharma SK, Vajpayee M, Kimura A, Mehra N.** 2013. Status of TIM-1 exon 4 haplotypes and CD4+T cell counts in HIV-1 seroprevalent North Indians. Hum Immunol **74:**163-165.

306. **Chen JP, Zhao WL, He NH, Gui Q, Xiong JP, Zhou HM, Wang Y, Chen S, Zhou P.** 2012. Association of Hepatitis A exposure and TIM-1 with childhood allergic asthma. J Asthma **49:**697-702.

307. **Neil SJD, Zang T, Bieniasz PD.** 2008. Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. Nature **451:**425-430.

308. **Van Damme N, Goff D, Katsura C, Jorgenson RL, Mitchell R, Johnson MC, Stephens EB, Guatelli J.** 2008. The interferon-induced protein BST-2 restricts HIV-1 release and is downregulated from the cell surface by the viral Vpu protein. Cell host & microbe **3:**245-252.

309. **Kondratowicz AS, Lennemann NJ, Sinn PL, Davey RA, Hunt CL, Moller-Tank S, Meyerholz DK, Rennert P, Mullins RF, Brindley M, Sandersfeld LM, Quinn K, Weller M, McCray PB, Chiorini J, Maury W.** 2011. T-cell immunoglobulin and mucin domain 1 (TIM-1) is a receptor for Zaire Ebolavirus and Lake Victoria Marburgvirus. Proceedings of the National Academy of Sciences **108:**8426-8431.

310. **Li M, Ablan SD, Miao C, Zheng Y-M, Fuller MS, Rennert PD, Maury W, Johnson MC, Freed EO, Liu S-L.** 2014. TIM-family proteins inhibit HIV-1 release. Proceedings of the National Academy of Sciences **111:**E3699-E3707.

311. **Hastings WD, Anderson DE, Kassam N, Koguchi K, Greenfield EA, Kent SC, Zheng XX, Strom TB, Hafler DA, Kuchroo VK.** 2009. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. European Journal of Immunology **39:**2492-2501.

312. **El-Andaloussi S, Lee Y, Lakhal-Littleton S, Li J, Seow Y, Gardiner C, Alvarez-Erviti L, Sargent IL, Wood MJ.** 2012. Exosome-mediated delivery of siRNA in vitro and in vivo. Nature protocols **7:**2112-2126.

313. **McNatt MW, Zang T, Bieniasz PD.** 2013. Vpu Binds Directly to Tetherin and Displaces It from Nascent Virions. PLoS Pathog **9:**e1003299.

314. **Le Tortorec A, Neil SJD.** 2009. Antagonism to and Intracellular Sequestration of Human Tetherin by the Human Immunodeficiency Virus Type 2 Envelope Glycoprotein. Journal of Virology **83:**11966-11978.

315. **Gupta RK, Mlcochova P, Pelchen-Matthews A, Petit SJ, Mattiuzzo G, Pillay D, Takeuchi Y, Marsh M, Towers GJ.** 2009. Simian immunodeficiency virus envelope glycoprotein counteracts tetherin/BST-2/CD317 by intracellular sequestration. Proceedings of the National Academy of Sciences **106:**20889-20894.

316. **Doyle T, Goujon C, Malim MH.** 2015. HIV-1 and interferons: who's interfering with whom? Nat Rev Micro **13:**403-413.

317. **Peeters M, D'Arc M, Delaporte E.** 2014. The origin and diversity of human retroviruses. AIDS reviews **16:**23.

318. **Deacon NJ, Tsykin A, Solomon A, Smith K, Ludford-Menting M, Hooker DJ, McPhee DA, Greenway AL, Ellett A, Chatfield C, Lawson VA, Crowe S, Maerz A, Sonza S, Learmont J, Sullivan JS, Cunningham A, Dwyer D, Dowton D, Mills J.** 1995. Genomic structure of an attenuated quasi species of HIV-1 from a blood transfusion donor and recipients. Science **270:**988-991.

319. **Li M, Ablan SD, Miao C, Zheng YM, Fuller MS, Rennert PD, Maury W, Johnson MC, Freed EO, Liu SL.** 2014. TIM-family proteins inhibit HIV-1 release. Proc Natl Acad Sci U S A **111:**E3699-3707.

320. **Richard AS, Zhang A, Park S-J, Farzan M, Zong M, Choe H.** 2015. Virion-associated phosphatidylethanolamine promotes TIM1-mediated infection by Ebola, dengue, and West Nile viruses. Proceedings of the National Academy of Sciences **112:**14682-14687.

321. **Callahan MK, Popernack PM, Tsutsui S, Truong L, Schlegel RA, Henderson AJ.** 2003. Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. The Journal of Immunology **170:**4840-4845.

322. **Adosraku R, Choi G, Constantinou-Kokotos V, Anderson M, Gibbons W.** 1994. NMR lipid profiles of cells, tissues, and body fluids: proton NMR analysis of human erythrocyte lipids. Journal of lipid research **35:**1925-1931.

323. **Dale BM, Alvarez RA, Chen BK.** 2013. Mechanisms of enhanced HIV spread through T‐cell virological synapses. Immunological reviews **251:**113-124.

324. **Zhong P, Agosto LM, Munro JB, Mothes W.** 2013. Cell-to-cell transmission of viruses. Current opinion in virology **3:**44-50.

325. **Casartelli N, Sourisseau M, Feldmann J, Guivel-Benhassine F, Mallet A, Marcelin A-G, Guatelli J, Schwartz O.** 2010. Tetherin restricts productive HIV-1 cell-to-cell transmission. PLoS Pathog **6:**e1000955.

326. **Puigdomènech I, Casartelli N, Porrot F, Schwartz O.** 2013. SAMHD1 restricts HIV-1 cell-to-cell transmission and limits immune detection in monocyte-derived dendritic cells. Journal of virology **87:**2846-2856.

327. **Mariani R, Chen D, Schröfelbauer B, Navarro F, König R, Bollman B, Münk C, Nymark-McMahon H, Landau NR.** 2003. Species-Specific Exclusion of APOBEC3G from HIV-1 Virions by Vif. Cell **114:**21-31.

328. **Sauter D, Schindler M, Specht A, Landford WN, Münch J, Kim K-A, Votteler J, Schubert U, Bibollet-Ruche F, Keele BF, Takehisa J, Ogando Y, Ochsenbauer C, Kappes JC, Ayouba A, Peeters M, Learn GH, Shaw G, Sharp PM, Bieniasz P, Hahn BH, Hatziioannou T, Kirchhoff F.** 2009. Tetherin-Driven Adaptation of Vpu and Nef Function and the Evolution of Pandemic and Nonpandemic HIV-1 Strains. Cell Host & Microbe **6:**409-421.

329. **Sauter D, Unterweger D, Vogl M, Usmani SM, Heigele A, Kluge SF, Hermkes E, Moll M, Barker E, Peeters M, Learn GH, Bibollet-Ruche F, Fritz JV, Fackler OT, Hahn BH, Kirchhoff F.** 2012. Human Tetherin Exerts Strong Selection Pressure on the HIV-1 Group N Vpu Protein. PLoS Pathog **8:**e1003093.

VITA

Minghua Li was born in December 1981 and grew up in Taiyuan, the capital city of Shanxi Province in China. Inspired by his mother, a doctor in the local hospital, he attended medical school and became a medical student, which was a dream since his childhood. During the five-year medical study, he had learned a great deal of knowledge in medicine and planned to be a doctor as his mother in a hospital. But things changed when SARS emerged as a serious problem in the world, especially in China in early 2003. It was this period of time that he became interested in virology and viral diseases.

After obtaining his medical degree, Minghua joined the Chinese Center for Disease Control and Prevention (China CDC) in 2005 as a staff scientist, working on Arbovirus. In 2009, Minghua obtained a Master's degree in Pathogen Biology from China CDC. Two years later, Minghua joined Dr. Shan-Lu Liu's lab and began his Ph.D. study at University of Missouri (MU). He is expected to graduate from MU in June or July, 2016 with a Ph.D. degree, and continues his training in Dr. Liu's lab as a postdoctoral fellow at The Ohio State University.