Understand the role of HIV-1 envelope glycoprotein in counteracting restriction factor SERINC5

By Saina Beitari

Department of Microbiology and Immunology

McGill University, Montreal, Québec, Canada

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Dedication

I dedicate this thesis to my mother, Susan, who has given up so much of her life so I can pursue my dreams. I am forever in your debt.

Abstract

Despite the success of the antiretroviral therapies in long-term suppression of HIV-1 viremia in the infected individuals, HIV-1 continues to infect two million people every year and causes close to one million deaths. Cure approaches and vaccines are the priorities of HIV research in an effort to end this decades-long pandemic. My research project is dedicated to understand the host restriction mechanisms that have posed a barrier for the cross-species transmission of HIV from non-human primates into humans. The goal is to elucidate the detailed molecular mechanisms and use the knowledge to develop HIV cure. A group of HIV-1 restriction factors inhibiting distinct steps of HIV-1 replication have been identified in the past two decades. In return, HIV-1 encodes accessory proteins to counter these host restriction factors and to warrant viral replication *in vivo*. One of the HIV-1 accessory proteins, Nef, plays a crucial role in HIV-1 pathogenesis. Recent findings reveal that Nef increases viral infectivity by countering a restriction factor called serine incorporator 5 (SERINC5), which belongs to a protein family with 10 transmembrane domains. Studies show that SERINC5 ablates HIV-1 infectivity through incorporating into progeny virions and blocking viral entry. I have discovered that in addition to Nef, HIV-1 envelope (Env) glycoprotein also overcomes SERINC5 inhibition. By testing a large panel of primary HIV-1 Env clones of different subtypes against ectopically expressed SERINC5, I found a high prevalence of SERINC5-resistant HIV-1 strains. These SERINC5-resistant HIV-1 Env proteins do not prevent SERINC5 incorporation into the virus particles. But the virus-bearing SERINC5 protein sensitizes HIV-1 to broadly neutralizing antibodies that target the membrane proximal external region (MPER) of Env. This finding explains the necessity of Nef-mediated removal of SERINC5 from HIV-1 particles so that HIV-1 can protect

itself from the MPER-targeting antibodies. I further tested the sensitivity of HIV-1 Env clones to the interferon-inducible transmembrane protein 3 (IFITM3) which also inhibits HIV-1 entry. In contrast to the resistance of primary HIV-1 Env clones to SERINC5, the Env clones from the chronic stage are sensitive to IFITM3 whereas the Env clones from the transmitted/founder HIV-1 are resistant. This suggests that IFITM3 and SERINC5 exert different levels of pressures on HIV-1 during the course of HIV-1 infection. Interestingly, I observed that SERINC5 incorporation into HIV-1 virions render particles more sensitive to entry inhibitors such as CD4 mimetic peptide M48U1.

Together, I have discovered the role of HIV-1 Env protein in countering SERINC5 restriction, observed that SERINC5 and IFITM3 exert differential inhibitory pressures on HIV-1 Env over different stages of HIV-1 progression, and HIV-1 Env uses varied strategies to resist these two restriction factors.

Résumé

Malgré le succès des thérapies antirétrovirales dans la suppression à long terme de la virémie du VIH-1 chez les personnes infectées, le VIH-1 continue d'infecter deux millions de personnes chaque année et provoque près d'un million de décès. Les approches de guérison et les vaccins sont les priorités de la recherche sur le VIH afin de mettre fin à cette pandémie de plusieurs décennies. Mon projet de recherche vise à comprendre les mécanismes de restriction de l'hôte qui ont posé une barrière à la transmission inter-espèces du VIH, des primates non-humains aux humains. Le but est d'élucider les mécanismes moléculaires détaillés et d'utiliser ces connaissances pour développer un traitement contre le VIH. Des facteurs cellulaires de restriction du VIH inhibant des étapes spécifiques de la réplication du virus ont été identifiés au cours des deux dernières décennies. Le VIH-1 code pour des protéines accessoires qui contrent ces facteurs de restriction et garantissent sa réplication virale *in vivo*. L'une de ces protéines accessoires, Nef, joue un rôle crucial dans la pathogenèse du VIH-1. De récentes découvertes ont révélé que Nef augmente l'infectiosité virale en bloquant un nouveau facteur de restriction appelé *serine incorporator 5* (SERINC5) qui fait partie d'une famille de protéines contenant 10 domaines transmembranaires. Des études montrent que SERINC5 élimine l'infectiosité du VIH-1 en s'incorporant dans la descendance des virions et en bloquant l'entrée virale. J'ai découvert qu'en plus de Nef, la glycoprotéine d'enveloppe (Env) du VIH-1, surmonte l'inhibition de SERINC5. En testant un large panel de clones primaires de Env de différents sous-types contre SERINC5 exprimée ectopiquement, j'ai découvert une forte prévalence de souches de VIH-1 résistantes à SERINC5. Ces protéines Env résistantes à SERINC5 n'empêchent pas l'incorporation de SERINC5 dans les particules virales. Cependant, elles le sensibilisent aux anticorps neutralisants à grande

échelle qui ciblent la région externe proximale de la membrane (*membrane proximal external region*; MPER) de Env. Cette découverte explique la nécessité d'éliminer SERINC5 des particules du VIH-1 par l'intermédiaire du Nef afin que le virus puisse se protéger contre les anticorps ciblant le MPER. De plus, j'ai testé la sensibilité des clones Env du VIH-1 à la protéine transmembranaire inductible par l'interféron 3 (IFITM3) qui inhibe également l'entrée du virus. Contrairement à la résistance des clones Env primaires du VIH-1 à SERINC5, les clones Env du stade chronique sont sensibles à IFITM3 tandis que les clones Env du VIH-1 transmetteur/fondateur sont résistants. Cela suggère que IFITM3 et SERINC5 exercent différents niveaux de pression sur le VIH-1 au cours de l'infection virale. De façon intéressante, j'ai observé que l'incorporation de SERINC5 dans les virions du VIH-1 rend les particules plus sensibles aux inhibiteurs d'entrée tels que le peptide M48U1, mime du récepteur CD4.

Ainsi, j'ai découvert et caractérisé le rôle de la protéine Env du VIH-1 contre la restriction de SERINC5. J'ai également observé que SERINC5 et IFITM3 exercent des pressions inhibitrices différentielles sur Env à des stades distincts de la progression du VIH-1, et enfin qu'Env utilise des stratégies variées pour résister à ces deux facteurs de restriction.

Preface

This thesis has been prepared in accordance with the guidance provided by McGill Graduate and Postdoctoral Studies. It is formatted as a manuscript-based (article-based) thesis. The author of this thesis is the first author of all the articles in this thesis. Each chapter of this thesis is linked together with a preface, and the references of each chapter are listed at the end of the thesis.

Chapter 1

This chapter presents a general discussion of HIV-1 discovery, HIV-1 replication cycle, and challenges we are facing in treating and curing HIV-1. In addition, a broad introduction about the role of the innate immunity and, in particular, restriction factors are discussed.

The following manuscripts have been adopted for this thesis:

Chapter 2

Beitari, S., Wang, Y., Liu, SL., Liang, C. HIV-1 envelope glycoprotein at the interface of host restriction and virus evasion. Viruses, 2019. 11(311).

This chapter is based on a published review which emphasizes on the growing body of research on restriction factors targeting HIV-1 Envelope (Env) protein and how HIV-1 Env evades the antiviral activities of these restriction factors

Chapter 3

Beitari, S., Ding, S., Pan, Q., Finzi, A. & Liang, C. *Effect of HIV-1 Env on SERINC5 antagonism*. J. Virol, 2017. 91(4).

This chapter is based on a published manuscript and it is the first study which reported the role of HIV-1 Env in countering SERINC5.

Chapter 4

Beitari, S., Pan, Q., Finzi, A., Liang, C. *Differential pressures of SERINC5 and IFITM3 on*

HIV-1envelope glycoprotein over the course of HIV-1 infection. J. Virol, 2020. Published Online. This chapter is adapted from a published manuscript. It is focused on characterizing the response of HIV-1 Env clones of different stages of HIV infection: transmission, acute, and chronic to the inhibition by SERINC5 and IFITM3. We showed that SERINC5 and IFITM3 exert differential inhibitory pressures on HIV-1 Env over different stages of HIV-1.

Chapter 5

This chapter provides a general discussion and contribution of this research to the current knowledge. In this chapter, remaining outstanding questions about this research is discussed.

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List of Abbreviations

3TC - Lamivudine

6HB - 6 helix bundle

ABC - Abacavir

ADCC - antibody dependent cellular cytotoxicity response

AIDS - Acquired Immune Deficiency Syndrome

APV - Amprenavir

ATV - Atazanavir

AZT - Azidothymidine

BST-2 - Bone marrow stromal antigen-2

CA - Capsid

CD4mc - CD4 mimetic

cPPT - Central PPT

CRF - Circulating recombinant forms

CRM1 - Chromosomal maintenance 1

CTD - C terminal domain

CTL - Cytotoxic T cells

d4T - Stadvudine

DC - dendritic cells

DC-SIGN - Dendritic cell specific intercellular adhesion molecule 3 grabbing

dCA - Didehydro-cortistatin A

ddI - Didanosine

DIS - Dimer initiation site

DLV - Delavirdine

dNTP - Deoxynucleotide triphosphates

DOR - Doravirine

DRV - Darunavir

DTG - Dolutegravir

EC - Elite controllers

EFV - Efavirenz

EIAV - Equine Infectious Anemia Virus

Env - Envelope

ER - Endoplasmic reticulum

ERAD - Endoplasmic-reticulum-associated protein degradation

ESCRT - Endosomal sorting complexes required for transport machinery

ESE - Exonic Splicing Enhancer

ESS - Exonic Splicing Silencer

ETG - Elvitegravie

ETR - Etravirine

FOS-APV - Fosamprenavir Calcium

FTC - Emtricitabine

GALT - Gut lymphoid tissue

GPI - Glycosylphosphatidylinositol

GRID - Gay-Related Immune Deficiency

HAART - Highly active antiretroviral treatments

HAV - Hepatitis A virus

HDAC - Histone deacetylase

HTLV-III - Human T lymphotropic retroviruses type III

IBD - IN binding domain

IDV - Indinavir

IFN - Interferon

IFN-I - Type I Interferon

IFN-II - Type II Interferon

IFN-III - Type III Interferon

IMD/TM1 - Intramembrane domain

IN - Integrase

INSTI - Integrase Inhibitor

IRF3 - Interferon regulatory factor 3

IRF7 - Interferon regulatory factor 7

IRF9 - Interferon regulatory factor 9

ISGF3 - Interferon stimulated gene factor 3

ISRE - Interferon stimulatory response element

ISS- Intronic Splicing Silencer

KS - Kaposi Sarcoma

LAMP1 - lysosomal-associated membrane protein 1

LEDGF - Lens epithelium-derived growth factor

LPV/RTV - Lopanavir and Ritonavir

LRA - Latency reversing agents

LTNP - Long term non-progressors

MA - Matrix

MHC-I - major histocompatibility complex I

MLV- Murine Leukemia Virus

MVC - Maraviroc

MxB - Myxovirus resistance gene B

NC - nucleocapsid protein

NE - Nuclear envelope

NES - nuclear export signal

NFV - Nelfinavir Mesylates

NHP - Non-human primate

NK - natural killer

NLS - nuclear localization signal

NNRTIS - Non- Nucleoside Reverse Transcriptase inhibitors

NRTI - nucleoside reverse transcriptase inhibitors

NTD - N terminal domain

NVP - Nevirapine

PBS - Primer binding site

PCP - Pneumocystis pneumonia

PI - Protease Inhibitor

PI(4,5)P2 - Plasma membrane-specific lipid phosphatidylinositol (4,5) biphosphate

PPT - Polypurine tract

PR - Protease

PrEP - Preexposure prophylaxis

PRR - Pathogen recognition receptors

PTEFb - positive transcription elongation factor b

Ptt - Pan troglodytes troglodytes

RAL - Raltegravir

RPV - Rilpivrine

RRE - Rev response element

RT - reverse transcriptase

RTC - reverse transcription complex

RTV - Ritonavir

SAHA - Vorinostat

SAMHDI - SAM domain HD domain-containg protein 1

SERINC5 - Serine incorporator 5

SIV - Simian Immunodeficiency Viruses

SQV - Saquinavir

SU - Surface protein

T-20 - Enfuvirtide

T/F - Transmitted/founder

TAR - Trans-activation element

Tat - Transactivator protein

TDF - Tenofovir Disoproxil Fumarate

TIM - T-cell immunoglobulin (Ig) and mucin domain

TM - Transmembrane protein

TMD2 - Tansmembrane domain

TPV - Tipranavir

TRIM5α - Tripartite-motif-containing 5α

TRN-SR2 - Transportin-SR2

Trp - tryptophan

UNAIDS - United Nations Programme on HIV/AIDS

vDNA - Viral DNA

Vif - Viral infectivity factor

VSV - Vesicular stomatitis virus

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Chapter 1

Introduction

1.1 Preface

A literature review of the topics relevant to this thesis is presented. In this chapter, the discovery of HIV-1, virology of the virus, detailed replication cycle, and AIDS disease progression are fully covered. Current HIV treatments and studies towards HIV cure and HIV vaccine are also discussed. Finally, this chapter ends with the research objectives of my PhD project.

1.2 Origins and Epidemiology of Human Immunodeficiency virus (HIV)

In 1981, cases of aggressive Kaposi's Sarcoma (KS) and Pneumocystis pneumonia (PCP) were reported among homosexual males in New York and California [1]. It was not known whether this increase in KS and PCP was restricted to homosexual men at that time. Hence, initially the term Gay-Related Immune Deficiency (GRID) was used to describe this new epidemic. In 1982, similar cases of immunodeficiency with KS and PCP were reported among heterosexual females, infants, recipients of blood transfusion, and drug users. After that, CDC used the term Acquired Immune Deficiency Syndrome (AIDS) for the first time to describe the ongoing epidemic [2, 3]. After two years of extensive research, two groups from France and the United States independently isolated a new class of cytopathic retroviruses, named human T lymphotropic retroviruses (HTLV-III), from patients with AIDS, thus identified the causative agent for AIDS [4, 5]. In 1986, the international committee on taxonomy of viruses renamed this new virus Human Immunodeficiency Virus (HIV) [6]. In 2008, Françoise Barré-Sinoussi and Luc Montagnier were awarded the noble prize in physiology along with Harald Zur Hausen (for his discovery of human papilloma virus causing cervical cancer). Extensive research in studying the origin of this sudden epidemic spread led to isolate a similar but antigenically different virus from West African patients with AIDS, termed HIV-2 [7].

HIV belongs to the family of lentiviruses. This family of viruses causes chronic and persistent infection. Extensive studies on the origin of HIV found that the HIV epidemic is the result of multiple cross-species transmissions from Simian Immunodeficiency Viruses (SIV) [8]. In 1986, a link between HIV-2 and SIV in sooty mangabeys, a non-human primate (NHP), was established, demonstrating that HIV-2 is the result of the early cross-species transfer of SIV from NHP [9]. Further studies discovered that cross-species transmission of SIV from chimpanzee

gave rise to HIV-1 [10]. To get insight into the origin of this deadly epidemic, scientists were able to isolate and amplify HIV-1 from an African patient from 1959 residing in Kinshasa in the Democratic Republic of Congo. This study reported the earliest case of HIV-1 infection [11]. According to the Joint United Nations Programme on HIV/AIDS (UNAIDS), there are currently 37 million people living with HIV, and every year almost 2 million new incidences of HIV infection are being reported. From the beginning of the HIV epidemic till the end of 2018, 75 million people have been infected with HIV, and over 35 million people died of AIDS-related diseases. However, with ongoing research and development of combination antiretroviral therapy (cART), life expectancy of HIV-infected people has dramatically increased compared to the beginning of the HIV epidemic [12].

In 2014, UNAIDS launched the 90-90-90 targets, which states that by 2020, 90% of people who are living with HIV-1 will be diagnosed, 90% of the HIV-1 positive people will be taking antiretroviral treatments, and 90% of people taking antiretroviral treatments will be under viral suppression. The 90-90-90 target was launched with the hope to end the AIDS epidemic by 2030 [13]. Currently, different regions around the world have progressed differently towards 90-90-90 target with six countries reported to have already achieved these targets, including Denmark, Netherlands, Cambodia, and three countries in South Africa, Botswana, Eswatini, and Namibia [14].

1.3 HIV-1 Classification and Diversification

Phylogenetic analysis suggests that HIV-1 is the result of independent transmission events mainly from chimpanzee subspecies Pan troglodytes troglodytes (Ptt) to humans. These transmission events gave rise to four HIV-1 groups, including groups M, N, O, and P [8, 15]. As mentioned earlier, strains from groups M and N are derived from chimpanzee. However, recent

studies suggest that group O and P may have originated from Gorillas [15-17]. The focus of this thesis is HIV-1 strains from group M, which is responsible for the global HIV-1 pandemic. This predominant HIV-1 group M has been classified into nine different subtypes, including subtypes A, B, C, D, F, G, H, J and K. Each subtype possesses a particular geographical location with subtype B mainly located in Western Europe, America, and Australia. Subtype C comprises 48% of the total global HIV-1 strains, and is predominantly found in southern Africa (Figure 1) [8]. Over the past decade, with the advances of full genome sequencing, recombination between strains has been identified as a frequent occurrence between different groups of HIV-1 or within one group of HIV-1, mostly group M [18]. These viral recombinations have been referred to as circulating recombinant forms (CRFs). It is worth mentioning that most of the HIV-1 research on drug resistance and treatment has been conducted using subtype B viruses which comprise 12% of the total epidemic. Furthermore, subtype B and non-B viruses demonstrate different pathogenesis, disease progression, and drug resistance mutations [19]. This viral diversity adds to the challenges of developing new treatments for HIV-1. Hence, cross-reactivity between subtype B and non-B viruses should be verified in new drug regimens and therapeutic approaches.

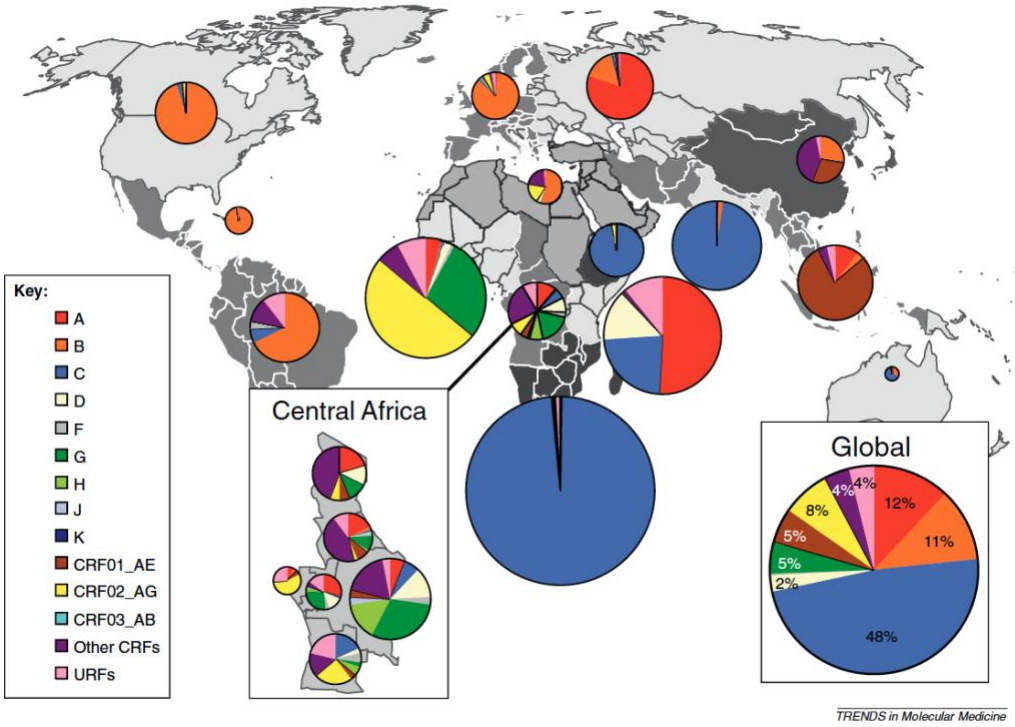
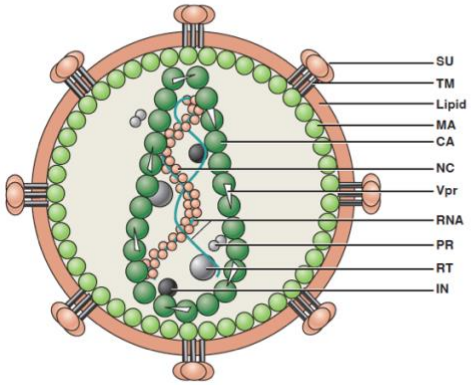


Figure 1. Global distribution of HIV-1 subtypes and recombinants (Adapted from [8]).

1.4 HIV-1 Particle

HIV-1 belongs to the family of Retroviridae and the genus of Lentivirus. It is a spherical, enveloped virion with a diameter of 100 nm. The envelope protein of the virus contains external surface protein (SU) called glycoprotein 120 (gp120) which is bound noncovalently to the transmembrane protein (TM) known as glycoprotein 41 (gp41) that traverse the lipid bilayer. The inner surface of the viral membrane is coated by matrix (MA) protein. Inside the virion, HIV-1 capsid (CA) protein known as p24 forms the conical viral capsid core caging viral enzymes protease (PR), reverse transcriptase (RT), integrase (IN) and two copies of viral genomic RNA. HIV-1 capsid also contain viral accessory proteins including Nef, Vpu, Vpr, and Vif. The 9.7 kb HIV-1 genomic RNA is covered by viral nucleocapsid (NC) protein (Figure 2) [20].

A



B

Name	Abbreviation	Alternative name (M. Wt. in KDa)
Matrix	MA	p17
Capsid	CA	p24
Nucleocapsid	NC	p7
Protease	PR	p14
Reverse transcriptase	RT	p66/51
Integrase	IN	p32
Surface protein	SU	gp120
Transmembrane protein	TM	gp41
Virion protein R	Vpr	p15

Figure 2. (A) Schematic illustration of HIV-1 particle. (B) HIV-1 proteins. (Adapted from [20]).

1.5 HIV Genome

HIV-1 genomic RNA is 9.7 kb. Similar to eukaryotes mRNA, HIV-1 RNA is capped at 5' end and polyadenylated at 3' terminus (Figure 3). Both ends of the viral RNA contain repeated sequence (R) with the length of 150-200 nt, adjacent to the unique regions designed as U5 and U3. Similar to other retroviruses, HIV-1 genome contains the *gag*, *pol*, and *env* genes encoding structural proteins Gag, Pol and gp160 [20]. However, HIV-1 is a complex retrovirus, it has six additional genes known as *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*. The role of these viral proteins in HIV-1 replication cycle and pathogenesis will be discussed later in this chapter.

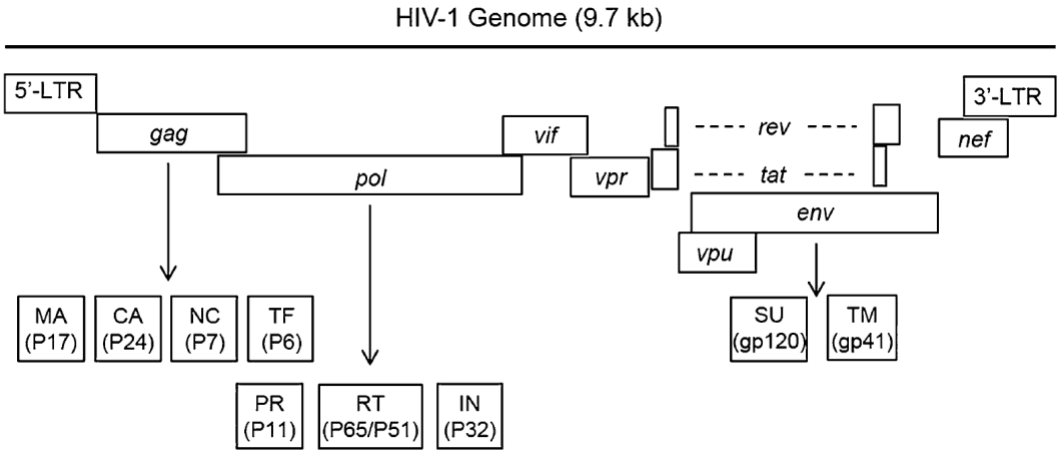


Figure 3. Schematic diagram of HIV-1 genome. Each box indicates an HIV-1 gene. The number in parenthesis indicates the molecular weight of the corresponding viral protein. (Adapted form [21]).

1.6 HIV Replication Cycle

Ever since the discovery of HIV-1 in 1983, details of the HIV replication cycle have been extensively studied. However, studying HIV-1 replication cycle is still essential and help us find new potential targets for HIV-1 treatment. In this section, HIV-1 replication cycle will be discussed in detail.

HIV-1 replication cycle consists of two major phases, the early stage and the late stage of HIV-1 replication cycle. The early stage includes all the steps from HIV-1 binding to CD4 to viral DNA integration. The late stage includes transcription of the HIV-1 DNA, translation of viral mRNA, virus assembly, and release of mature virions [20] (Figure 4). This section describes the events of the HIV-1 replication cycle from virus entry to release of the mature HIV-1 particles.

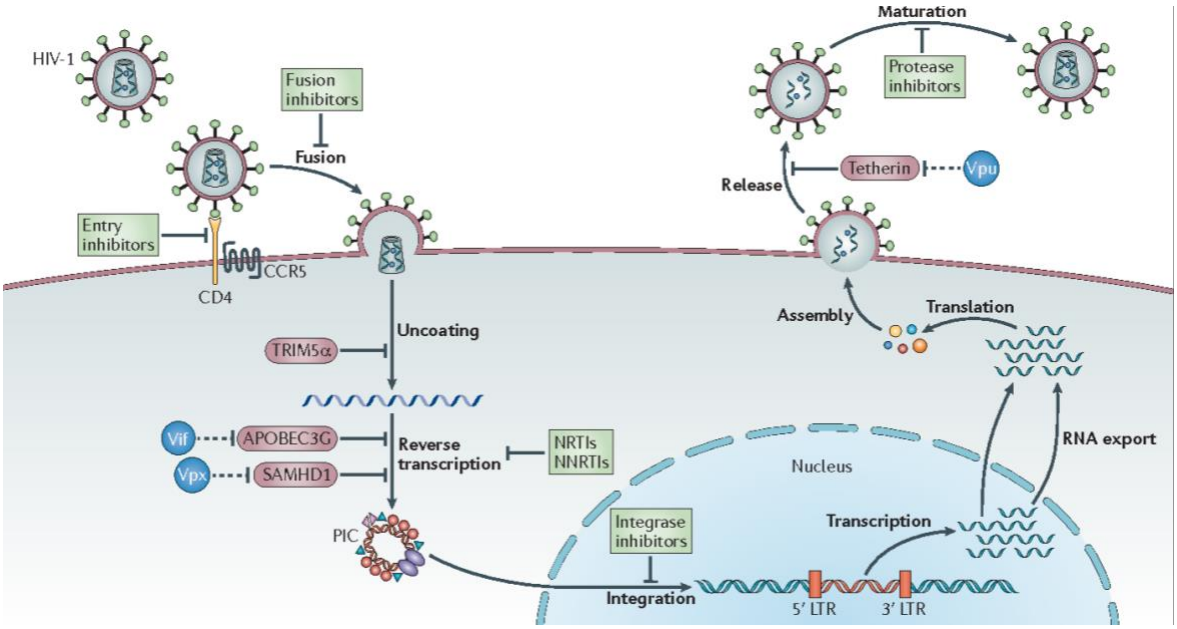


Figure 4. Overview of HIV-1 replication cycle from entry of the virus to the release of mature virus particles. The blue circles indicate the viral accessory proteins, the green boxes indicate different classes of antiretroviral therapies, and the red boxes indicate the innate immunity restriction factors which will be discussed in detail (Adopted from [22]).

1.6.1 Early Stage: Entry

HIV-1 infection begins with the entry of the virus to the target cell. HIV-1 entry consists of three sequential steps, including attachment of HIV-1 to the host cell, binding of the viral Env protein to the CD4 receptor and the CXCR4 or CCR5 coreceptor, and fusion between the cellular and viral membrane [23]. These steps lead to the delivery of the viral core into the target cell. HIV-1 entry is solely dependent on the Env trimer which is the only viral protein exposed on the surface of the virus particles. The Env precursor gp160 is synthesized at the endoplasmic reticulum (ER), followed by complex glycosylation and furin protease cleavage in trans-Golgi network complex, which leads to the formation of gp120/gp41 Env trimer [24]. Each HIV-1 virion carries an average of fewer than 15 Env trimers on the surface. However, most of the HIV-1 strains require an average of only 2-3 trimers to initiate infection [25, 26]. Being the only viral protein at the

surface of the HIV-1 particles, Env is the chief target for both host adaptive immunity as well as innate immunity, which will be discussed in detail in Chapter 2.

The HIV-1 Env gp120/gp41 heterodimer is kept by non-covalent bonds between gp120 and gp41. HIV-1 gp120 consists of five conserved regions called C1 to C5 and five variable regions, V1 to V5. The conserved regions form the core of the Env trimer, while the variable regions form the exterior of the gp120 ectodomain. The HIV-1 gp41 has three major regions including ectodomain, transmembrane domain, and C terminal cytoplasmic domain. The gp41 ectodomain contains regions essential for HIV-1 entry, including the fusion peptide, two hydrophobic heptad repeated regions HR1 and HR2, and the membrane-proximal external region rich in tryptophan (Trp) [24] (Figure 5).

The first step of HIV-1 entry is the attachment of the virus particle to the target cell. Several host factors have been reported to mediate the attachment of the virus to the target cell, thus facilitate Env-mediated entry of the virus. Integrin receptors are one of these host factors. The interaction between $\alpha 4\beta 7$, a gut homing receptor, and viral protein gp120 enhances HIV-1 dissemination in gut associated lymphoid tissue (GALT) [27, 28]. However, the necessity of $\alpha 4\beta 7$-gp120 interaction for viral entry in vivo is still under investigation. Another host factor that mediates the viral entry is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) which binds to the HIV-1 Env and facilitates the trans-infection of CD4+T cells [29].

In contrast to the auxiliary role of the attachment of Env to these host factors, binding of gp120 to the CD4 receptor is essential for virus entry. During the early years of HIV-1 epidemic, strong depletion of CD4+ T cells in HIV-1 infected individuals suggested CD4 as the main receptor for HIV-1 entry [30-33]. Upon binding of CD4 to the conserved domains C1 and C2 in HIV-1

gp120, Env undergoes conformational changes [23, 34]. These conformational changes include the rearrangements of V1/V2 and formation of the four-stranded β sheet (bridging sheet), which leads to exposure of the V3 loop region of gp120 [23]. The exposure of the V3 loop allows for the binding to the coreceptor CXCR4 or CCR5 [35, 36]. HIV-1 can be categorized based on their usage of the coreceptors, the R5-tropic HIV-1, X4-tropic HIV-1 and dual tropic HIV-1 [35].

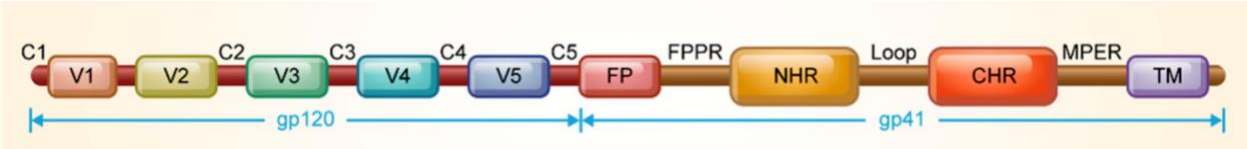


Figure 5. Domain structure of HIV-1 Env glycoprotein including gp120 and gp41(Adapted from [37]).

After binding of HIV-1 to coreceptors, the fusion between the viral membrane and host membrane is initiated. This virus fusion step is dependent on gp41, and begins by the exposure of the fusion peptide and insertion of the fusion peptide to the target cell membrane. Afterward, the fusion peptide folds and brings the HR1 and HR2 from each gp41 subunit together to form a 6 helix bundle (6HB), which brings the viral and host membrane together. Formation of 6HB leads to the formation and stabilization of the fusion pore which allows the delivery of the viral core into the cytoplasm of the target cell (Figure 6).

In contrast to enveloped viruses such as vesicular stomatitis virus or influenza virus that enter cells through endocytosis in a pH-dependent manner, HIV-1 entry is pH-independent. HIV-1 entry often occurs at the plasma membrane, but may also take place via endocytosis in certain cell types including macrophages [38, 39].

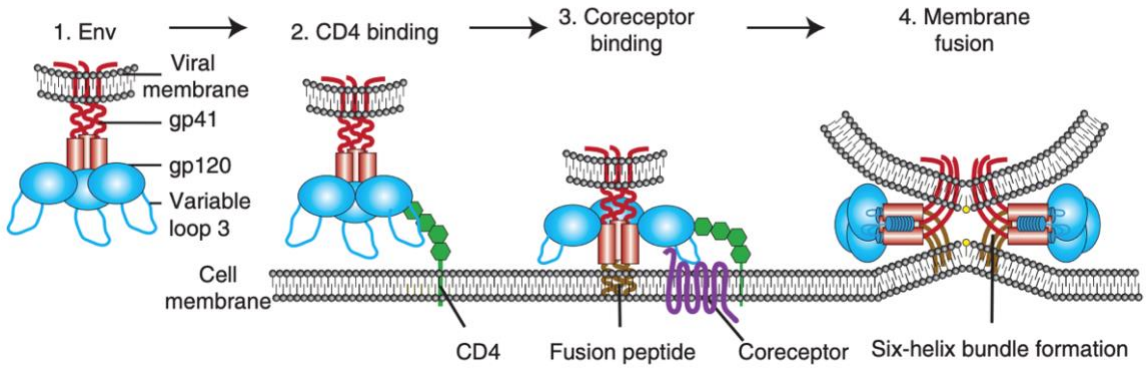


Figure 6. Overview of the HIV-1 entry. First, V3 loop of HIV-1 gp120 binds to the CD4 receptor, this interaction induces a conformational change in Env which leads to the exposure of the coreceptor binding site. Following the binding of the HIV-1 Env to coreceptors, fusion peptide is formed which leads to the fusion between the viral membrane and cellular membrane; thus, HIV-1 entry is mediated (Adapted from [23]).

1.6.2 Early Stage: Uncoating and Reverse Transcription

Following the delivery of the HIV-1 viral core into the cytoplasm of the target cell, hexameric CA rings covering the viral core will slowly dissociate under a tightly regulated process called uncoating. This step accompanies the transition between reverse transcription complex (RTC) to pre-integration complex (PTC). In RTC, reverse transcription of viral RNA occurs, while PTC mediates the integration of viral DNA into the host genome [40]. The exact time, duration, and location of the uncoating process is still debatable. Studies of the viral mutants with mathematical modeling support that the uncoating process is initiated after reverse transcription [41]. Furthermore, single HIV-1 imaging reveals that viral CA protects the core from degradation and mediates the docking of the viral core to the nuclear envelope (NE) for the integration process [42], suggesting that the uncoating process does not occur until reverse transcription is completed.

The conversion of single-stranded viral RNA to double-stranded viral DNA occurs in the cytoplasm. The HIV-1 reverse transcriptase (RT) carries two enzymatic activities, including

DNA polymerase activity to produce viral DNA and RNase H activity to degrade viral RNA in the RNA-DNA complex [43, 44]. The DNA polymerase activity of RT is highly error-prone, which contributes to the high mutation rate in HIV-1 [45]. Therefore, rather than a uniform pool of HIV-1, there is a collection of HIV-1 variants circulating in infected individuals known as quasispecies [46]. The reverse transcription process can be divided into multiple steps as follows [47] (Figure 7). The cellular $tRNA_{Lys3}$ acts as the primer for viral RT and binds to the primer binding site (PBS) at the 5'end of HIV-1 plus-strand RNA, this leads to the formation of minus-strand strong-stop DNA. Due to the formation of RNA-DNA complex, RNase H digests the RNA part of the newly formed complex. The minus-strand strong DNA hybridizes with 3'end of viral RNA through complementary R sequence. The " first strand transfer" of DNA mediates the synthesis of the full-length genome, while removing the template RNA except for the polypurine tract (PPT) which locates immediatly upstream of the U3 element. PPT serves as the primer to initiate the synthesis of plus-strand strong DNA. It is worth mentioning that Lentiviruses also initiate plus strand synthesis at a second PPT, known as the central PPT(cPPT) locating on the intergrase ORF [48]. After the second strand transfer, $tRNA_{Lys3}$ is degraded and RT completes the synthesis of both minus and positive strands, forming the double-stranded viral DNA.

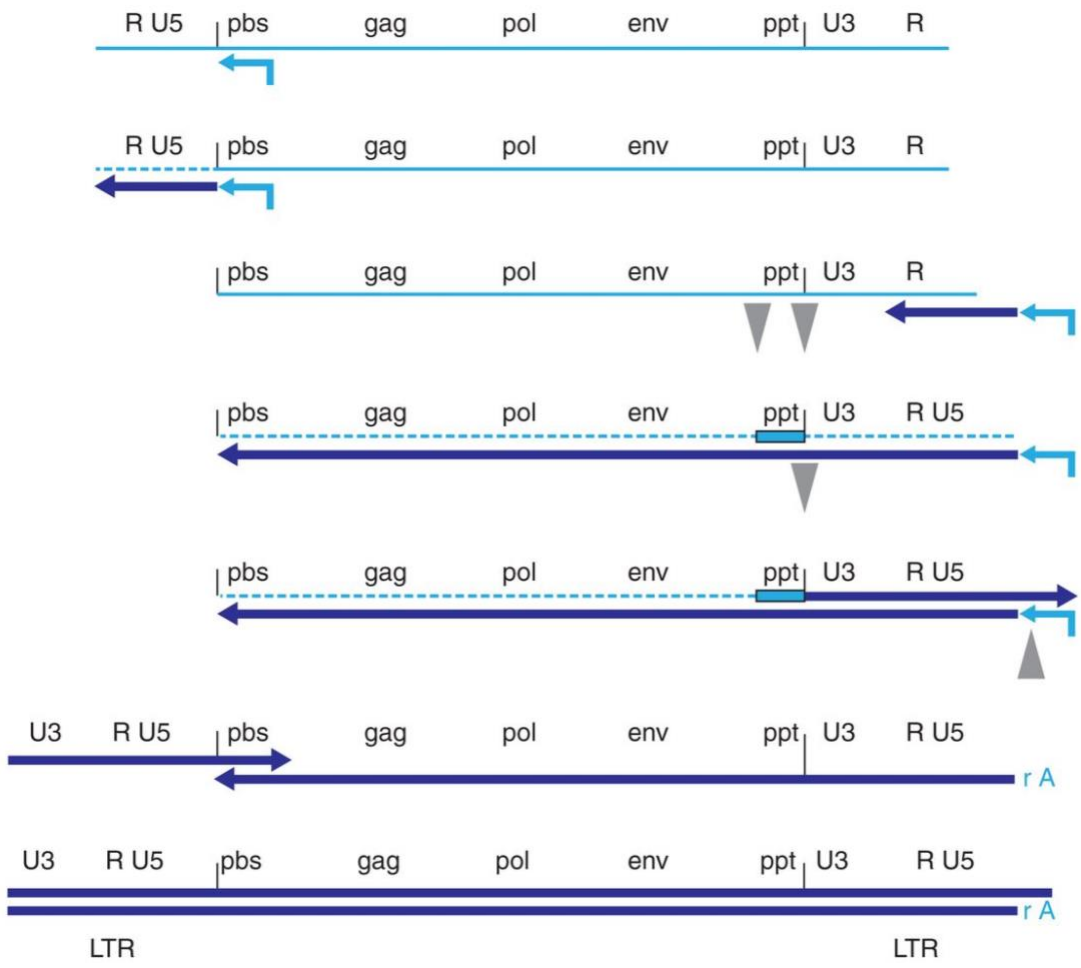


Figure 7. Overview of the reverse transcription step (Adapted from [47]).

1.6.3 Early Stage: Nuclear Import and Viral DNA Integration

HIV-1 infects non-dividing cells with intact nuclear envelope. To do so, HIV-1 has evolved several strategies to transport the viral DNA (vDNA), which is packed inside the PIC, into the nucleus. This allows the integration of vDNA into the host genome. Viral proteins including MA, CA, IN and Vpr are known to be essential for transport of PIC into the nucleus [49]. In addition to the viral proteins, genome-wide screen studies have reported the requirement of several host proteins to mediate nuclear import of PIC. This includes the nuclear import factors

such as transportin-SR2 (TNPO3/TRN-SR2), importin 7, and nuclear pore complex proteins including Nup358 and Nup158 [50-54].

The last step of the early infection in the HIV-1 replication cycle is vDNA integration. Viral enzyme integrase (IN) mediates the integration step and enters the target cell as a component of the HIV-1 viral core. Viral integration does not occur at random sites. A growing body of evidence suggests that HIV-1 integration preferably occurs at transcription-active chromosomal regions [55]. Moreover, studies using fluorescently labeled PICs show that integration occurs at decondensed euchromatin regions of the nuclear periphery [55, 56].

During vDNA integration, IN removes two nucleotides from 3'end of the viral DNA. Next, the 3'end attacks the phosphodiester bonds of the opposite strands of the host gene. This step is known as DNA strand-transfer. Subsequently, the two 3'ends of the viral DNA covalently joint the host DNA. Finally, a cellular enzyme repairs the two nucleotides overhangs of viral DNA and fix the single strand gaps to complete viral integration [49]. Like many other viral steps, integration also engages different host factors. One example is lens epithelium-derived growth factor (LEDGF) which binds to IN binding domain (IBD) and forms a stable tetramer [57]. LEDGF binding to IN mediates targeting of DNA into the hot spots of transcription and increases the efficiency of viral integration [58, 59]. Following integration, late stage of HIV-1 replication cycle begins.

1.6.4 Late Stage: Viral Transcription

Following the integration of the viral genome into the host genome, the late stage of HIV-1 replication cycle begins from viral transcription to the release of mature HIV-1 virions . Similar to the expression of the host genes, the expression of the viral genome requires the presence of a set of transcription factors. In the absence of the appropriate transcription factors, the viral

genome will not be expressed, this outcome is called latent infection [60, 61]. Although latently infected cells do not actively produce viral proteins, but they act as a barrier to the HIV-1 cure, which will be discussed throughout this chapter.

Similar to the transcription of the host genes, HIV-1 transcription is also dependent on the RNA polymerase II which binds to the 5' LTR promoter. Two viral regulatory proteins transactivator of transcription protein (Tat) and regulator of the expression of viral proteins (Rev) are essential for viral RNA expression [62]. In the presence of the transcription factors, initially, the genomic RNA gives rise to a fully spliced viral mRNA of 2 kb which is produced and exported to the cytoplasm to synthesize Rev, Tat, and Nef [62]. HIV-1 Tat binds to Trans-activation response element (TAR) located in R and regulates HIV-1 transcription by recruiting the positive transcription elongation factors composed of P-TEFB, CDK9 kinase, and Cyclin T1 [63-66].

1.6.5 Late Stage: Viral RNA Splicing

The splicing of HIV-1 viral RNA adds more complexity. Unlike other retroviruses, HIV-1 produces three types of viral RNA, unspliced, singly spliced, and doubly-spliced. HIV-1 splicing generates over 40 different mRNA species [67]. Several factors contribute to this complexity of HIV-1 viral RNA including existence of multiple splicing sites (ss) on both 5' (4 sites) and 3' (7 sites) ends of viral RNA and the combination of positively acting exonic splicing enhancer (ESE) elements and negatively acting exonic splicing silencer (ESS) and intronic splicing silencer (ISS) elements [67]. These mRNA species fall into three main classes, the unspliced 9-kb which encodes for Gag and Pol, singly spliced 4-kb encodes for Env, Vif, Vpr, Vpu, and multiply spliced 2-kb encoding for Tat, Rev, and Nef (Figure 8).

1.6.6 Late Stage: Nuclear Export

Nuclear export of full-length viral mRNA and singly spliced species requires Rev. Rev binds to Rev response element (RRE) and interacts with chromosomal maintenance 1 (CRM1), also known as exportin 1 through nuclear export signal (NES) [68-70]. Once Rev is exported into the cytoplasm, it shuttles back to the nucleus through the engagement of Rev nuclear localization signal motif (NLS) with importin β. Hence, this shuttling mechanism allows Rev to export more viral mRNA into the cytoplasm [71].

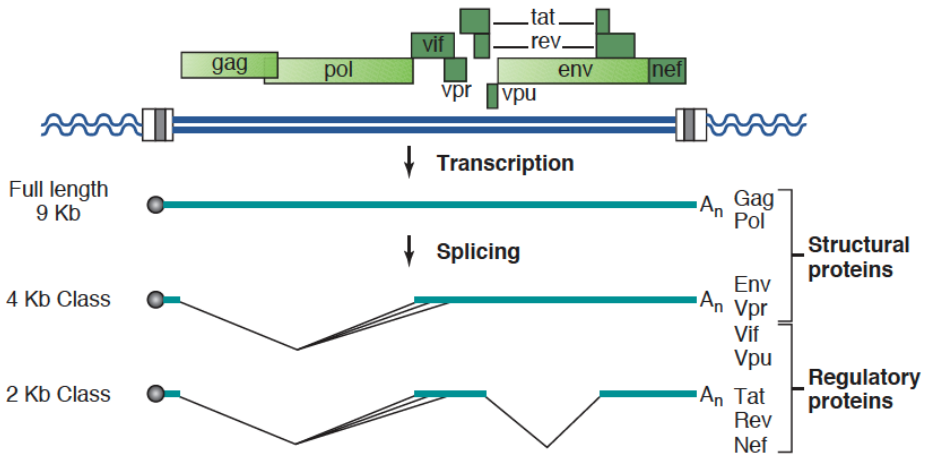


Figure 8. HIV-1 genome and splicing of HIV-1 genome (adapted from [20]).

1.6.7 Late Stage: Viral Translation and Protein Assembly

Once HIV-1 mRNA is inside the cytoplasm, HIV-1 uses the host translation machinery to generate viral proteins. Synthesis of Gag, Gag-pol polyprotein and other viral accessory proteins occur in the cytosolic polysome [72]. Gag protein is the main structural protein which drives the assembly of new infectious HIV-1 particles (Figure 9) [73]. Gag-Pol polyprotein arises from programmed -1 ribosomal frameshifting. Upon translation in the cytoplasm, Gag traffics via a cellular endosomal pathway to the plasma membrane and accumulates at the lipid raft. Lipid raft

is the cholesterol-enriched microdomains at the plasma membrane where HIV-1 assembly takes place [74]. Assembly of Gag at the plasma membrane requires the binding of matrix domain (MA) of Gag to the plasma membrane-specific lipid phosphatidylinositol (4,5) biphosphate ($PI(4,5)P_2$). This binding leads to the conformation changes and exposure of the myristylation site of the MA [75]. Hence, Gag anchors to the inner leaflet of the plasma membrane and multimerizes through Gag-Gag interactions [75].

Unlike Gag-Pol polyprotein, translation of Env and Vpu occur via in the ER [24, 76]. Processed Env trimers are targeted to the lipid raft via palmitoylation of the cytoplasmic domain of gp41. Whether the localization of the Env protein to the lipid raft depends on the MA is yet to be determined [77]. Once in the lipid raft, MA binds to Env and induces the incorporation of Env into the newly synthesized virions [24].

HIV-1 incorporates two copies of viral RNA into the newly synthesized virions. To do so, full-length unspliced RNA is non-covalently dimerized via a kissing loop at the dimer initiation site (DIS). This dimerization facilitates the packaging of two copies of viral RNA into the progeny virion [78]. Upon dimerization, the nucleocapsid (NC) region of Gag binds to the packaging signal (ψ) region of RNA. This RNA-Gag interaction is essential for the viral RNA packaging into the HIV-1 particles [79].

Figure 9. Schematic diagram of the pr55 Gag protein. SP1 and SP2 refer to spacer peptides, which separate CA and NC, NC and P6, respectively.

1.6.8 Late Stage: Virion Budding and Maturation

Following the formation of HIV-1 particles, HIV-1 hijacks the host endosomal sorting complexes required for transport machinery (ESCRT) to bud off from the virus producing cells [80]. In order for HIV-1 to bud off, the p6 domain of Gag binds to ESCRT factors including ALIX and TSG101 [81]. As the immature HIV-1 virion buds off from the cell, viral protease (PR) becomes activated and cleaves Gag and Gag-Pol polyprotein into MA, CA, NC, P6, PR, RT, and IN [37]. Proteolysis by PR induces morphological changes of the virion, which is required for the conversion of the immature HIV-1 virion into a mature and infectious one [37, 72]. It is worth mentioning that downregulation of CD4 receptor by viral accessory proteins Nef and Vpu is required for HIV-1 release from the cells. Downregulation of CD4 prevents the interaction between CD4 and gp120 on the newly synthesized virion [82, 83]. Details on how the interaction between the Env protein and CD4 induces conformational changes of Env will be discussed in Chapter 2.

1.7 HIV-1 Infection Progression and Pathogenesis

HIV-1 transmission predominantly occurs at the mucosal membrane during the heterosexual intercourse. However, other routes of transmission such as men who has sex with men (MSM), direct blood transmission and transmission from mother to child during pregnancy, birth, and breast milk have been established as well [20, 84, 85]. Transmission via heterosexual route accounts for almost 70% of HIV-1 infections worldwide. Interestingly, transmission probability per exposure event is lowest for heterosexual intercourse route with the probability of 1 in 200 to 1 in 2000. However, other routes of transmission including MSM and drug injections have higher probability per exposure event, 1 in 20 to 1 in 300 and 1 in 150, respectively [86].

During the exposure event, HIV-1 needs to cross the mucosal barrier consisting of squamous epithelial cells to reach the CD4+ T-cells which are the main target cells for HIV-1 infection [23]. A number of environmental factors may alter the susceptibility or the number of CD4+ T-cells, including inflammation and micro-abrasion in the mucosal surface [85]. In addition to inflammatory cascades, a network of dendritic cells (DCs) residing under epithelial cells may facilitate HIV-1 transfer to CD4+ T-cells. These DCs capture HIV-1 via binding to the gp120 viral protein and transfer HIV-1 across epithelial cells to T-cells [85, 87, 88]. Upon transmission and dissemination of the HIV-1 infected T cells to draining lymph nodes, the acute infection is established which occurs within 24 hours post exposure [89]. It is known that 80% of the heterosexual HIV-1 transmission events are established from one single HIV-1 variant called transmitted/founder (T/F) strain [90]. These T/F viruses are mostly R5 tropic, meaning that in addition to the CD4 receptor, they also require the coreceptor CCR5 on the target cells to initiate the infection [90].

HIV-1 infection is a dynamic process and is divided into three main phases: the acute stage (asymptomatic), the chronic stage, and AIDS progression (symptomatic). The highest level of viremia is detected during the acute stage and AIDS progression stage. Figure 1-10 depicts the natural course of HIV-1 infection [91].

Following HIV-1 exposure and transmission, viral RNA is undetectable in the body for almost 10 days. This period is known as the eclipse phase [92]. At the end of the eclipse phase, HIV-1 or infected cells reach the drain lymph nodes and disseminate by infecting more CD4+T cells. During the acute phase, which lasts up to 12 weeks after initial exposure, more than 80% of the CD4+ T cells are depleted [93]. Meanwhile, HIV-1 keeps replicating, and the viral load reaches one hundred million particles per milliliter in the plasma [94]. During the acute phase, most

patients do not show any symptoms or they show mild flu-like symptoms such as fever, skin rash, headache anorexia, and diarrhea [95]. These symptoms indicate the activation of the immune system by HIV-1. At the end of the acute phase, viral load decreases due to an increase in the immune response including HIV-1 specific CD8+ T cells activation. Finally, with an increase in the immune response, seroconversion will occur and HIV-specific antibodies are detected [96].

As shown in Figure 1-10, the gradual decrease in CD4+T cells and chronic infection are the hallmarks of HIV-1 infection. Following the acute infection, HIV-1 viremia decreases to the set point viral load. This set point viral load is critical for determination of the disease progression and AIDS [97]. The chronic phase ensues due to the systematic activation of the immune system [97]. During the chronic phase, which lasts up to 15 years, an equilibrium between HIV-1 viral load and CD4 depletion is reached and the outcome of this balance determines the fate of the disease progression. The systematic activation of the immune system during the chronic phase increases more target CD4+T cells for HIV-1 which in turn leads to chronic inflammation [98]. The main reason of the variation in the chronic phase from 2 years to 15 years among individuals remains to be elucidated. This variation in length of chronic phase might be due to variabilities in HIV-1 and host genetics [98].

The late phase of HIV-1 pathogenesis is AIDS progression, which is a symptomatic stage. This symptomatic phase arises in non-treated individuals where HIV-1 viral load rises and CD4 count goes below 200 per mm^3 [99]. Due to the loss of CD4+ T cells, opportunistic infections such as tuberculosis and pneumatosis emerge. Furthermore, neurological complications might also occur [100]. This profound immunosuppression that occurs during the late phase of infection leads to death of the infected individual. Without antiretroviral therapeutic (ART) interventions, the

survival of infected individuals was limited to three years. Fortunately, ART has drastically increased the life expectancy of HIV-1 positive individuals. However, it is still shorted than the general population [101].

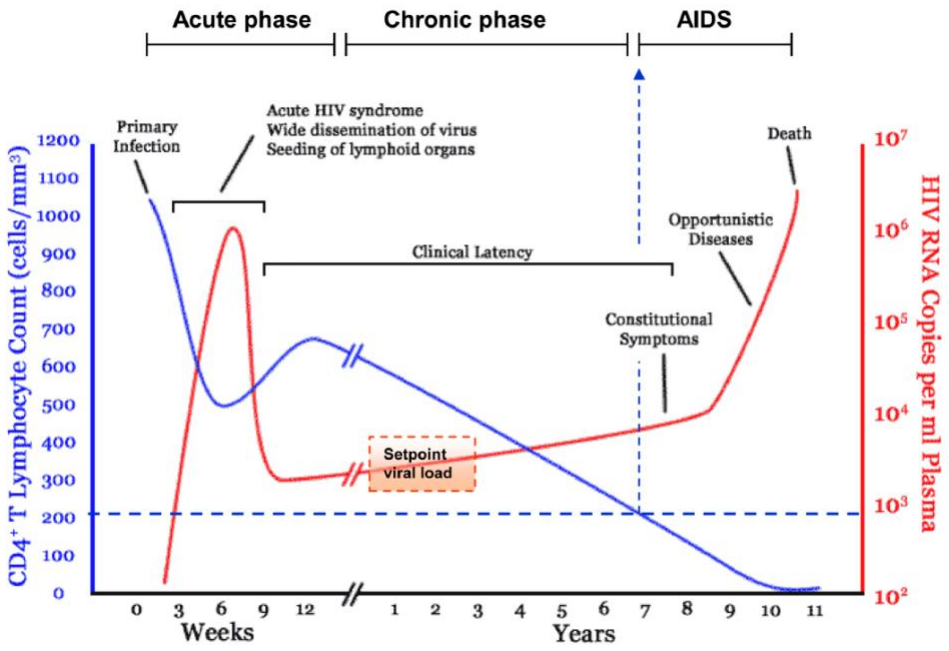


Figure 10. HIV-1 disease progression. The typical course of HIV-1 disease progression is depicted here with two main characterization of HIV-1 infection including CD4+ T lymphocyte counts (shown in blue) and HIV RNA copies also known as viral load (shown in red) (Adapted from [91]).

1.8 Elite Controllers

As mentioned earlier, without ART interventions, the majority of HIV-1 infected individuals suffer from ongoing viral replication which results in an increase in viral load with a gradual decrease in CD4+ T cells. However, 5%-15% of individuals, known as long term non-progressors (LTNP), are immunologically and clinically stable with high levels of CD4+ T cells count [102]. Later on, with advances in diagnosis tests, researchers and doctors found a subset

of LTNP individuals with a viral load below the levels of detection (less than 50 copies of HIV-1 RNA/ml). These individuals are referred to elite controllers (EC) with a natural ability to control HIV-1 replication and a prevalence ranges from 0.15 to 1.5 % [103].

The exact mechanism to explain the control of HIV-1 infection in EC remains unclear. It is worth mentioning that studying EC is important, since it can further our understanding of HIV-1 pathogenesis and help us develop novel vaccine strategies to control HIV-1 infection [104]. Recently, a new study has found that HIV-specific CD8+ T cells have a distinct transcriptional profile in EC with a more efficient translation of proteins [105]. This finding shows the important role of CD8+ T cell response in controlling HIV-1 infection and disease progression.

1.9 HIV-1 Latency

As mentioned earlier, ART interventions increase the life expectancy of HIV-1 infected individuals. However, once discontinued, latently infected memory CD4+ T cells are capable of producing infectious HIV-1 particles [106]. HIV latency is a hallmark of HIV-1 infection and poses a major obstacle to achieve HIV-1 cure. HIV-1 latency refers to the non-productive state of HIV-1 infection. The cells that harbor replicative competent forms of HIV-1 are called reservoirs [106]. Resting memory CD4+ T cells are the most recognizable HIV-1 reservoir. Furthermore, other immune cells including monocyte-derived macrophages and dendritic cells also contribute to HIV-1 latency, but their role as HIV-1 reservoir is debatable [107]. Studies found that it will take over 70 years to eliminate HIV-1 reservoirs under ART. As a result, HIV-1 latency renders infection a chronic disease that requires life-long treatment with ART [60, 108, 109].

HIV-1 latency occurs during the early infection. Studies show that early ART restricts the size of reservoirs. However, ART cannot prevent the establishment of HIV-1 latency [110]. Different molecular mechanisms contribute to the establishment of HIV-1 latency, including site and

orientation of HIV-1 DNA integration, absence of essential transcription factors in HIV transcription, or epigenetic silencing such as DNA methylation [111].

1.10 Treatment and Antiretroviral Therapy

At the beginning of the AIDS epidemic in 1981, no treatment or medication was available to control the HIV-1 infection except interferon which was used to alleviate AIDS symptoms [112]. In 1987, treatment with azidothymidine (AZT), a nucleoside reverse transcriptase inhibitors (NRTI), was approved despite its limitations and enormous side effects [113]. Following approval of AZT, other NRTIs medications such as cytidine analog lamivudine (3TC) was developed and approved [114]. Nowadays, different classes of antiretroviral treatments have been developed targeting various steps of HIV-1 replication cycle as mentioned earlier in this thesis (Figure 11).

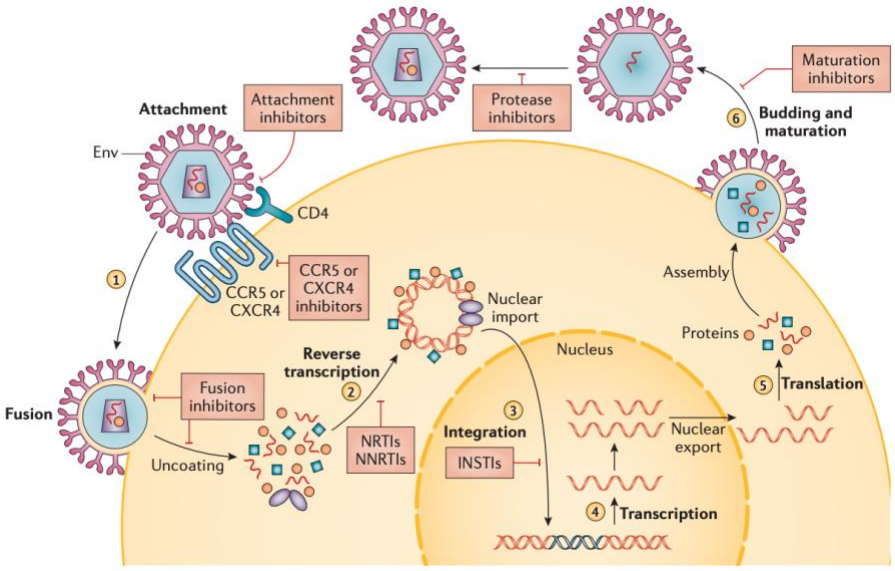


Figure 11. Schematic diagram of different antiretroviral drugs and their target sites (Adapted from [115]).

In 1996, combination antiretroviral therapies known as highly active antiretroviral treatments (HAART) was introduced [116]. HAART reduces mortality and morbidity by dramatically suppressing HIV-1 viral load to below detection level [116]. Usually, HAART involves the combinations of at least three different active antiretroviral agents from two distinct classes [22]. Table 1 summarizes currently available FDA approved ART.

Ever since HAART development, the life expectancy of HIV-1 infected individuals has been increasing. Hence, HIV-1 infection is no longer a deadly disease [117]. Moreover, HAART intervention is also effective in reducing transmission of HIV-1. This is done by preventing the transmission from mother to child or preventing HIV-1 transmission to individuals at high risk using preexposure prophylaxis (PrEP) [118]. Another ground breaking success of HAART is zero HIV-1 transmission when the infected partner has undetectable viral load due to adherence to antiretroviral treatments [119].

Despite the aforementioned advantages, HAART comes with its own disadvantages. For example, HAART does not cure HIV-1 infection and once interrupted, HIV-1 viral load rebounds [120]. Therefore, patients are required to adhere to HAART their entire lifetime. This comes with complications such as toxicity and the emergence of viral drug resistance mutations [121]. Another drawback with HAART is accessibility. According to UNAIDS, 20 million people out of 37 milling people living with HIV-1 have access to HAART (UNAIDS). It is therefore of great interest to continue research on finding a preventive or treatment vaccine to eradicate or cure HIV-1.

Table 1. Summary of currently available FDA approved ART

Generic Name (Abbreviation)	Brand name	Year of FDA approval
CCR5 Inhibitor		
Maraviroc (MVC)	Selzentry	August 2007
Fusion Inhibitor		
Enfuvirtide (T-20)	Fuzeon	March 2003
Nucleotide Reverse Transcriptase inhibitors (NRTIS)		
Zidovudine (AZT)	Retrovir	March 1987
Didanosine (ddI)	Videx EC	October 1991
Stadvudine (d4T)	Zerit	June 1994
Lamivudine (3TC)	Epivir	November 1995
Abacavir (ABC)	Ziagen	December 1998
Tenofovir Disoproxil Fumarate (TDF)	Viread	October 2001
Emtricitabine (FTC)	Emtriva	July 2003
Non- Nucleotide Reverse Transcriptase inhibitors (NNRTIS)		
Nevirapine (NVP)	Viramune	June 1996
Delavirdine (DLV)	Rescriptor	April 1997
Efavirenz (EFV)	Sustiva	September 1998
Rilpivrine (RPV)	Edurant	May 2011
Etravirine (ETR)	Intelence	January 2018
Doravirine (DOR)	Pifeltro	August 2018
Integrase Inhibitor (INSTI)		
Raltegravir (RAL)	Isentress	Octiber 2007
Dolutegravir (DTG)	Tivacay	August 2013
Elvitegravie (ETG)	Vitekta	November 2015
Protease Inhibitor (PI)		
Saquinavir (SQV)	Invirase	December 1995
Ritonavir (RTV)	Norvir	March 1996
Indinavir (IDV)	Crixivan	March 1996
Nelfinavir Mesylates (NFV)	Viracept	March 1997
Amprenavir (APV)	Agenerase	April 1999
Lopanavir and Ritonavir (LPV/RTV)	Kaletra	September 2000
Atazanavir (ATV)	Reyataz	June 2003
Fosamprenavir Calcium (FOS-APV)	Lexiva	October 2003
Tipranavir (TPV)	Aptivus	June 2005
Darunavir (DRV)	Prezista	June 2006

1.11 HIV-1 Vaccine

From the discovery of HIV-1 in 1983 until now, the knowledge of the scientific community on HIV-1 virology, viral pathogenesis, and the immune response against HIV-1 has increased tremendously. However, the development of an HIV-1 vaccine is yet challenging. Six vaccine trials have been completed; however, only one vaccine trial in Thailand, RV144, showed a modest vaccine efficacy of 31.2 % [122].

There are two main challenges in developing an effective preventative or therapeutic vaccine. One major challenge is the high genetic diversity of HIV-1, in particular, the HIV-1 Env protein. Env, as the only exposed viral protein on the surface of HIV-1, acts as the main target for vaccine development. However, due to high variability of Env, targeting Env for vaccine development is challenging [123]. Another challenge is the lack of conclusive immune mediated response to clear HIV-1 infection [124]. Most of the HIV-1 preventative vaccines are focused on developing an immunogen to elicit broadly neutralizing antibody response to prevent HIV-1 infection [125]. However, the results from RV144 vaccine trial highlighted the role of non-neutralizing antibodies to elicit antibody dependent cellular cytotoxicity response (ADCC) by stimulation of CD8+ T cells [126]. Hence, research on characterizing neutralizing antibodies as well as non-neutralizing antibodies to elicit ADCC response to clear HIV-1 infection are of great interest [127].

1.12 HIV-1 Cure

Despite the advances in the antiretroviral treatments which render HIV-1 infection from a deadly disease to a chronic manageable disease, finding a cure for HIV-1 remains a key priority of research. One of the main obstacles of finding a cure for HIV-1 is the presence of latently

infected cells. Presence of HIV-1 reservoirs increase the risk of HIV-1 reactivation upon interruption of antiretroviral treatments [109]. There are two main strategies under investigation to achieve HIV-1 cure. The first avenue is sterilizing cure. In this strategy, all the infected cells are eliminated and there are no more reservoir and latently infected cells in the HIV-1 infected individual. The second strategy is the functional cure, which is defined as the control of viral replication wherein HIV-1 remains undetectable even with discontinues of antiretroviral treatments. The ultimate goal of functional cure is to achieve prolonged HIV-1 remission [128]. As mentioned, the key obstacle of finding a cure for HIV-1 is the presence of viral reservoirs and the goal of sterilising cure is to eliminate all the infected cells and viral reservoirs. One strategy to achieve sterilising cure is called "shock and kill" strategy. This strategy depends on latency-reversing agents (LRA) which activate HIV-1 replication in latently HIV-1 infected cells. As the name indicates, this strategy consists of two steps: during the "shock" step, HIV-1 expression is activated in latently infected cells using LRAs, subsequently during the "kill" step, these activated HIV-1 expressing cells are eliminated by being the target of the immune system as well as by viral cytopathic effects and antiretroviral treatments [129]. One of the well-studied groups of LRAs are histone deacetylase (HDAC) inhibitors. It is known that deacetylation of histones by HDAC induces a heterochromatin environment and makes host DNA less accessible to the transcription factors [130]. Therefore, HDAC inhibitors increase DNA transcription which includes the transcription of HIV-1 proviral DNA. Many potent HDAC inhibitors including Vorinostat (SAHA) have been well characterized by different research groups using CD4+ T cells both in vivo or ex-vivo [131]. Despite the advances of LRAs to increase HIV-1 DNA transcription, LRAs are not effective in reducing viral reservoirs [132].

Due to challenges of eliminating all the infected cells using the sterilizing cure, the functional cure is a more feasible and favorable approach. As discussed earlier in section 1.8, elite controllers have the natural ability to control HIV-1 viral load below detectable levels in the absence of antiretroviral treatments [103]. Therefore, elite controllers act as a natural and valuable model in achieving functional cure which is to achieve prolonged HIV-1 remission in the absence of ART [133]. One strategy in achieving HIV-1 remission is to reduce the size of viral reservoirs by early treatment of HIV-1. One recent case of HIV-1 remission using early treatment is known as the "Mississippi baby" [134]. In this case study, researchers were able to reduce the size of HIV-1 reservoir in a baby from Mississippi by initiating ART within 30 hours after the baby was born. However, after 2 years, viral rebound was reported in this child [134] . This study indicates that early ART initiation may restrict but not eradicate HIV-1 reservoirs [135]. An earlier study also looked into long term HIV-1 remission in perinatally infected children who receive early ART. In this study, HIV-1 remission lasted for more than 12 years despite of treatment interruption [136]. Furthermore, a study known as VISCONTI cohort standing for Viro-Immunologic Sustained Control After Treatment Interruption, also reported long term HIV-1 remission in some individuals after post-early ART interruption [137].
As mentioned early ART, may restrict but not eradicate HIV-1 reservoirs. Another study also provided a proof of concept that viral rebound occurs despite the early antiretroviral treatment [120]. Therefore it remains questionable on how much shrinking of viral reservoir is required and how early antiretroviral therapy should be initiated to achieve prolonged remission.
Another exciting avenue to investigate to achieve functional cure is through gene therapy. In 2009, the first official case of HIV-1 remission was reported in the "Berlin patient", who achieved HIV-1 cure through hematopoietic stem cell transplant by receiving cells from a donor

with homozygous CCR5Δ32 [138]. As mentioned earlier, CCR5 are the main coreceptors for entry of R5-tropic HIV-1, and 32 base pair deletion in CCR5 prevents the expression of these receptors. Therefore, this mutation interferes with the entry of R5-tropic HIV-1 into the target cells [139]. The homozygous CCR5Δ32 deletion is observed in 1% of Caucasian demographic which provides them with a natural protection and resistant to HIV-1 infection [139]. Another case of HIV-1 cure using hematopoietic stem cell transplant with homozygous CCR5Δ32 donor was achieved in 2019 known as the "London patient" [140]. Due to the risks associated with hematopoietic stem cell transplant and also the rarity of homozygous CCR5Δ32 donors, this approach is not scalable. However, the mentioned successful cases of HIV-1 remission prompts researchers to use gene therapy approach to modify CCR5 receptor using zinc-finger nuclease or CRISPR/Cas9 gRNA [141, 142]. Like other cure approaches, gene therapy is also associated with limitations including unknown long term safety and low transduction efficiency. Finally, this approach is not effective for people infected with X4-tropic HIV-1 which uses CXCR4 as the coreceptor [143].

Another novel strategy for the functional cure is called the "block and lock" strategy. In opposite to the "shock and kill" strategy in sterilizing cure which aims at eliminating all the infected cells, the "block and lock" strategy aims at inducing permanent silence of all the HIV-1 infected cells [144]. This strategy targets multiple factors of HIV-1 transcription machinery using different inhibitors such as Tat inhibitor didehydro-cortistatin A (dCA) [145]. Moreover, targeting the interaction between HIV-1 IN and LEDGF using small molecule LEDGIN is also a favourable strategy to silence HIV-1 transcription [146]. One of the main advantages of the "block and lock" strategy over gene therapy is the scalability of this approach, since most of the HIV-1 transcription inhibitors are small molecules which can be administrated at low cost [144]. The

next step of the "block and lock" strategy is to investigate the safety and effectiveness of these small molecules in clinical trials.

1.13 Innate Immune Response to HIV-1 Infection

During HIV-1 infection, the response from the adaptive immunity which includes generation of neutralizing antibodies will not appear until later on when HIV-1 infection has been established. Like other viral pathogens, HIV-1 infection leads to activation of the innate immune response during the acute early infection [147]. Activation of the innate immune response begins with activation of pathogen recognition receptors (PRR) including Toll like receptors (TLR). TLR7 and TLR8 sense HIV-1 viral ssRNA in endosomes of dendritic cells; while, TLR2 and TLR4 recognize HIV-1 structural proteins such as HIV-1 gp120 on the surface of the female genital epithelial cells in the presence of heparan sulfate [148, 149]. After sensing HIV-1 via TLRs, transcription factors including NF-KB and interferon regulatory factors 3 and 7 (IRF3 & IRF7) are activated which in turn promote the expression of the antiviral cytokines in particular type I interferon (IFN).

1.14 IFN Family and IFN Activation Pathway

IFNs are glycoproteins that are produced in response to infectious agents including HIV-1. Production of IFN leads to the activation and differentiation of immune cells including T cells, B cells, and natural killer (NK) cells. Three types of IFN has been characterized, type I, II, and III with types I and III being the essential component of the antiviral defense [150, 151]. In humans, type I IFN (IFN-I) compromises of IFN-α, IFN-β, IFN-ω, IFN-κ, and IFN-ε [150]. IFN-α consists of 13 subtypes which are secreted by mainly by leukocytes, while IFN-β is produced mainly by fibroblasts. Secreted type IFN-I binds to IFNAR, a heterodimer receptors composed of IFNAR1 and IFNAR2 [152]. Type III IFN (IFN-III) consists of IFN-λ which

impairs HIV-1 replication in macrophages [153, 154]. Compared to IFN-I, IFN-III use different receptors. Upon secretion of IFN-III, they bind to different heterodimer receptors composed of IFN-λR1 and IL-10R2 [152]. Recently, a growing body of studies show that in addition to type I and type III IFN, type II IFN-γ also inhibits HIV-1 replication in primary CD4+ T cells. However, transmitted/founder (T/F) HIV-1 viruses are able to resist IFN-γ and this resistant phenotype is mapped to HIV-1 Env region [155].

Upon secretion, IFNs bind to their corresponding receptors, either on the IFN producing cells or other cells. This binding stimulates the JAK/STAT signaling pathway. Induction of JAK/STAT signaling pathway includes phosphorylation of TYK2 and JAK1. Subsequently this promotes dimerization and phosphorylation of STAT1 and STAT2. Next, phosphorylated STAT proteins recruit IRF9 which triggers the formation of the Interferon Stimulated Gene Factor 3 (ISGF3) complex. The ISGF3 complex translocates into the nucleus and binds to the Interferon Stimulatory Response Element (ISRE) which leads to the generation of hundreds of Interferon Stimulated Genes (ISGs) [156] (Figure 12). Induction of ISGs promotes an early innate immune response in cells. Several of these produced ISG proteins are capable of inhibiting HIV-1 replication which are discussed in this section [157].

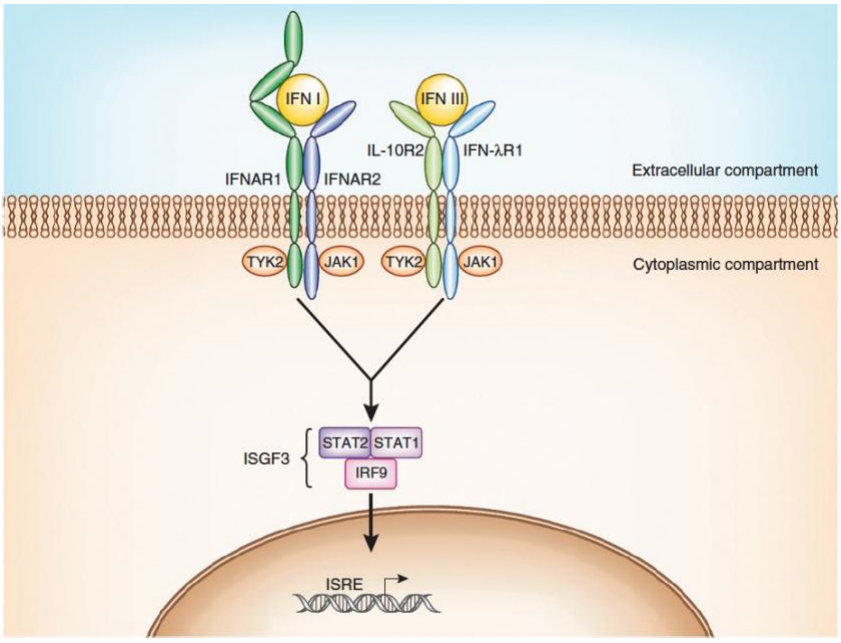


Figure 12. JAK/STAT signaling pathway. Binding of the IFN to the corresponding heterodimeric receptors triggers the downstream JAK/STAT signaling pathway which leads to the assembly of ISGF3 transcription factor and expression of the genes containing ISRE (Adapted from[152]).

It is not a surprise that HIV-1 has evolved different strategies to counter IFN signalling pathways for its own benefit to escape from IFN induced innate immunity response. HIV-1 uses accessory proteins such as Nef and Vpu to disrupt STAT1 phosphorylation. Furthermore, HIV-1 Vif protein interacts with STAT1 and promotes the ubiquitination of this protein to inhibit the induction of ISGs [158, 159].

1.15 Restriction Factors

As mentioned earlier some of the ISGs exert antiviral activities by targeting multiple steps of the HIV-1 replication cycle, these ISGs are known as restriction factors. It's been nearly two decades since the first HIV-1 restriction factor APOBEC3G was discovered. Over these years, extensive studies have been conducted to elucidate the antiviral mechanism of these restriction factors and the strategies that HIV-1 employs to evade or antagonize their antiviral activities. Most of these

restriction factors share common features such as IFN-inducibility. Most of the restriction factors are unable to restrict wildtype HIV-1, this indicates a strong positive genetic selection that has pressured HIV-1 to evolve mechanisms to antagonize these antiviral proteins. Therefore, host-pathogen coevolution can be detected in these restriction factors [160]. In addition, some restriction factors control cross-species transmission of HIV-1. In this section, details about restriction factors and how HIV-1 evades or antagonizes their antiviral activities will be discussed.

1.15.1 Apoliprotein B mRNA-editing Enzyme Catalytic Polypeptide-like 3G (APOBEC3G)

Initial studies demonstrate that Vif (Viral Infectivity Factor) deficient HIV-1 is not capable of replicating in CD4+ T cells, referred to as nonpermissive cells [161]. However, in some lymphocyte cell cultures such as SupT1 and Jurkat T cells, known as permissive cells, Vif deficient HIV-1 is able to replicate and generate infectious virus particles [162]. Further studies using heterokaryons formed between these nonpermissive and permissive cell lines demonstrate that nonpermissive cell lines produce an antiviral protein called APOBEC3G, and HIV-1 Vif protein antagonizes this host factor; thus, the HIV-1 carrying functional Vif replicates in nonpermissive cell lines [163] . APOBEC3G belongs to the family of APOBEC3 with cytidine deaminase activity and is known to have the most potent anti-HIV-1 activity among other family members. In the absence of Vif, APOBEC3G incorporates into the progeny virions, and induces G to A hypermutation in the newly synthesized HIV-1 DNA [164]. To antagonize APOBEC3G, Vif binds to the Vif binding domain of APOBEC3G and induces its proteasomal degradation by recruiting ubiquitin ligase complex [165, 166]. Thus, Vif prevents the packaging of APOBEC3G into the HIV-1 particles.

1.15.2 Tripartite-motif-containing 5α (TRIM5α)

As mentioned earlier, some restriction factors are known to act as a cross species barrier and control HIV-1 transmission among species. The inability of HIV-1 to successfully infect non-human primate species is due to the expression of TRIM5α in the old world monkeys and TRIM5Cyp in the new world monkeys such as owl monkeys. These proteins are components of cytoplasmic bodies [167, 168]. Like other members of the family of TRIM5, TRIM5α acts in a dimeric form and contains the following domains: RING domain with ubiquitin ligase activity, coiled-coil domain which is essential for dimerization, B-box domain to promote assembly of the protein, and B30.2/SPRY to recognize the viral capsid. In the case of TRIM5Cyp, B30.2/SPRY domain is substituted with CypA. Following HIV-1 entry, TRIM5α binds to HIV-1 capsid and induces the proteasome dependent degradation [169, 170]. As a consequence, the reverse transcription step including the formation of RTC is inhibited [171]. Unlike TRIM5α from non-human primates, human TRIM5α is unable to restrict HIV-1, since it is a poor binder to the capsid protein [167].

1.15.3 Bone Marrow Stromal Antigen-2 (BST-2)/ Tetherin

Studies of cells infected with Vpu-deleted HIV-1 showed that in the absence of Vpu accessory protein, fully matured HIV-1 particles are accumulated and trapped on the cell surface, suggesting that Vpu is essential for efficient HIV-1 release [172]. Microarray analyses of messenger RNA (mRNA) revealed a transmembrane protein with unknown function called Tetherin/CD37/ BST-2 which inhibits HIV-1 release in the absence of Vpu [173]. Thus, HIV-1 requires Vpu to antagonize tetherin for efficient viral release and replication. As a type II transmembrane protein, tetherin has an unusual topology which contributes to its antiviral activity. Tetherin contains a transmembrane domain, N-terminal, ectodomain, and C terminal glycosylphosphatidylinositol (GPI) anchor. These domains are all crucial in the antiviral function

of tetherin [174]. Accumulating data demonstrate that tetherin directly incorporates into HIV-1 particle, and bridges the viral membrane and the cellular membrane. Tetherin traps HIV-1 particles by forming a dimer through coiled coil regions in the ectodomain [174, 175]. Similar to other mentioned restriction factors, tetherin is IFN-inducible. Tetherin is expressed in cholesterol enriched lipid raft membrane where it is colocalized with HIV-1 Gag [176]. HIV-1 Vpu antagonizes tetherin by binding to its transmembrane domain and induces internalization and degradation of tetherin via the ubiquitin-proteasome machinery in βTrCP-dependent manner [177, 178].

1.15.4 SAM Domain and HD Domain-containing protein 1 (SAMHDI-1)

Earlier observation revealed that HIV-1 poorly infects dendritic cells and non-dividing myeloid cells including macrophages. In contrast, SIV and HIV-2 are able to infect these cells efficiently. This observation leads to the discovery of Vpx protein as the viral determinant of this viral function [179, 180]. Unlike SIV and HIV-2, *Vpx* gene is absent in HIV-1 genome [181]. Mass spectrometry analyses identified SAM domain HD domain-containing protein 1 (SAMHD1) host protein that is counteracted by Vpx viral protein [181]. SAMHD1 is IFN-inducible and highly expressed in dendritic cells and macrophages. Expression of SAMHD1 interferes with the efficiency of reverse transcription step of HIV-1 by reducing the intracellular pool of deoxynucleotide triphosphates (dNTP) [181, 182]. To antagonize SAMHDI, Vpx recruits CRL4 DCAF1 E3 ubiquitin ligase and induces proteasome-dependent degradation of SAMHD1 [183].

1.15.5 Myxovirus Resistance Gene B (MxB)

Myxovirus resistance gene B (MxB, also called Mx2) is an ISG, belongs to the family of dynamin-like large GTPases. Humans have two Mx proteins, MxA and MxB [184]. MxA is known to inhibit a range of different viruses including hepatitis B virus, influenza virus, and

measles virus [185]. The anti-HIV-1 activity of MxB was reported by three groups including our group in 2013 [186-188]. Further studies of endogenous MxB in CD4+ T cells using Cas9/gRNA to knockout MxB demonstrate an important contribution of MxB to IFN-mediated HIV-1 infection [189]. The association of MxB with HIV-1 capsid revealed that HIV-1 capsid is the main target of MxB antiviral activity [190]. MxB binds to HIV-1 capsid and reduces the production of HIV-1 2-LTR circles. Furthermore, MxB interferes with nuclear accumulation of HIV-1 DNA and the viral DNA integration step [186-188]. Mutagenesis studies of MxB demonstrate that oligomerization capacity of MxB is essential for its antiviral activity and mediates the binding of MxB to HIV-1 capsid [191]. Recently, a new study has shown that MxB expression is required for the ability of SAMHD1 to block HIV-1 infection [192].

1.15.6 T-cell Immunoglobulin (Ig) and Mucin Domain (TIM)

T- cell immunoglobin (Ig) and mucin domain (TIM) proteins are known as regulators of both the innate immunity and the adaptive immunity including T cell activation [193]. The TIM family proteins are type 1 glycoproteins and unlike other discussed restriction factors, these proteins are not IFN- inducible [193]. In 1996, human TIM-1 protein was identified as the main receptor for Hepatitis A virus (HAV) [194]. Later studies identified human TIM family proteins as entry cofactors for several enveloped viruses including Ebola virus, Marburg virus, and Dengue virus [195, 196]. The entry-mediating function of TIM family of proteins is linked to the phosphatidylserine (PS) binding capacity of TIM [197]. It is known that virus particles have PS. Thus, TIM proteins bind to virion-associated PS and increase the viral infectivity by enhancing the entry of enveloped viruses [197].

Recent studies show that TIM proteins block HIV-1 release by inducing the accumulation of HIV-1 Gag and mature HIV virions on the plasma membrane [198]. The anti-HIV-1 function of

TIM proteins is dependent on its PS-binding capacity, since mutations in PS binding domains of TIM-1 lead to an inefficient block of the HIV-1 release [198].

1.15.7 Interferon-Inducible Transmembrane Protein-3 (IFITM3)

Interferon-Inducible Transmembrane proteins (IFITMs) were among the first ISG proteins that were discovered in 1984 [199]. Five *IFITM* genes are located on chromosome 11 of humans, including IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10 [200]. The IFITM proteins are ubiquitously expressed in primary human tissues and are involved in many biological processes, including cell adhesion, germ cell differentiation, embryonic development, and tumor suppression [201]. As the name indicates, IFITM proteins are IFN-inducible, with the exception of IFITM5 [202]. This family of proteins have distinct subcellular localization. IFITM1 is mainly localized on lipid raft and early endosomes, and interacts with membrane bound proteins such as CD81 and CD19 [203]. IFITM2 and IFITM3 localize in endosomal compartments positive for Rab7 and lysosomal-associated membrane protein 1 (LAMP1) [204].

The IFITM proteins contain five major domains, N terminal domain (NTD), a conserved hydrophobic intramembrane domain (IMD/TM1), a conserved intracellular domain (CIL), a variable hydrophobic transmembrane domain (TMD2), and C terminal domain (CTD). The topology of IFITM proteins is controversial. Currently, there are three proposed topology models for IFITM proteins as shown in Figure 13. Earlier studies using tagged NTD and CTD following by detection with flow cytometry showed that both NTD and CTD of IFITM is localized extracellularly (Figure 13-i) [205]. However, later studies by characterizing the S-palmitoylation patterns of IFITM3 suggest that both NTD and CTD are located intracellularly (Figure 13-ii) [206]. Later on, using a variety of assays including protease cleavage assay and immuno-electromicroscopy analyses, Marsh group showed that NTD of IFITM is located inside the

cytoplasm while CTD is localized extracellularly. Together, these evidences suggest that IFITM proteins are type II transmembrane proteins (Figure 13-iii) [207]. The most recent topology model for IFITM proteins (Figure 13-iii) is the most acceptable IFITM topology in the field which is further supported by other groups. It is shown that CTD of IFITM3 is readily accessible at the cell surface, and adding ER retention motif KDEL to IFITM3 CTD causes sequestration of IFTIM3 into the ER [208]. Finally, Tian's group in 2016 illustrated the topology of IFITM3 using combination approaches of EPR and NMR [209]. This finding provides further support for the most recent accepted IFITM topology where NTD is intracellular and CTD is extracellular.

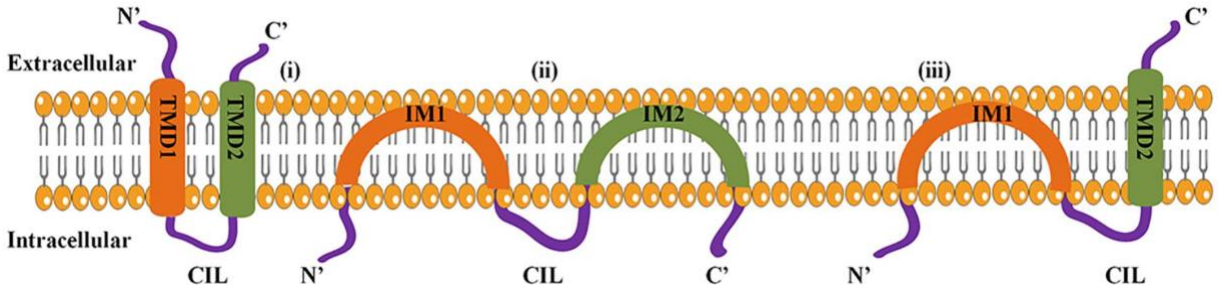


Figure 13. Overview of three suggested topology for IFITM proteins (Adapted from [210].

The antiviral function of IFITM proteins was not discovered until 1996, when mouse cells expressing IFITM proteins, in particular IFITM1, were found partially resistant to infection with vesicular stomatitis virus (VSV) [211]. Later on, the antiviral spectrum of the IFITM family of proteins greatly expanded. This suggests that IFITM proteins are important cellular antiviral factors with the capacity to inhibit over 10 different families of viruses including both DNA and RNA viruses, as summarized in the Table 2. The antiviral activity of IFITM3 has also been tested in animal models. Studies of IFITM3 knockout mice reveal the susceptibly of these mice

to several viruses including Influenza A Virus (IAV), West Nile virus (WNV), and Respiratory Syncytial Virus (RSV) [212].

The antiviral activity of IFITM proteins against HIV-1 was first reported by our group in 2011 [213]. In this study we showed that knockdown of IFITM1 increases HIV-1 production and infectivity. Furthermore, overexpression of IFITM proteins in CD4+ SupT1 cells profoundly inhibit HIV-1 replication by interfering with virus entry [213]. Later studies shed more light on the antiviral mechanism of IFITM proteins. IFITM proteins get incorporated into HIV-1 virions and impair the fusogenecity of the HIV-1 particles [214]. Furthermore, HIV-1 Env determines the susceptibility of the virus to IFITM3 inhibition [215]. Details on how IFITM proteins inhibit HIV-1 infection by targeting HIV-1 Env and how HIV-1 Env evades the inhibition by IFITM proteins will be discussed in Chapter 2.

Table 2. Summary of viruses inhibited by IFITM proteins. (Adapted from [210]).

Family	Name of the Virus	Envelope	References
DNA Viruses			
Asfarviridae	African swine fever virus	Yes	[216]
Poxviridae	Vaccinia virus	Yes	[217]
Irodiviridae	Rana grylio virus	Yes	[218]
RNA Viruses			
Orthomyxoviridae	Influenza A and B virus,	Yes	[219, 220]
Flaviviridae	West Niles virus, Dengue virus, Hepatitis C virus, Avian tembusu virus, Zika virus	Yes	[204, 219]
Rhabdoviridae	Rabies virus, Lagos Bat virus,	Yes	[220] [205]
Bunyaviridae	La Crosse virus, Hantaan virus, Andes Virus Rift Valley fever	Yes	[221]
Filoviridae	Ebola virus, Marburg virus	Yes	[222]
Alphaviridae	Sindbis and Semliki Forest virus	Yes	[203]
Coronaviridae	SARS Corona virus	Yes	[222]
Retroviridae	HIV-1, Jaagsiekte sheep retrovirus	Yes	[219, 223]
Paramyxoviridae	Human respiratory syncytial virus	Yes	[224]
Reovirus	Reovirus	No	[225]

1.15.8 Serine-Incorporator 5 (SERINC5)

In 1994, an initial report showed that HIV-1 lacking Nef protein has lower infectivity compared to Nef-positive viruses [226]. HIV-1 Nef is a myristoylated, 27 kDa accessory protein which contributes to the maintenance of high viral load in patients [227]. Furthermore, the effect of Nef on HIV-1 infectivity is maintained by strong selective pressure during the disease progression [228]. The role of Nef in viral pathogenesis is manifested by the association of Nef mutants with slow disease progression in both HIV-1 and SIV infections [229]. Nef interacts with a large set of cellular proteins to downregulate important immune molecules on the surface of HIV-1 infected cells, thus protects the infected cell from immune surveillance to promote viral dissemination [230]. Nef downregulates cell surface CD4 receptor to prevent re-infection and avoids antibody-dependent cell-mediated cytotoxicity (ADCC) [231, 232]. Furthermore, Nef also downregulates cell surface major histocompatibility complex I (MHC-I) to protect the infected cells from killing by the cytotoxic T cells (CTLs) [233, 234]. Another strategy that Nef employs to protect HIV-1 from CTLs is by inducing apoptosis in bystander cells by upregulation of FasL [234]. Nef also interacts with several kinases, and thereby interferes with various signaling pathways in T cells such as serine kinases [235].

Early studies suggested that Nef enhances the infectivity of HIV-1 by downregulating CD4 and increasing the levels of Env in the virus particles [236]. However, it was later shown that the effect of Nef on HIV-1 infectivity was maintained in CD4-negative virus producer cells and Nef restricts the susceptibility of HIV-1 particles to proteasomal degradation [237]. More recent findings show that Nef responsiveness is associated with Env variable regions and that the effect of Nef on the infectivity of HIV-1 is more pronounced in X4-tropic Env containing virions compared to R5-tropic HIV-1 [238]. After twenty years of extensive studies on elucidating how

Nef increases HIV-1 infectivity, two groups identified Serine-Incorporator 5 (SERINC5) as the cellular protein that inhibits HIV infectivity and is antagonized by Nef [239, 240].

SERINC5 belongs to the family of SERINC proteins that contains 5 members, including SERINC1 to 5 with 58%-31% amino acid homology. This family of proteins are predicated to contain 10-12 transmembrane domains, and they lack homology to any other known proteins [241]. The expression of SERINC proteins is highly conserved from yeasts to mammals. At the physiological level, SERINC proteins are known to mediate the incorporation of serine amino acid into sphingolipids and phosphatidylserine [241]. Recently, using Cryo-EM approaches, the structure of human SERINC5 was deciphered (Figure 14). In this study, it is shown that SERINC5 contains 10 transmembrane helicases that are organized into two subdomains [242].

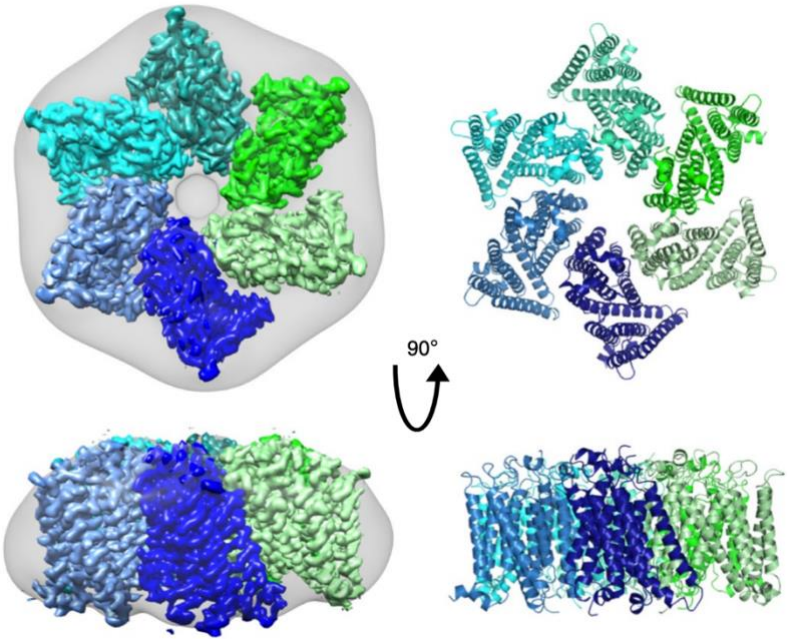


Figure 14. Cryo-EM map of recombinant human SERINC5 and Drosophila melanogaster SERINC (DmSERINC). Each protomer is coloured individually. The grey indicates detergent micelle [242].

Two distinct approaches from two different groups lead to the discovery of the antiviral activity of SERINC5 in 2015. Göttlinger's group identified SERINC5 by proteomic analysis of HIV-1 virions produced by T-lymphoid cells infected with Nef+ or Nef- HIV-1 particles. SERINC3 and SERINC5 were not identified in Nef+ virion samples [239]. On the other hand, Pizzato's group identified SERINC5 by comparing the infectivity of Nef+ and Nef- viruses from 31 different human cell lines. Next, by generating heterokaryons derived from cell lines with different Nef responses, his group was able to identify SERINC5 whose expression correlates with the Nef response to HIV-1 infectivity [240]. Knockout and overexpression experiments of SERINC5 illustrate that in the absence of Nef, SERINC5 incorporates into the virus particles, impairs the fusogenicity of the virions, and reduces HIV infectivity up to 100-fold [239, 240]. The antiviral activity of SERINC5 is counteracted by Nef. Nef prevents SERINC5 association into HIV-1 virions by inducing the endocytosis of SERINC5 into Rab7-positive endosomes [239, 240]. Later on, studies show that Nef uses a similar mechanism to downregulate SERINC5 and CD4 and targets them to lysosomes for degradation [243]. Furthermore, the interaction between Nef and clatherin adapted AP-2 is indispensable for the ability of Nef to internalize SERINC5 [243]. Studies by the Kirchhoff group show that the anti-SERINC5 activity of Nef is a fundamental property of lentiviral Nef proteins, which is species-independent. Furthermore, the potency of SERINC5 antagonism of Nef correlates with the prevalence of SIV, which suggest SERINC5 as a determinant of viral spread [244].

SERINC5 does not follow the characteristics of a traditional host restriction factor such as Tetherin or IFITM3. Unlike other known restriction factors, SERINC5 is not IFN-inducible. Moreover, evolutionary arm race study did not detect strong positive selection on SERINC5 [245]. Therefore, SERINC5 is known as an unconventional restriction factor [246].

It is reported that SERINC5 interferes with the HIV-1 entry. However, the exact mechanism of how SERINC5 impairs HIV-1 entry is not completely known. Details on SERINC5 antiviral activity and how HIV-1 evades SERINC5 antiviral function will be further discussed in Chapter 2.

In addition to Nef, Env of some HIV-1 strains has been reported to overcome SERINC5 inhibition. However, the mechanism by which Env resists SERINC5 is not known [239, 240]. Furthermore, the inhibitory pressure of SERINC5 is not limited to HIV-1, since the glycosylated Gag protein of murine leukemia virus (MLV), glycoproteins of VSV and Ebola virus counteract SERINC5 [240]. Recent work by the Pizzato group showed that the accessory protein of lentivirus equine infectious anemia virus (EIAV), called S2, also antagonizes t SERINC5 [247]. They showed that similar to Nef, S2 recruits the AP-2 complex to exclude SERINC5 from virus particle [247]. Furthermore, Glycogag protein from MLV downregulates SERINC5 via the endosome/lysosome pathway [248]. These findings further support the critical role of SERINC5 in restricting diverse retroviruses infecting different species.

1.16 Thesis objectives

The goal of my PhD project is to characterize the antiviral activity of SERINC5 and elucidate how HIV-Env antagonizes SERINC5 inhibition. In Chapter 2, I will provide a comprehensive review on SERINC5, IFITM3, and other restriction factors that target HIV-1 Env. I will discuss how HIV-1 Env has evolved different strategies to evade or escape from these restriction factors. The objectives of my PhD research are three folds:

1. To determine HIV-1 Env resistance to SERINC5 (Chapter 3).

The first objective of this thesis is to determine the ability of HIV-1 Env to antagonize SERINC5 inhibition. This is the first study to show that in addition to Nef, HIV-1 Env also

counters SERINC5 inhibition. Furthermore, I will present the results of characterizing the inhibitory effect of SERINC5 on HIV-1 Env by showing that virion-associated SERINC5 sensitizes HIV-1 to neutralizing antibodies.

2. To characterize the susceptibility profile of primary HIV-1 Env isolates to SERINC5 inhibition (Chapter 4).

After finding the anti-SERINC5 activity of HIV-1 Env in Chapter 3, the second objective of this thesis is to measure the effect of SERINC5 on a large panel of primary HIV-1 Env clones that were isolated from different stages of HIV-1 infection, including transmission, acute, and chronic stages. I observed that all these Env clones resist SERINC5 inhibition. In contrast, when I examined the responses of these Env clones to another restriction factor IFITM3, I observed that while the T/F HIV-1 Env showed resistance to IFITM3, this resistance phenotype was gradually lost as infection progressed into the chronic stage.

3. To determine which HIV-1 Env property confers resistance to SERINC5 (Chapter 4).

I have investigated whether the efficiency of using CD4 and/or CCR5 by Env has correlation with its susceptibility to SERINC5 inhibition. I observed that SERINC5- resistant HIV-1 Env had lower affinity to CD4. This finding led me to test CD4 mimetic M48U1 which induces Env trimer to an "open" conformation. Excitingly, M48U1 renders SERINC5-resistant HIV-1 Env sensitive.

Chapter 2

HIV-1 Envelope Glycoprotein at the Interface of Host Restriction and Virus Evasion.

2.1 Preface

This chapter is adapted from the following published review article:

Beitari, S., Wang, Y., Liu, SL., Liang, C. HIV-1 envelope glycoprotein at the interface of host restriction and virus evasion. Viruses, 2019. 11(311). DOI: 10.3390/v11040311

In chapter 2, I will review the growing body of research unveiling host restriction factors targeting viral entry and the HIV-1 Env protein. I will discuss the molecular mechanisms on how restriction factors inhibit HIV-1 Env protein. Furthermore, I will focus on strategies employed by the HIV-1 Env protein to counter these host restriction factors. Finally, I will highlight the cooperation between innate immunity and adaptive immunity to effectively inhibit and suppress HIV-1 infection.

Author contributions:

S.B., Y.W., S.-L.L., and C.L. prepared the manuscript.

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2.2 Introduction

The HIV-1 envelope (Env) protein is synthesized as a gp160 precursor at the endoplasmic reticulum (ER). Following complex glycosylation and cleavage by furin proteases at the trans-Golgi complex, the mature gp120/gp41 trimer travels to the plasma membrane where it joins HIV-1 Gag proteins, forming infectious virus particle [24]. To start a new round of infection, gp120 binds to the CD4 receptor on the surface of the target cell, which triggers conformational changes of the gp120/gp41 trimer, exposing the binding site in gp120 for the CCR5 or the CXCR4 co-receptor. Engagement of the co-receptor leads to the exposure of the fusion peptide followed by the assembly of the 6-helix bundle in gp41, causing the fusion of viral and cellular membranes. Membrane fusion begins with the joining of both membranes' outer lipid leaflets in a process called hemifusion. Continued fusion of the two membranes leads to the formation of the fusion pore. The fusion pore further dilates to an adequate size for the delivery of HIV-1 RNA within the core structure into the cytoplasm.

As the only viral protein on the surface of HIV-1 particles, the Env protein represents the chief target for recognition by the host adaptive immune system, leading to the production of antibodies that recognize and bind to Env protein. Despite the various strategies that HIV-1 has exploited to evade neutralizing antibodies, including heavy glycosylation to mask the epitopes [249]. a series of antibodies that are able to neutralize diverse HIV-1 strains has been isolated from HIV patients, called broadly neutralizing antibodies (bnAbs) [250]. The discovery of bnAbs has fueled HIV vaccine research through the characterization of bnAb epitopes on the Env protein and the elucidation of the B cell generation of bnAbs [251].

In addition to neutralizing HIV-1 particles, some Env-targeting antibodies are able to recruit natural killer (NK) cells through engaging the FC receptor, and kill HIV-1 infected cells by

antibody-dependent cell-mediated cytotoxicity (ADCC) [252]. The ADCC-mediating Env Abs were isolated from subjects enrolled in the RV144 vaccine trial, which has shown a modest 31.2% protection efficacy [122]. Not surprisingly, HIV-1 has mechanisms to evade ADCC, including the use of accessory proteins Vpu and Nef to downregulate CD4, which otherwise interacts with the Env protein to expose epitopes of ADCC-triggering antibodies [253].

2.3 HIV-1 Env Is Attacked by Host Innate Immunity

In addition to the constitutively active intrinsic innate immune mechanisms, the main stream of innate immunity is elicited upon host recognition of pathogen-associated molecular patterns (PAMPs) by a group of host proteins that act as pathogen recognition receptors (PRRs). This recognition event triggers signaling cascades that lead to the expression of cytokines, including interferons (IFNs). One function of these cytokines is to induce the expression of proteins that can directly restrict viral infections. The other equally important function of cytokines is to activate immune cells and initiate the pathogen-specific adaptive immune response.

By acting together, the constitutively expressed and interferon-induced antiviral proteins form the first line of host defense against viral infections. These antiviral proteins operate by a variety of molecular mechanisms to target distinct steps in the virus replication cycle. As viral nucleic acids are the main PAMPs to induce innate immune response, they have also become the target of many cellular antiviral factors (reviewed in [254]). Examples include (1) nucleases, such as ISG20, OAS/RNase L, and three primer repair exonuclease 1 (TREX1) that degrade viral RNA or DNA; (2) deaminases, such as adenosine deaminases acting on RNA (ADAR) and apoliprotein B editing complex 3 (APOBEC3) proteins that edit and mutate the viral genome; (3) dNTP hydrolase SAM domain and HD domain-containing protein 1 (SAMHD1), which diminishes the cellular DNA pool and inhibits viral DNA synthesis; (4) factors, such as

Myxovirus resistance 1, 2 (Mx1, Mx2), and Tripartite motif-containing protein 5 (Trim5alpha) that target the replication complex of viral genome and block viral multiplication; and (5) factors such as schlafen 11 (SLFN11) and protein kinase activated by RNA (PKR), which inhibit the translation of viral RNA [182, 213, 255-260]

In addition to these diverse cellular mechanisms that attack viral nucleic acids, recent studies have discovered an array of cellular proteins that contribute to the control of viral infection by targeting viral Env and inhibiting viral entry, which began to unravel a new layer of host antiviral defense. Through these studies, we have come to appreciate the combinatorial strategy that cells have evolved to restrict viral entry by targeting virtually every stage of Env's life, from Env synthesis and maturation, to its incorporation into virus particles, to its execution of membrane fusion.

2.4 A Long and Challenging Journey for Env Protein to Reach Virus Particles

From its de novo synthesis at the ER, HIV-1 Env protein travels through the trans-Golgi complex, arriving at the plasma membrane to join HIV-1 Gag proteins, together forming infectious progeny virions. Env can choose to detour to endosome recycle compartments (ERC), where it reaches the particle assembly site on the plasma membrane by interacting with FIP1C and Rab14 [261, 262]. Along this journey, Env undergoes complex glycosylation, trimerization, and cleavage by furin, providing ample opportunities for the host cell to attack (Figure 15).

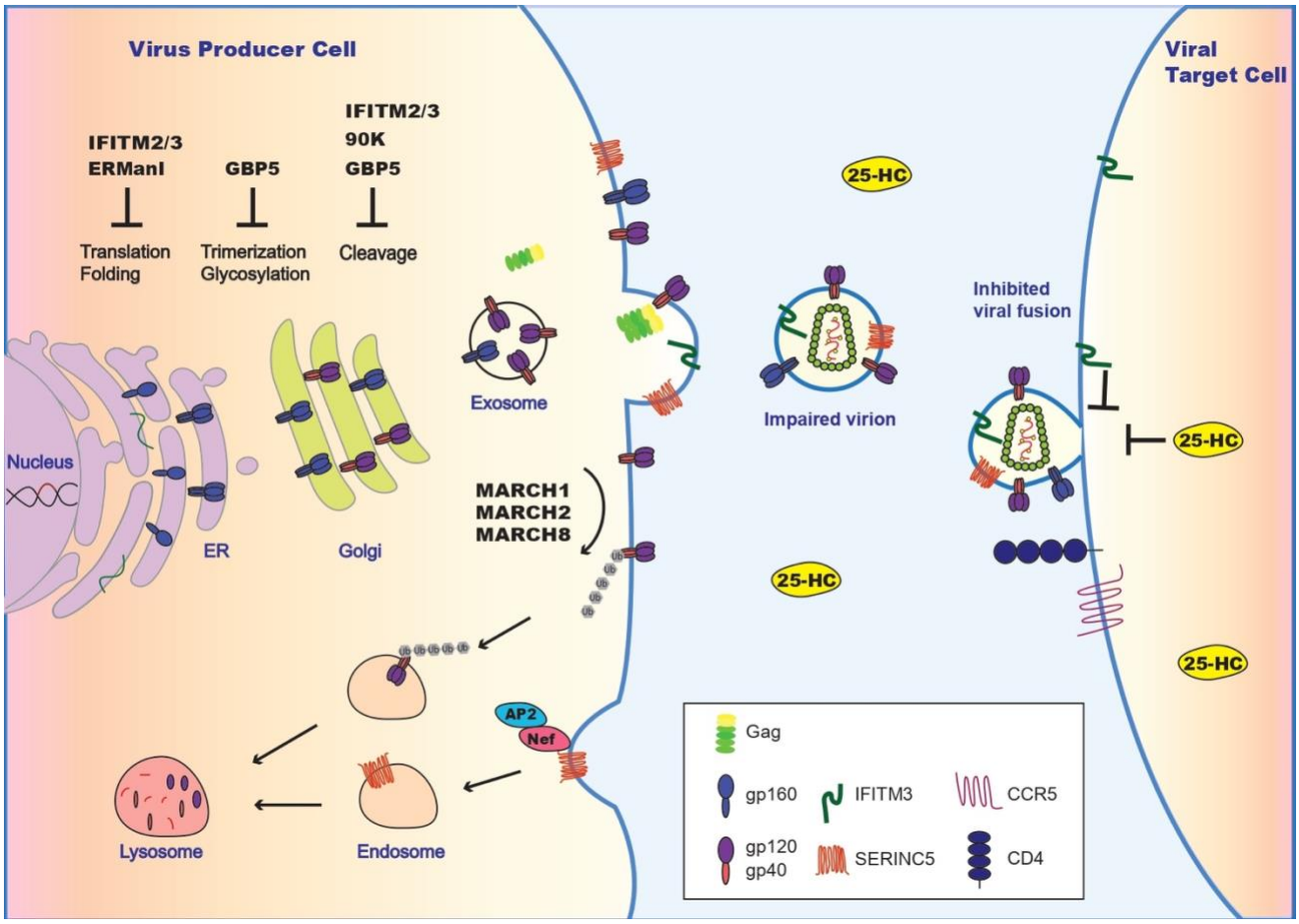

Figure 15. Inhibition of HIV-1 entry by restriction factors and viral counter measures. Illustrated are restriction factors that operate in virus producer cells and inhibit Env synthesis at the endoplasmic reticulum (ER) (by IFITM2/IFITM3 and ERManI), impair Env maturation at Golgi (by IFITM2/IFITM3, GBP5, and 90K), and downregulate Env at the plasma membrane (by MARCH1/MRCH2/MARCH8). IFITM2/IFITM3 and SERINC5 get incorporated into HIV-1 particles and impair viral membrane fusion. In virus target cells, IFITM2/IFITM3 and 25-HC deter viral entry. HIV-1 uses Nef to downregulate SERINC5. The other viral countering strategies are summarized in Table 1.

2.4.1 ER-Associated Degradation: Traps at Its Place of Birth

The newly synthesized Env precursor, gp160, folds at the ER with low efficiency The often misfolded Env protein is subjected to ER-associated degradation (ERAD), a process that controls and eliminates misfolded proteins [263]. One ER protein, called ERManI, has been shown to

promote degradation of ER-associated proteins by ERAD. ERManI is a class I α-mannosidase and belongs to the glycosidehydrolase family 47 (GH47) α-mannosidases, which are carbohydrate-active enzymes [264]. GH47 α-mannosidases mediate the trimming of α-1,2-mannose residues from Man9GlcNAc2, among which ERManI is the first enzyme to generate Man8GlcNAc2 [265]. ERManl interacts with HIV-1 Env via its luminal catalytic domain, and mutation of the catalytic sites ablates its activity in degrading HIV-1 Env protein [266]. This function of ERManl is specific, because overexpression of other α-mannosidases from the family of GH47 α-mannosidases, such as ER-degradation enhancing α-mannosidase-like (EDEM) proteins 1, 2, and 3, does not affect HIV-1 Env expression [267]. It is thus speculated that ERManI operates by modulating glycosylation of HIV-1 Env. Interestingly, ERManI is required for the function of a mitochondrial translocator protein called TSPO in diminishing HIV-1 Env expression via ERAD since ERManI depletion abolished TSPO-mediated Env degradation [267, 268].

The antiviral activity of ERManI is not limited to the degradation of HIV-1 Env protein, and the hemagglutinin (HA) glycoprotein of influenza virus is also a target of ERManI and the ERAD pathway [269]. To date, viral countermeasures of ERManI have not been reported [267]. However, HIV-1 Vpr has been shown to increase Env expression [270]. In the absence of Vpr, Env tends to misfold and is targeted to the ERAD pathway for degradation. The N-terminal region of Vpr controls this activity, since a single A30L mutation disrupts the function of Vpr to increase Env expression [270]. It remains to be determined whether Vpr directly interacts with ERManI to save Env from degradation at the ER.

The ERAD-mediated degradation of HIV-1 Env can be exploited for therapeutic purposes. One example is the depletion of HIV-1 gp160 precursor from the ER via the ERAD pathway by an

engineered molecule called degradin, which contains gp120-targeting antibody chains and the C-terminal sequence of ER-resident protein SEL1L [271]. By a similar mechanism, the small peptide glycine-prolyl-glycine amide (GPG-NH2) abolishes HIV-1 infectivity by targeting viral Env to the ERAD pathway for degradation [272]. The ERAD pathway appears to be a double-edged sword, since HIV-1 Vpu hijacks this protein degradation mechanism to remove CD4 from the ER [273]. Premature contact of Env with CD4 is thus avoided to ensure that Env is safely transported to the plasma membrane for virus assembly.

2.4.2 GBP5, 90K, and IFITM3: Blocks Along Env's Route to the Virus Assembly Site

The precursor of HIV-1 Env, gp160, trimerizes at the ER then moves to the trans-Golgi apparatus where it is cleaved to become gp120/gp41, forming a mature Env trimer [24]. Products of three interferon-stimulated genes (ISGs), GBP5 (guanylate binding protein 5), 90K, and IFITM2/3 (IFN-induced transmembrane protein 2 and 3), have been shown to obstruct gp160 cleavage and diminish the incorporation of functional mature gp120/gp41 trimers into HIV-1 particles [274-276].

GBP5 is a member of IFN-inducible guanosine triphosphatases (GTPases) [277]. Members of the GBP family have been reported to antagonize a variety of invading pathogens including viruses, bacteria, and protozoa [278]. GBP1, a protein that is closely related to GBP5, inhibits a number of viruses including dengue virus, hepatitis C virus (HCV), encephalomyocarditis virus, and vesicular stomatitis virus (VSV) [279, 280]. GBP5 was identified as a potential anti-HIV-1 factor in a genome-wide study for human genes sharing evolutionary signature of known restriction factors [281]. To supplement this identification, levels of GBP5 in primary CD4+ T cells and macrophages are enhanced by IFN-α, IFN-γ, IL-2, and TCR activation [274, 277, 282]. Not only does ectopic expression of GBP5 reduce the infectivity of HIV-1 particles by

diminishing virion incorporation of gp120/gp41, depletion of GBP5 in primary macrophages elevates HIV-1 infectivity by enhancing the incorporation of mature gp120/gp41 into virions [274]. GBP5's location is crucial to its inhibitory effect on HIV-1; the intact C-terminal domain responsible of localizing GBP5 in the Golgi apparatus is required in lieu of GTPase activity. Ectopically introduced GBP5 causes two defects to HIV-1 Env protein as detected with Western blotting; gp160 cleavage is impaired, and glycosylation of HIV-1 Env is altered. it was thought that within the GBP family, this anti-HIV-1 function appears to be specific to GBP5, as GBP1 does not affect HIV-1 Env despite its trans-Golgi localization. However, recent studies demonstrate that GPB2 also shares this antiviral activity with GBP5 [283]. Efforts to understand the molecular mechanism of GBP2/5 reveal that these proteins reduce the proteolytic activity of cellular protein furin. Hence, in addition to retroviruses, GBP2/5 target diverse viral glycoproteins including but not limited to ZIKA, Ebola, and influenza. However, viruses carrying VSV glycoprotein are immune from inhibition by GBP2/5 since they do not require host protease [283].

HIV-1 has a "trade-off" mechanism to partially overcome GBP5 inhibition through shutting down Vpu expression. Since Vpu and Env are synthesized from a single bicistronic mRNA, shutting down Vpu expression increases Env expression, which confers partial resistance to GBP5 [284]. Interestingly, the Vpu mutation that causes a loss of Vpu expression was identified in macrophage-tropic HIV-1 and some brain-derived HIV-1 strains, indicating that HIV-1 might have been pressured to resist high levels of GBP5 in macrophages [274, 285].

The 90K protein (also known as Mac-2BP or LGAL3SBP) is an IFN-inducible, secreted immunostimulatory glycoprotein; it belongs to the family of scavenger receptor cysteine-rich (SRCR) domain-containing proteins [286]. In response to IFN-α stimulation, levels of 90K

increase in various T cell lines, primary CD4+ T cells, and primary macrophages. Elevated levels of 90K have been reported in HIV-1 infected patients; hence, it was proposed as a serological marker of disease progression to AIDS [287, 288]. 90K is N-glycosylated in the ER and Golgi complex before entry to the secretory pathway, thereby sharing the same route of trafficking and modification with HIV-1 Env protein [289] Ectopic expression of 90K causes an accumulation of gp160, concomitant reduction of gp120, and loss of mature gp120/gp41 in HIV-1 particles, which result in impaired infectivity of nascent HIV-1 particles [275]. In addition, knockdown of 90K with siRNA in primary macrophages increases virion-associated gp120 and HIV-1 infectivity. It is also noted that both GBP5 and 90K are highly expressed in macrophages and may contribute to the low infection of macrophages by HIV-1. 90K inhibits both R5 and X4 HIV viruses [275]. While 90K also affects the furin-dependent maturation of Ebola GP, it minimally changes the processing of influenza virus HA0 protein and cellular glypican-3, suggesting a selectivity of 90K action on furin substrates. Lastly, the inhibitory effect of 90K on HIV-1 Env might be indirect, given the lack of detectable interaction of 90K with HIV-1 Env [275].

The anti-HIV-1 activity of 90K has been further mapped to the two central protein-binding domains of BTB-POZ and IVR, whereas the N-terminal scavenger receptor cysteine rich (SRCR)-like domain is dispensable [275]. However, a mutagenesis study by a different group showed that one truncation mutant of 90K (1-95) inhibits Env processing, while another 90K mutant (124-585) inhibits virion production [290]. Studies by Wang and colleagues also showed that 90K inhibits HIV-1 virion production by interacting with Gag and vimentin (VIM) in trapping HIV-1 Gag to VIM filaments, suggesting an alternative anti-HIV-1 mechanism by 90K [290]. It is unknown whether HIV-1 has adopted any mechanisms to counter this factor. The antiviral function of 90K is conserved among primates except the rhesus macaque [291].

Interestingly, 90K impairs gp160 processing and reduces levels of gp120 on the plasma membrane in other species, including the rhesus macaque; however, these functions do not always reduce the infectivity of nascent HIV-1 virions [291]. Further studies have shown that the impairment of mature gp120 incorporation into virions might also contribute to the antiviral action of 90K [291].

IFITM3 also causes the accumulation of gp160 and loss of mature gp120/gp41 in HIV-1 particles [276]. IFITM3 is a member of the IFITM family, including IFITM1, IFITM2, IFITM3, IFITM5, and IFITM10. Among these, IFITM1, 2, and 3 are interferon-inducible and have been shown to inhibit a wide range of viruses (Reviewed in [292]). Ectopic expression of IFITM3, and to a lesser extent IFITM2, impairs gp160 cleavage, promotes gp120 shedding, and diminishes the level of mature gp120/gp41 in HIV-1 particles, thus reducing HIV-1 infectivity [215, 276]. In departure from GBP5 and 90K, IFITM3 has been shown to associate with both gp160 and gp120/gp41, which may allow IFITM3 to directly interfere with processing of the gp160 precursor. In addition to interfering with the processing of gp160, IFITM3 expression in virus producing cells decreases the amount of Env in virions [293]. More work is required to investigate whether IFITM3 inhibits the formation of the Env trimer or the clustering of the trimer into the virions.

2.4.3 MARCH1, MARCH2, and MARCH8: Removing HIV-1 Env from the Cell Surface

Reaching the plasma membrane does not warrant safety for Env. MARCH8 (membrane-associated RING-CH 8) has been recently reported to modify HIV-1 Env and envelope proteins of other viruses to further downregulate them from the cell surface. MARCH8 is one of the 11 members of the MARCH family of RING-finger E3 ubiquitin ligases. As a transmembrane protein, MARCH8 bears a C4HC3 RING finger domain in the N-terminal cytoplasmic tail that

recruits the E2 enzyme [294, 295]. MARCH8 is involved in downregulating multiple transmembrane proteins, including but not limited to MHC-II [296], TRAIL receptor 1, and transferrin receptor [297]. Association of MARCH8 with these cellular transmembrane proteins often causes polyubiquitination of the target protein, followed by its trafficking to lysosomes for degradation. Another recently reported substrate for MARCH8 is BST-2, and MARCH8 regulates its ubiquitination, trafficking, and turnover [298]. Given its prolific regulation of cellular transmembrane proteins, it is thus not surprising that MARCH8 also targets viral envelope proteins and downregulates them from the cell surface [299].

When ectopically expressed, MARCH8 antagonizes not only HIV-1 Env but also glycoproteins of HIV-2, SIV, MLV, xenotropic MLV-related virus (XMRV), and VSV, suggesting a broad antiviral function. Mutating the RING domain, such as CS and W114A mutations, abrogates the antiviral activity of MARCH8, which demonstrates its dependence on E3 ligase activity. It is important to note that MARCH8 may impair different viral glycoproteins by different mechanisms. For example, MARCH8 removes HIV-1 Env from the cell surface, which is then retained within lysosomes without degradation. As a result, the total level of Env protein in cells does not change. In contrast, VSV G protein is downregulated by MARCH8 both at the cell surface and within the cell due to its degradation in lysosomes. Regardless of mechanistic details, MARCH8 interacts with both HIV-1 Env and VSV G proteins and likely alters their levels through ubiquitination. Further examination of antiviral activity exhibited by other members of the MARCH family revealed that MARCH1 and MARCH2 have antiviral functions similar to those observed in MARCH8; these members of MARCH family inhibit HIV-1 infectivity by downregulating HIV-1 Env from the cellular surface and reducing the levels of Env incorporated into the virions [300, 301]. Similar to MARCH8, MARCH1 and MARCH2 are also localized at

the plasma membrane [300]. As observed for MARCH8, MARCH1 also gets incorporated into virions; however, virion incorporation of MARCH2 remains controversial [300]. One group showed that HIV-1 infection increased MARHC2 expression but MARCH2 was not detected in the released virus particles [301], whereas another group showed that, similar to MARCH1 and MARCH8, MARCH2 is also found in progeny virions [300].

Higher levels of MARCH1, MARCH2, and MARCH8 were detected in myeloid cells such as monocyte-derived macrophages (MDM) and monocyte-derived dendritic cells (MDDCs) in comparison to primary CD4+ T cells. Unlike MARCH8, expression of MARCH1 and MARHC2 is highly inducible by type I IFN in MDM and MDDCs [299, 300]. Knockdown or knockout of MARCH8 in myeloid cells increases HIV-1 infectivity, suggesting MARCH8 as one of the cellular factors that restrains HIV-1 infection of macrophages and dendritic cells. HIV-1 Vpr, Nef, and Vpu do not antagonize MARCH proteins; as a result, it remains to be determined how HIV-1 and other viruses, especially those that replicate in macrophages and dendritic cells, evade inhibition by MARCH1, MARCH2, and MARCH8. Since these MARCH proteins remove HIV-1 Env from the plasma membrane, it is speculated that HIV-1 takes advantage of these proteins to escape immunosurveillance.

2.4.4 Cellular Antagonists of Env Protein in HIV-1 Particles

In addition to targeting Env in the infected cells and preventing its incorporation into virus particles, some cellular factors such as IFITM3 and SERINC5 also find their way into virus particles and block the fusion of viral membrane and cellular membrane.

Beyond its impairment of gp160 processing in HIV-1 producing cells, IFITM3 is incorporated into HIV-1 particles. Virion incorporation of IFITM3 is at least partially due to its interaction with HIV-1 Env protein, as shown by co-immunoprecipitation [276, 302]. Compared to IFITM3,

IFITM2 demonstrates weaker inhibitory activity, whereas IFITM1 shows the least anti-HIV-1 activity [214, 215, 276]. One mechanism behind this impairment of HIV-1 infectivity is the reduction of gp120 in HIV-1 particles when HIV-1 is produced from 293T cells transfected with IFITM3 DNA and proviral DNA [276]. Alternative mechanisms may also exist, since IFITM3-bearing HIV-1 particles produced from CD4+ U87 cells are also less infectious but without detectable defects in viral Env protein [303]. In addition to HIV-1, IFITM proteins have also been detected in the particles of a large group of enveloped viruses, namely, murine leukemia virus (MLV), Mason–Pfizer monkey virus (MPMV), VSV, measles virus (MeV), Ebola virus (EBOV), West Nile virus (WNV), dengue virus (DENV), and Epstein–Barr virus (EBV), all leading to a decreased viral infectivity [304]. IFITM proteins' broad spectrum of antiviral activity suggests a general mechanism that recruits IFITM proteins into different viruses to dampen viral infectivity.

Another factor in virions, SERINC5, is a member of the serine incorporator (SERINC) family. As its name indicates, SERINC proteins are involved in the synthesis of two serine-containing lipids: phosphatidylserine and sphingolipids [241]. In 2015, two groups reported that in the absence of HIV-1 Nef protein, SERINC5, and to a lesser extent SERINC3, is incorporated into HIV-1 particles and impairs HIV-1 infectivity [239, 240]. SERINC5 contains 11 transmembrane domains, and is associated with lipid rafts where HIV-1 particles often form. Presence of SERINC5 in HIV-1 particles obstructs the formation of the viral fusion pore [305], thus inhibiting HIV-1 entry into target cells. This mechanism of action likely results from an increased rigidity of viral membrane that bears clustered SERINC5, rather than altered lipid composition of viral membrane by SERINC5 [306]. SERINC5 interferes with the conformation of the MPER region of Env, which may contribute to its inhibition of Env-mediated cellular

entry [305, 307]. This is further supported by new study demonstrating the interaction between Env and SERINC5 and reporting that SERINC5 inhibits Env clustering and induces Env to an open conformation .

HIV-1 and other viruses have evolved countermeasures to antagonize SERINC5. HIV-1 Nef protein downregulates SERINC5 from the plasma membrane via the endosome/lysosome pathway, thus preventing SERINC5 incorporation into HIV-1 particles [243]. Interestingly, the Env protein of some HIV-1 strains are refractory to SERINC5 inhibition, which is analogous to the SERINC5-resistant property of VSV G, Ebola GP, and other viral envelope proteins [239]. In addition to these viral antagonists, the glycoGag protein of gammaretroviruses (such as MLV) and the S2 protein of equine infectious anemia virus (EIAV) have also been reported to overcome the antiviral function of SERINC5 [247, 308]. The anti-SERINC5 strategies from different viruses indicate its broad antiviral function.

2.4.5 Env Antagonists in the Membrane of Target Cells: the Other Half of the Fusion Story

HIV-1 entry is marked by the fusion of viral and target cell membranes; this process is driven by the sequential conformational changes of Env trimer as a result of binding to receptor CD4 and co-receptor CCR5 or CXCR4. In addition to the series of host inhibitory mechanisms discussed above present in virus producing cells that target and disable Env in the viral membrane, target cell membrane is also equipped with mechanisms to prevent fusion with viral membrane. One prominent example is the antiviral function of IFITM proteins, which was discovered in a genome-wide siRNA screen for host factors that modulate the infection of influenza A virus [219]. Subsequently, IFITM proteins were shown to inhibit HIV-1 entry in a shRNA-based screen aiming to identify anti-HIV-1 ISGs [213]. Mechanistic studies further revealed that these IFITM proteins hamper viral membrane hemi-fusion and/or block the formation of fusion pore in

virus target cells, due to the increased rigidity and altered curvature of IFITM-bearing cellular membranes [223, 309]. IFITM proteins may modulate membrane fluidity by interfering with intracellular cholesterol homeostasis [223, 309-311], although the involvement of cholesterol is still controversial [311]. Entry deterrence of incoming viruses also benefits from the subcellular localization of IFITM proteins at the plasma membrane and in endosomes/lysosomes, which covers the route of virus entry [312-314]. The N-terminal sequences of IFITM2 and IFITM3 bear the YMEL motif that binds to AP-2, and guides its endosomal and lysosomal localization via the endocytic pathway [314]. In contrast, IFITM1 lacks this endocytic motif; rather, it carries a KR-sorting motif at the C-terminus, which guides IFITM1 trafficking to recycling/early endosomes [208, 315].

IFITM proteins are not the sole defense in target cells against virus entry. Cholesterol-25-Hydroxylase (CH25H), another ISG, protects target cells from viral infection [316]. CH25H produces a soluble oxysterol, 25-hydroxycholesterol (25-HC), which inhibits a large group of viruses including murine herpesvirus 68 (MHV68), VSV, Zika virus, HIV-1, herpes simplex virus 1 (HSV-1), EBOV, Nipah virus, Russian Spring–summer encephalitis virus, Rift Valley fever virus, and hepatitis C virus [317-320]. In addition to protecting its producer cells, 25-HC can be secreted to restrict virus entry into surrounding uninfected cells [318]. While 25-HC has been reported to regulate cholesterol biosynthesis and maintain cholesterol homeostasis, this function has been challenged by the observation that CH25H-deficient mice demonstrated normal cholesterol metabolism [321-323]. Clinical evidence from patients suffering from a hereditary disease, spastic paresis, that displays a high level of 25-HC but a normal level of cholesterol further disputes the role of 25-HC in cholesterol regulation. In contrast, accumulating evidence suggests an upregulation of CH25H in macrophages and dendritic cells upon

inflammatory stimulation [316, 324, 325] Furthermore, it was reported that accumulation of 25-HC instead of cholesterol in the lipid membrane prevents HIV-1 Env-mediated membrane fusion by modifying the secondary structure of the HIV-fusion peptide [326]. Analogous to IFITM proteins, 25-HC also operates in virus producing cells by altering the glycosylation of Lassa virus glycoprotein [327]. Interestingly, 25-HC does not spare non-enveloped viruses, unlike IFITM proteins [328]. For example, 25-HC was shown to hamper reovirus uncoating [328] Again, IFITM3 and 25-HC are thematically resonant in their antiviral actions, given the observation that IFITM3 likely prevents reovirus entry by delaying the proteolytic processing of reovirus particles within late endosomes [225]. Independent to producing 25-HC, CH25H also operates by directly acting on viral proteins. For example, the catalytically inactive CH25H mutant retains its antiviral function against HCV and porcine reproductive and respiratory syndrome virus (PRRSV) through direct interactions with NS5A of HCV and nsp1α of PRRSV [319].

2.5 Env Protein Fights Back: Evasion of Host Restriction on Virus Entry and Beyond

In order to replicate and transmit, viruses need to counter and evade the multi-layered host restriction defense system. Identification of viral antagonism against a host restriction factor also demonstrates the presence of this restriction in the context of in vivo viral infections, which have driven the selection and evolution of specific viral counter measures. Indeed, viral antagonistic strategies have been discovered for some host restriction mechanisms targeting HIV-1 entry, which began to illuminate the diversity of viral evolution in evading host restriction.

One viral countermeasure is to use a viral protein to target and downregulate the host restriction factor, which is well illustrated by the downregulation of SERINC5 by HIV-1 Nef, MLV

glycoGag, and EIAV S2 proteins. A second strategy is to counter restriction factor inhibition through an indirect escape mechanism. One example of this mechanism is to increase the level of Env expression to counter host inhibition of virus entry, exemplified by HIV-1 escape from GBP5 inhibition through shutting down Vpu to elevate Env expression. Another example of viral adaptation and escape has been shown through Vif-null HIV-1 viruses conferring full resistance to APOBEC3G (A3G), which has been linked to a novel Env-dependent mechanism [329]. Env adaptation in Vif-null HIV-1 virus decreases virus fusogenicity and leads to higher levels of Gag-pol packaging into virions, which increases the levels of reverse transcriptase (RT). This Env-mediated elevation in RT levels prevents A3G-mediated hypermutation [329].

The third known strategy is to change Env protein sequence and thus adjust its entry function to gain resistance to host restriction of virus entry. We observed this viral escape mechanism when passaging HIV-1 in IFITM1-expressing SupT1 cells with the goal to select IFITM1-resistant viruses. The resistance mutations that enhanced HIV-1 cell-to-cell transmission were identified in viral Vpu and Env proteins, and they rescued HIV-1 replication in IFITM1-expression SupT1 cells [330]. Similarly, we identified Env mutations that enhance HIV-1 replication in IFITM3-expresing cells [276]. We later found that changing the V3 loop alone in Env can confer resistance to IFITM3 inhibition[215] . The ability of HIV-1 Env protein to resist IFITM3 was also observed in the transmitted founder HIV-1 strains [303]. This study convincingly showed that Env mutations, which arise to evade autologous antibodies 6 months after infection, transform the IFITM3-resistant HIV-1 to an IFITM3-sensitive one. Molecular and structural features of HIV-1 Env that determine its susceptibility and resistance to IFITM3 inhibition remain to be fully elucidated. A related envelope protein-mediated evasion was reported for influenza A virus [331]. It is known that IAV tends to finalize membrane fusion at a low pH in

late endosomes/lysosomes, where IFITM3 is abundantly present. To escape from IFITM3 restriction, IAV HA protein can adapt to mediate membrane fusion in early endosomes where pH is relatively less acidic and has low levels of IFITM3 [331].

It appears that viral envelope protein resists more than just IFITM proteins. We and others have found that Env proteins of some HIV-1 strains, including transmitted founder HIV-1 isolates, are able to resist SERINC5 inhibition even in the absence of viral Nef protein which acts as a SERINC5 antagonist [239, 307]. We further mapped the resistant determinant to the V3 loop of Env. Viral envelope has also demonstrated the capacity of overcoming host restrictions beyond virus entry. For example, passage of SIV/HIV chimeric virus (SHIV) in macaques in the presence of interferon-α led to the selection of interferon-α-resistant SHIV [332]. This resistance phenotype was mapped to viral Env protein that had a higher level of expression from the resistant virus. A separate study reported resistance of transmitted founder HIV-1 to type II interferon (interferon-γ), and this resistance activity was also mapped to viral Env [155]. In support of Env's role in countering interferon-γ, replication of the sensitive HIV-1 strain in the presence of interferon-γ selected for resistance mutations in viral Env protein [155]. These studies report a general role of HIV-1 Env protein in generating resistance to interferon suppression. One possibility is that HIV-1 changes Env to acquire higher replication capacity in compensating for the loss of infectivity as a result of interferon inhibition.

In addition to this compensatory mechanism, viral envelope proteins are able to directly counter specific host restriction factors. One example is the antagonization of tetherin by HIV-2 Env, HERV-K Env, and Ebola glycoprotein [333-335]. Tetherin is known to inhibit the release of HIV-1 and many other enveloped viruses by tethering the progeny virions to the cell surface [173, 176]. HIV-1 uses Vpu to nullify tetherin, while some primate lentiviruses such as SIVs

from chimpanzee, sooty mangabeys, and African green monkeys use Nef to antagonize tetherin [336]. In contrast, HIV-2 and some SIV lineages including SIV tetanus do not encode for Vpu, they instead use Env as a tetherin antagonist [140, 333]. Functional domain analysis revealed that Env's antagonist activity against tetherin depends on a tyrosine based motif (YXXin the cytoplasmic tail of the gp41 membrane proximal region [333]. Upon direct interaction between the extracellular domain of Env and the ectodomain of tetherin, the tyrosine motif (YXX of Env gp41 recruits AP-2 complex and induces intracellular sequestration of tetherin from the cell surface and its accumulation in the trans-Golgi network [333, 337, 338]. Recent studies on HIV-2 isolates from different patients reveal that the anti-tetherin activity is a conserved function of HIV-2 Env . Some ancient retroviruses might have also used their Env proteins to overcome tetherin inhibition, since the youngest and most active endogenous retrovirus (ERV) in human genomes, HERV-K, still preserves this function through its Env protein [334]. In addition to retroviruses, the glycoproteins (GPs) of Ebola virus and Lluvia virus are also antagonists of tetherin. Ebola GP does not remove tetherin from the cell surface but appears to depend on a GxxxA motif in its transmembrane domain [339].

2.6 Viral Envelope Protein under the Suppressive Pressure of Both Adaptive and Innate Immunity

Adaptive and innate immunity cooperate to create a higher genetic barrier for viral envelope protein to escape compared to either immune response alone. For example, HIV-1 Env is engaged in a constant battle with the antibody-mediated adaptive immune response. The ability of Env to evade an innate immunity might be limited by the need to resist antibody attack. This scenario is illustrated by the loss of IFITM3 resistance in transmitted founder HIV-1 strains, which mutate Env in order to resist autologous antibodies as infection progresses [303]. One

implication of this finding is that relatively high level of IFITM3 may mount high enough inhibitory pressure to limit Env mutation pathways in evading inhibition by antibodies, thus creating a synergistic control of HIV-1 infection. It is equally possible that given the interferon-inducible nature of IFITM3 expression, subsidence of interferon response after the acute stage of HIV-1 infection may lead to reduction in IFITM3 level, thus mounting less pressure on HIV-1 Env and allowing Env to change and resist neutralizing antibodies.

The potential interplay of adaptive and innate immunity may also explain the need for Nef to antagonize SERINC5, even though HIV-1 Env has full capacity of SERINC5 resistance. This requirement is likely because certain types of neutralizing antibodies, such as those targeting the MPER sequence of Env, are able to sensitize the otherwise resistant Env to SERINC5 inhibition. This example demonstrates that dual pressures from SERINC5 (innate immunity) and neutralizing antibodies (adaptive immunity) have driven HIV-1 to evolve Nef's antagonism against SERINC5. Further research could illuminate the synergistic suppression on viral envelope protein from both adaptive and innate immunity, and the seemingly endless evolution of viral counter measures to ensure viral survival.

2.7 Conclusions

A growing body of studies demonstrate that HIV-1 Env protein is not only the primary antigen of adaptive immunity but also the main target of innate immunity. An arsenal of antiviral proteins have already been discovered that either limit the synthesis of Env protein, deregulate Env glycosylation, impair Env cleavage by furin, or impede the incorporation of mature Env trimers into HIV-1 particles. In addition, some antiviral factors, such as SERINC5, IFITM3, and 25-HC restrict HIV-1 entry not by acting on Env directly but by altering the physical property of viral membranes or cellular membranes, which often enable them to inhibit a broad range of viruses.

Cells use these diverse molecular mechanisms to inhibit the entry of many viruses far beyond HIV-1, which illustrates that targeting virus entry is a general and important host antiviral strategy (Figure 15, Table 3). Future research is expected to provide further insights into the molecular mechanisms by which each of these antiviral proteins restricts virus entry, to elucidate how these factors function together in vivo to create an optimal antiviral effect, and to understand viral countermeasures and escape mechanisms. It will also be interesting to investigate how these antiviral proteins, through altering the Env glycoprotein, modulate adaptive immune responses. Similar to the broadly neutralizing antibodies, key effectors in adaptive immunity, which are now being tested in clinical trials as a new HIV treatment [340], and restriction factors, as a key layer of innate immunity, also promise new approaches to treat and even cure HIV infection, such as the application of TRIM5α in gene therapy to create HIV-resistant hematopoietic stem cells [341, 342].

Table 3. Summary of the restriction factors that target HIV.

Restriction Factor	Impact on HIV-1 Env	Other Enveloped Viruses Affected	Virus Escape Mechanism	References
ErManI	Decrease Env expression via ERAD pathway; modulate glycosylation of HIV-1 Env	IAV	HIV Vpr increases Env expression	[267, 269, 270]
GBP5	Impair cleavage of gp160; alter glycolysation of HIV-1 Env	MLV	Viral trade-off mechanism to increase Env expression by shutting down Vpu expression	[274, 281]
90K	Prevent gp160 processing; decrease mature gp120/gp41 in virions	EBOV	TBD*	[275, 291]
IFITM2/3	Deter viral entry into virus target cells; impair gp160 processing; promote gp120 shedding; decrease mature gp120/gp41 in virions; incorporate into virions and impair viral entry;	MLV, WNV, MPMV, EBOV, EBV, MeV, DENV	Overcome by HIV-1 Env	[276, 304]
MARCH1/2/8	Downregulate Env from the plasma membrane	HIV-2, SIV, MLV, VSV	TBD	[299-301]
SERINC5	Impair virus infectivity; incorporate into virus particles; affect the conformation of the MPER region of Env	MLV, EIAV, EBOV	Downregulated by Nef from plasma membrane; countered by HIV-1 Env	[239, 240, 305, 307]
25-HC	Modify the secondary structure of the HIV-fusion peptide; prevents membrane fusion	VSV, ZIKV, EBOV, NiV, HCV, RVF	TBD	[318-320, 326-328]

Chapter 3

Effect of HIV-1 Env on SERINC5 Antagonism.

3.1 Preface

This chapter is adapted from the following published research article:

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In Chapter 3, we show that HIV-1 Env protein of some HIV-1 strains is able to resist high levels of SERINC5 without excluding SERINC5 from incorporation into HIV-1 particles. We further show that the virion-associated SERINC5 renders HIV-1 more sensitive to some broadly neutralizing antibodies, in particular those that target MPER of Env. Results from this chapter identify a new role for Env which is to overcome SERINC5 inhibition. Findings from this chapter support the necessity of Nef to remove SERINC5 from HIV-1 particles, even though Env is able to resist virion-associated SERINC5.

Author contributions:

C.L. conceived the study. S.B., S.D., and Q.P. performed the experiments. S.B., A.F., and C.L. analyzed the data and prepared the manuscript.

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3.2 Introduction

The HIV-1 Nef protein plays important roles in viral pathogenesis and disease progression. This is manifested by the association of Nef mutants with slow disease progression in both HIV-1 and simian immunodeficiency virus infections [227, 343, 344]. Nef is known to downregulate important immune molecules on the surface of HIV-1-infected cells. Examples include Nef downregulation of cell surface CD4 [231, 345] , which prevents reinfection and avoids antibody-dependent cell-mediated cytotoxicity [232, 346], and downregulation of cell surface major histocompatibility complex class I (MHC-I) [233]which protects the infected cells from killing by cytotoxic T cells [347]. Nef interacts with several kinases and thereby interferes with various signaling pathways in T cells [235, 348, 349]. Nef also enhances the infectivity of HIV-1 particles [226]. The latter function of Nef is conserved across HIV-1 strains and contributes to the maintenance of a high viral load in patients [228, 229]. Since the initial report that Nef enhances HIV-1 infectivity in 1994 [226], the molecular mechanism remained elusive until the SERINC5 (serine incorporator 5) protein was discovered to profoundly impair HIV-1 infectivity and it was found that its activity is countered by Nef [239, 240]. The human SERINC family has five members, all of which contain multiple transmembrane domains and may be involved in incorporating serine into phospholipids to produce phosphatidylserine and sphingolipids [241]. Recent studies have shown that SERINC5 and, to a lesser extent, SERINC3 ablate HIV-1 infectivity by blocking viral entry, likely through restricting the expansion of the viral fusion pore and thus preventing the release of the viral core into the cytoplasm [239, 240]. In addition to Nef, which antagonizes SERINC5 through downregulating cell surface SERINC5 and thus preventing SERINC5 incorporation into HIV-1 particles, the viral envelope (Env) protein of

some HIV-1 strains has also been reported to overcome SERINC5 inhibition [239, 240]. SERINC5 must exert inhibitory pressure on viruses other than HIV-1 as well, since the glycosylated Gag protein of murine leukemia virus and the glycoproteins of vesicular stomatitis virus (VSV) and Ebola virus all counteract SERINC5 [239, 240]. In contrast to Nef, much less is known about how HIV-1 Env overcomes SERINC5. It is also unclear why HIV-1 has evolved two means, Nef and Env, to resist SERINC5. The results of this study demonstrate that HIV-1 Env, but not Nef, is able to resist high levels of SERINC5 without excluding SERINC5 from incorporation into viral particles. In spite of the greater ability of Env than Nef to counter SERINC5, virion-associated.

3.3 Materials and Methods

3.3.1 Plasmids

pNL4-3, pYU-2, and a panel of infectious molecular clones of transmitted founder viruses were obtained from the NIH AIDS Reagent Program. pAD8-1 and pNL(AD8Env) proviral DNA clones were kindly provided by Eric O. Freed [350]. NL4-3 V3 chimeric plasmids were generated by inserting into NL4-3 DNA a synthesized env fragment of NL4-3 containing the V3 regions of different HIV-1 strains, including AD8-1, YU-2, RHPA, WITO, and THRO. The NL4-3 ΔEnv mutation was engineered by replacing 2 amino acids at positions 39 and 40 into two consecutive termination codons through site-directed mutagenesis. The NL4-3 ΔNef mutation was generated by replacing Nef codons 31 to 33 into three consecutive termination codons. The HIV-1 Env-expressing DNA clones EnvHxB2 and EnvYU-2 were kindly provided by Joseph Sodroski. Panels of HIV-1 Env-expressing plasmids, including HIV-1 subtype A, C, and D Env clones, were obtained from the NIH AIDS Reagent Program (catalog number 11947) [351]. The panel of SGA HIV-1 subtype B clones was obtained from the NIH AIDS Reagent

Program (catalog number 11663) [90]. The cDNAs of SERINC genes were purchased from OriGene (SERINC1, catalog number RC206001; SERINC2, catalog number RC210091; SERINC3, catalog number RC202866; SERINC4, catalog number RC216546; SERINC5, catalog number RC230125). SERINC DNA sequences were amplified and cloned into the pQCXIP retroviral expression vector (catalog number 631516; Clontech). A Flag tag was added to the C terminus of each SERINC protein. Nef DNA was amplified from NL4-3, AD8-1, and YU-2 proviral DNA and inserted into pQCXIP. A hemagglutinin (HA) tag was added to the C terminus of each Nef protein. pMX-hCD4 (catalog number 16416; Addgene) expresses human CD4.

3.3.2 Cell lines

Parental Jurkat cells, SERINC3/5-knockout Jurkat cells, and SERINC3/5-reconstituted KO cells were kindly provided by Heinrich Gottlinger [239] Parental Jurkat cells and SERINC3/5-knockout Jurkat cells were grown in RPMI containing 5% fetal bovine serum (FBS). SERINC3/5-reconstituted KO cells were grown in RPMI containing 5% FBS in the presence of 2 g/ml puromycin (Sigma) and 150g/ml hygromycin B (Roche Diagnostics).

3.3.3 Virus Production

HIV-1 was produced by transfecting cells of the human embryonic kidney cell line HEK293T with HIV-1 proviral DNA. Viruses in the supernatants were clarified by centrifugation in a CS-6R centrifuge (Beckman Coulter) at 3,000 rpm for 25 min at 4ºC. The amounts of viruses were determined by measuring viral reverse transcriptase (RT) activity. To investigate the effect of SERINC5 on HIV-1 infectivity, 500 ng of HIV-1 proviral DNA was cotransfected with different amounts of SERINC5 DNA into HEK293T cells that were seeded in 6-well plates. In experiments in which HIV-1 Env was supplied in trans from an Env-expressing

plasmid to the NL4-3(ΔEnv) virus, after testing different doses of Env plasmid DNA, an amount of 25 ng Env DNA produced an infectivity similar to that of wild-type NL4-3 and was thus used in the NL4-3(ΔEnv) and Env DNA cotransfection experiments. For virus production in parental Jurkat cells, SERINC3/5-knockout Jurkat cells, and SERINC3/5-reconstituted KO cells, DNA clones of NL4-3, NL(AD8V3), and NL(YU2V3), which express either wild-type Nef or the mutated NefG2A protein, were cotransfected with the VSV G protein DNA into HEK293T cells. Forty-eight hours later, viruses were harvested and concentrated by ultracentrifugation (29,000 rpm, 1 h) before they were used to infect the three Jurkat cell lines described above. After 24 h, the infected cells were washed 3 times with phosphate-buffered saline to remove free viruses and were resuspended in fresh medium. The supernatant was harvested 24 h later, and the amounts of viruses were determined by measuring viral RT activity.

3.3.4 Measuring Viral Infectivity

Viral infectivity was measured by infecting TZM-bl indicator cells, which contain an HIV-1 LTR-luciferase expression cassette. These cells were obtained from the NIH AIDS Reagent Program (catalog number 8129). TZM-bl cells were first seeded into 24-well plates (40,000 cells per well) before being infected with HIV-1. At 48 h after viral infection, the TZM-bl cells were lysed in 1x passive lysis buffer (catalog number E1941; Promega). Cell lysates were mixed with luciferase substrate (catalog number E4530; Promega), and luciferase activity was measured using a luminometer. The levels of luciferase activity were normalized by the relative quantities of viral RT activity, and the results represent the infectivity of the virus particles.To measure the inhibition of HIV-1 infection by the CCR5 inhibitor maraviroc (catalog number 11580; NIH AIDS Reagent Program), TZM-bl cells were incubated with different concentrations of maraviroc for 1 h at 37 C before they were infected with HIV-1. The fusion inhibitor T20

(catalog number 9845; NIH AIDS Reagent Program) was first mixed with HIV-1 and then immediately used to infect TZM-bl cells. Virus particle analysis. To detect the incorporation of SERINC5 into HIV-1 particles, HEK293T cells were transfected with HIV-1 proviral DNA together with SERINC5 DNA. At 48 h posttransfection, culture supernatants were first clarified by passage through a 0.2-_m-pore-size filter (VWR) to remove the cell debris. HIV-1 particles were pelleted through 20% sucrose by ultracentrifugation in an Optima L-100XP ultracentrifuge (Beckman Coulter) at 35,000 rpm for 1 h at 4 C. The pelleted virus particles were suspended in phosphate-buffered saline. The amounts of viruses were determined by measuring viral RT activity. Viruses with the same amounts of viral RT activity were examined by Western blotting using antibodies against HIV-1 p24 and Flag (to detect SERINC5-Flag).

3.3.5 Western Blotting

Transfected cells were lysed in Cytobuster protein extraction reagent (catalog number 71009; EMD Millipore Novagen) containing protease inhibitors (catalog number 11836153001; Roche) on ice for 20 min. After clarification by centrifugation, cell lysates were mixed with 4X Laemmli buffer. Protein samples were separated by electrophoresis in SDS-polyacrylamide gels, followed by transfer onto a polyvinylidene difluoride (PVDF) membrane (catalog number 3010040; Roche). The membranes were blocked in 5% skim milk (in phosphate-buffered saline) containing 0.1% Tween 20 (catalog number TWN510; BioShop) for 1 h at room temperature. The membranes were incubated with primary antibodies, including rabbit anti-p24 antibody (catalog number SAB3500946; Sigma-Aldrich), sheep anti-gp120 (catalog number 11710; NIH AIDS Reagent Program), rabbit anti-HA (catalog number H6908; Sigma-Aldrich), mouse anti-Flag (catalog number F1804-1MG; Sigma-Aldrich), mouse anti tubulin (catalog number sc-23948; Santa Cruz

Biotechnology), and mouse anti-HIV-1 Nef (catalog number 3689; NIH AIDS Reagent Program). After the membranes were washed, they were incubated with secondary horseradish peroxide (HRP)-conjugated antibodies consisting of either donkey anti-rabbit IgG (catalog number NA934V; GE Healthcare Life Science), sheep anti-mouse IgG (catalog number NA931; GE Healthcare Life Science), or rabbit anti-sheep IgG (catalog number 618620; Invitrogen). The membranes were treated with enhanced chemiluminescence (ECL) reagents (catalog number NEL105001EA; Perkin Elmer), and the signals were visualized by exposure to X-ray films. The intensities of the protein bands in the Western blots were determined using ImageJ software (NIH).

3.3.6 Immunostaining of Cell Surface CD4

HEK293T cells were cotransfected with 50 ng of Nef-HA DNA and 50 ng of pMX-hCD4 DNA. At 48 h after transfection, the cells were harvested and washed twice in phosphate-buffered saline containing 2% fetal bovine serum (FBS). Samples were fixed in 2% paraformaldehyde (PFA) and incubated on ice for 20 min, followed by incubation with phycoerythrin (PE)-conjugated anti-human CD4 antibody (catalog number 555342; BD Pharmingen) for 1 h. The cells were washed with phosphate-buffered saline containing 2% FBS, and cell surface CD4 was detected using a flow cytometer. The flow cytometry data were analyzed using FlowJo software.

3.3.7 Antibody Neutralization Assay

We obtained from the NIH AIDS Reagent Program a panel of HIV-1 neutralizing antibodies, including 4E10 (catalog number 10091) [352], 10E8 (catalog number 12294) [353], 2F5 (catalog number 1475)[354] , Z13e1 (catalog number 11557) [355], 35O22 (catalog number 12586) [356], VRC03 (catalog number 12032) [357], 17b (catalog number 4091) [358], 7H6 (catalog number 12295) [353], 447-52D (catalog number 4020) [359], PG16 (catalog number 12150)

[360], and 10-1074 (catalog number 12477) [361]. Viruses were incubated with different concentrations of each antibody for 1 h at 37 C and then used to infect TZM-bl cells. At 48 h after infection, TZM-bl cells were harvested and the levels of luciferase activity in the cell lysates were measured. For neutralization assays with viruses that were produced from parental Jurkat cells, SERINC3/5-knockout Jurkat cells, and SERINC3/5-reconstituted KO cells, the same amount of virus (as measured from the viral RT activity) was incubated with different concentrations of neutralizing antibody 35O22 for 1 h at 37ÅãC before infecting TZM-bl cells. Forty-eight hours later, the luciferase activity from the cell lysate was measured.

3.3.8 Statistical Analysis.

The P values of parametric data sets were calculated by an unpaired, two-tailed Student's t test. The P values of normalized data sets were calculated using the Wilcoxon signed-rank test.

3.4 Results

3.4.1 HIV-1 strains YU-2 and AD8-1 But Not NL4-3 Are Resistant to High Levels of Ectopic SERINC5.

The Pizzato group reported that Nef-negative and Nef-positive HIV-1 NL4-3 strains were equally inhibited by high levels of ectopic SERINC5 [240]. In agreement with this observation, when we measured the infectivity of wild-type HIV-1 NL4-3 and NL4-3 carrying the nonfunctional NefG2A mutation, which were produced in the presence of increasing levels of SERINC5, both viruses were strongly inhibited in a SERINC5 dose-dependent manner (Fig. 16A). As a control, SERINC1 did not exhibit any anti-HIV-1 activity (Fig. 16A). Both the SERINC1 and SERINC5 proteins were well expressed in the transfected cells (Fig. 16B). We then asked whether there exist any HIV-1 strains that are able to resist these high levels of

ectopically expressed SERINC5. Three primary HIV-1 strains, 89.6, YU-2, and AD8-1, as well as 10 transmitted founder viruses were tested by cotransfection together with SERINC5 DNA. The infectivity of these viruses was determined by infecting TZM-bl indicator cells. The results showed that, in contrast to NL4-3 and 89.6, which were profoundly inhibited by SERINC5, all transmitted founder viruses were much less inhibited and viruses AD8-1 and YU-2 showed complete resistance to SERINC5 (Fig. 16C). It is possible that the observed resistance phenotype of AD8-1 and YU-2 is a result of the ability of their Nef proteins to counter high levels of SERINC5. To test this, we cloned the Nef genes of strains NL4-3, AD8-1, and YU-2 into expression vectors and first tested their function in downregulating cell surface CD4 (Fig. 16D and E). All three Nef proteins were expressed to similar levels (Fig. 16E). The Nef proteins of both strain NL4-3(NefNL4-3) and strain AD8-1 (NefAD8-1) were equally effective in diminishing cell surface CD4, whereas the Nef of strain YU-2 (NefYU-2) was less effective, likely as a result of a sequence inserted in its N-terminal region (Fig. 16D and E) (19). We then cotransfected these Nef DNA clones with NL4-3(ΔNef) and SERINC5 DNA and observed that none of the three Nef proteins were able to markedly overcome the inhibition by the overexpressed SERINC5 (Fig. 16F). These data suggest that AD8-1 and YU-2 have a Nef independent mechanism to resist high levels of ectopic SERINC5.

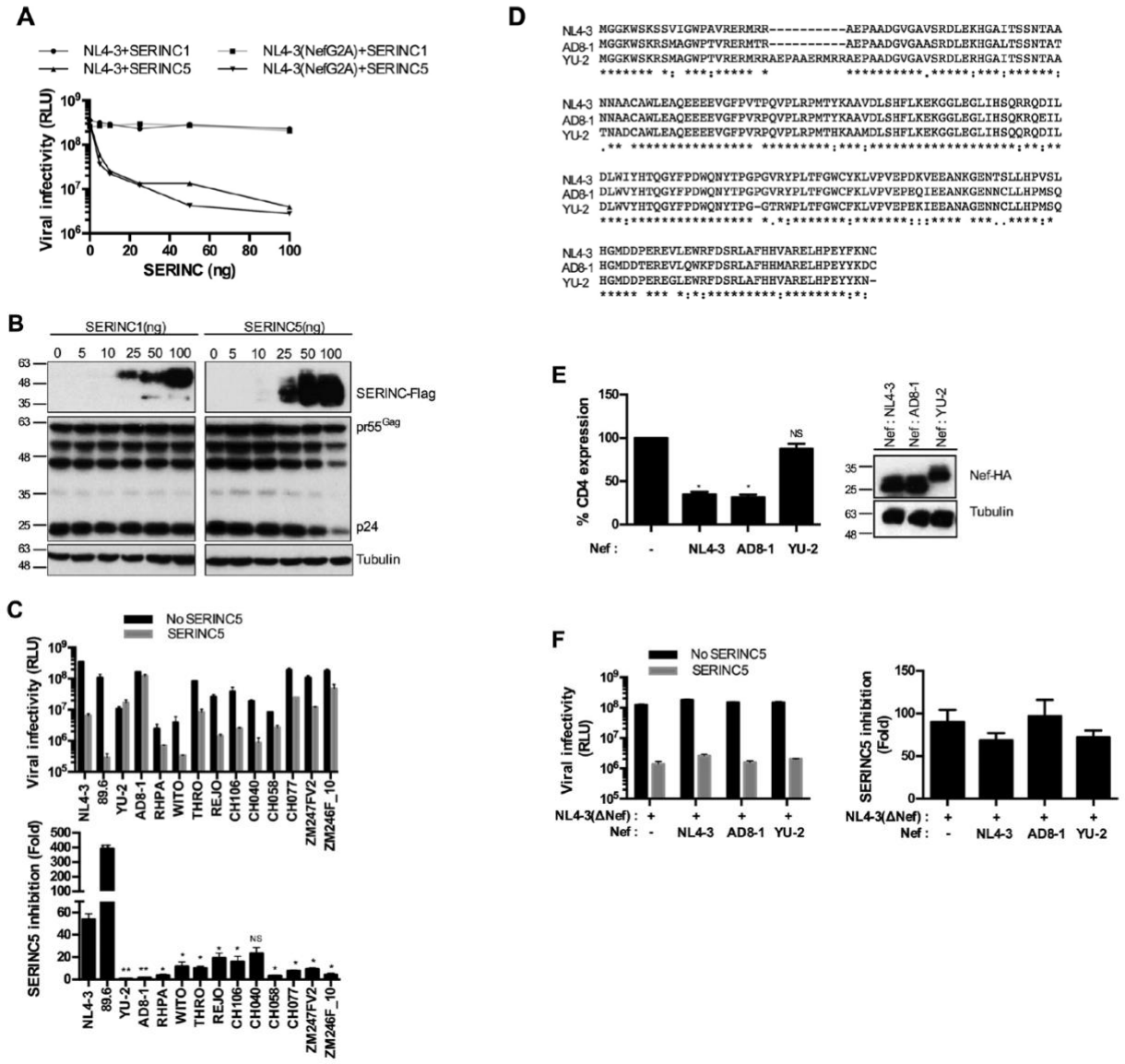

Figure 16. Susceptibility of different HIV-1 strains to inhibition by SERINC5.

(A) Inhibition of NL4-3 and NL4-3(NefG2A) by different amounts of SERINC1 and SERINC5. HIV-1 DNA was cotransfected with increasing doses of SERINC1 or SERINC5 DNA. The infectivity of the viruses was determined by infecting TZM-bl cells. The results shown represent those from three independent experiments. RLU, relative light units. (B) Detection of SERINC1-Flag and SERINC5-Flag expression in cotransfected cells. Western blotting was performed using antibodies against the Flag tag, HIV-1 p24, and tubulin. The numbers on the left are molecular masses (in kilodaltons). (C) Inhibition of different HIV-1 strains by SERINC5. The DNA of

different HIV-1 strains (500 ng) and SERINC5 DNA (25 ng) were cotransfected into HEK293T cells. The infectivity of the viruses was measured by infecting TZM-bl cells. (Top) The results of viral infectivity from one representative experiment are shown. (Bottom) The fold inhibition by SERINC5 from three independent experiments is summarized. (D) Alignment of the amino acid sequences of Nef from strains NL4-3, AD8-1, and YU-2. Conserved amino acids are indicated by asterisks; a colon indicates strong conservation of amino acids; a period indicates weak conservation. (E) Nef downregulates cell surface CD4 protein. Plasmid DNA that expressed NefNL4-3, NefAD8-1, and NefYU-2 and CD4 DNA were cotransfected into HEK293T cells. Cell surface CD4 protein was stained with PE-conjugated anti-CD4 antibody and detected by flow cytometry. (Left) The results of three experiments are summarized, with the level of CD4 expression in the absence of Nef being set equal to 100. P values were calculated using the Wilcoxon signed-rank test. (Right) The levels of Nef proteins were assessed by Western blotting. (F) The Nef proteins of strains NL4-3, AD8-1, and YU-2 did not overcome the inhibition by ectopic SERINC5. NL4-3(ΔNef) DNA was cotransfected with SERINC5 DNA as well as plasmid DNA that expressed NefNL4-3, NefAD8-1 or NefYU-2. (Left) The infectivity of the viruses was determined by infecting TZM-bl cells. (Right) The fold inhibition by SERINC5 from three independent transfections is summarized. *, P _ 0.05; **, P _ 0.01; NS, not significant.

3.4.2 The V3 loop determines the ability of HIV-1 Env to counter SERINC5.

The Env protein of some HIV-1 strains has been reported to resist SERINC5 inhibition [239, 240]. We therefore measured SERINC5 inhibition of an NL(AD8Env) virus that had the NL4-3 Env replaced by the Env of AD8-1 [350]. The results showed that NL(AD8Env) was as resistant to SERINC5 as AD8-1, suggesting that the AD8-1 Env is sufficient to render the otherwise sensitive NL4-3 virus resistant to high levels of SERINC5 (Fig. 17A). The V1 and V2 loops of Env have been shown to contribute to SERINC5 resistance [239]. Accordingly, we replaced the V1, V2, and V3 loop sequences in NL4-3 Env with their counterparts in the AD8-1 Env. The resultant viruses, NL(AD8V1V2V3), which had all three V loops replaced, and NL(AD8V3), which had only the V3 loop replaced, were resistant to the overexpressed SERINC5 (Fig. 17A). The NL(AD8V1) and NL(AD8V2) viruses were not viable and were not tested for SERINC5 inhibition. We further investigated the role of the V3 loop in resisting SERINC5 inhibition by inserting into NL4-3 Env the V3 loop sequences from HIV-1 strains YU-2, RHPA, WITO, and THRO. The phenotypes of these V3 loop chimeric viruses, NL(YU2V3), NL(RHPAV3),

NL(WITOV3), and NL(THROV3), respectively, recapitulated the SERINC5 resistance phenotype of the parental viruses from which the V3 loop was derived (Fig. 17B). We also generated V3 chimeric viruses that had a nonfunctional mutant NefG2A protein and observed that these chimeric viruses were also resistant to SERINC5 inhibition (Fig. 17C).

We expected that resistance to high levels of ectopic SERINC5 should also enable viral resistance to endogenous SERINC5. We therefore used viruses NL4-3, NL(AD8V3), and NL(YU2V3) to infect Jurkat cells, SERINC3 and SERINC5 (SERINC3/5)-knockout (KO) Jurkat cells, and knockout Jurkat cells that were reconstituted to express SERINC3 and SERINC5 [239]. The infectivity of the viruses that were produced from these Jurkat cells was determined by infecting reporter TZM-bl cells. As expected, Nef-mutated NL4-3 acquired an approximately 30-fold higher level of infectivity when it was produced from the SERINC3/5-knockout Jurkat cells than when it was produced from either control Jurkat cells or SERINC3/5-reconstituted Jurkat cells (Fig. 17D). In contrast, similar levels of viral infectivity were measured for the NL(AD8V3) or NL(YU2V3) viruses, regardless of whether the viruses were produced from the control, the SERINC3/5-knockout Jurkat cells, or the SERINC3/5-reconstituted Jurkat cells in the absence of functional Nef (Fig. 17D). We therefore conclude that the V3 loop represents a key determinant in Env that enables viral resistance to both ectopically expressed SERINC5 and endogenous SERINC5.

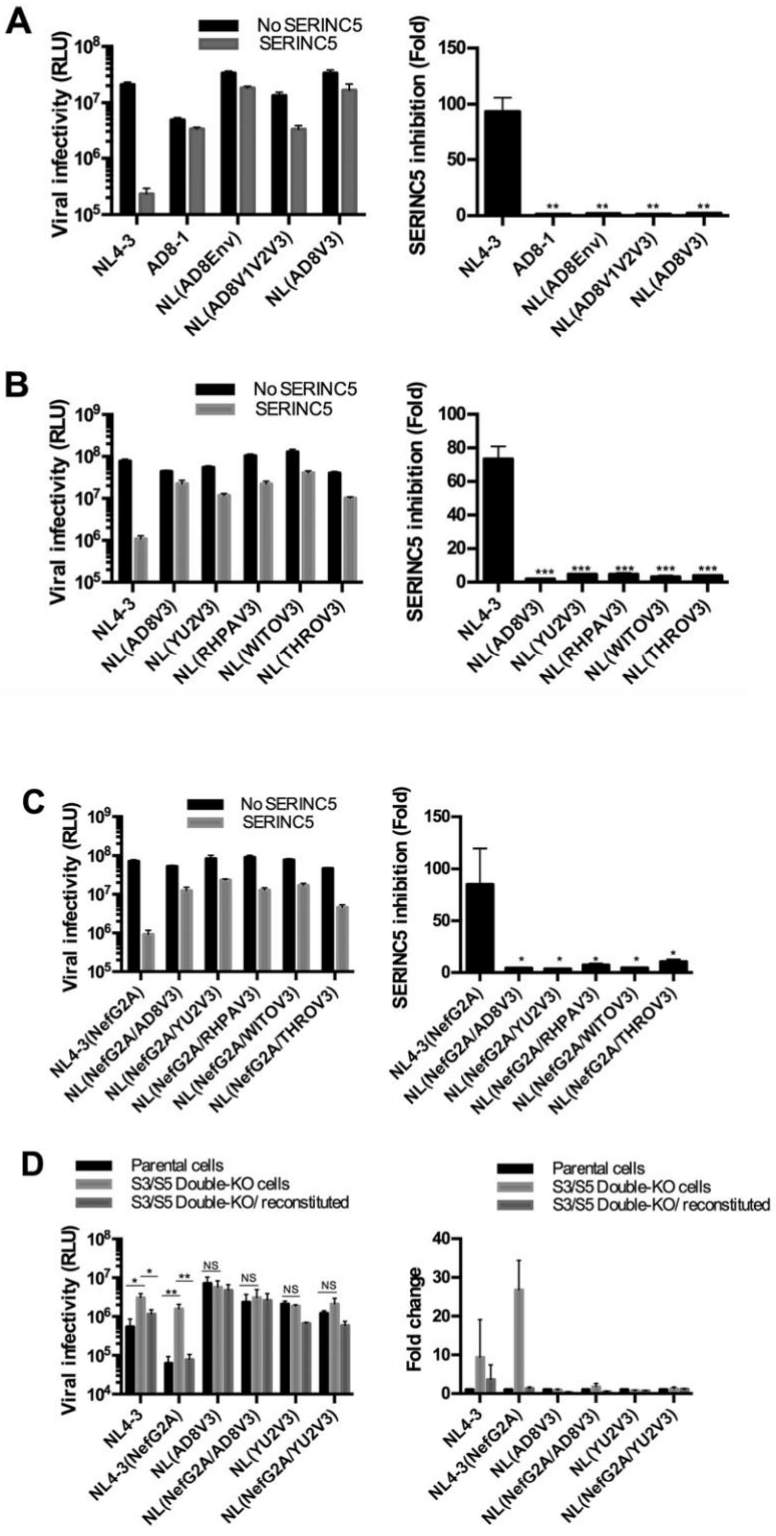

Figure 17. HIV-1 Env resists SERINC5 inhibition.

(A) SERINC5 DNA was cotransfected with NL4-3 DNA that bears either the Env of AD8-1 (AD8Env), the V1, V2, and V3 loop sequences of AD8-1 Env (AD8V1V2V3), or only the V3

loop sequence of AD8-1 Env (AD8V3). (Left) The infectivity of these viruses was determined by infecting TZM-bl cells. (Right) The fold inhibition by SERINC5 from three independent transfections is summarized. (B) HEK293T cells were transfected with SERINC5 DNA together with NL4-3 DNA that had the V3 loop sequence from either the AD8-1, YU-2, RHPA, WITO, or THRO virus strain. (Left) The infectivity of these viruses in the absence or presence of SERINC5 was determined by infecting TZM-bl cells. (Right) The fold inhibition by SERINC5 from three transfections is summarized. (C) SERINC5 inhibition of NL4-3(NefG2A) carrying the V3 loop from different viruses. (D) Infectivity of viruses that were produced from parental Jurkat cells, SERINC3/5-knockout Jurkat cells, and SERINC3/5-reconstituted KO cells. These three different Jurkat cell lines were infected with NL4-3, NL(AD8V3), and NL(YU2V3) viruses that expressed either the wild-type or the mutated Nef protein. Viruses that were produced from these Jurkat cells were used to infect TZM-bl cells. Viral infectivity was calculated by normalizing luciferase activity (in relative light units) to viral RT activity. The fold change for four infections was calculated, with the infectivity of each virus from the parental Jurkat cells being set equal to 1. *, P _ 0.05; **, P _ 0.01; ***, P _ 0.001; NS, not significant.

3.4.3 HIV-1 Env is as Potent as the VSV G Protein in Overcoming SERINC5 Restriction.

Next, we asked whether HIV-1 Env is superior to other viral antagonists, such as the VSV G protein, in countering SERINC5. To answer this question, we transfected HEK293T cells with ΔEnv/NefG2A NL4-3 viral DNA together with EnvHxB2, EnvYU-2, or VSV G protein DNA as well as different doses of SERINC5 DNA. In contrast to the profound SERINC5 inhibition of HIV-1 carrying the EnvHxB2 protein, EnvYU-2 resisted this inhibition as effectively as the VSV G protein (Fig. 18A). We then investigated to what extent this SERINC5 resistance activity is conserved in HIV-1 Env proteins across different HIV-1 strains. Cotransfection experiments were thus conducted with a panel of HIV-1 Env DNA clones of different HIV-1 subtypes. A broad range of sensitivity to SERINC5 inhibition, from complete resistance to 70-fold inhibition, was observed (Fig. 18B and C). The results also revealed that the Env proteins of subtype A, C, and D strains were much more resistant to SERINC5 inhibition than those of subtype B strains, which suggests a possible subtype-specific resistance of HIV-1 Env to SERINC5.

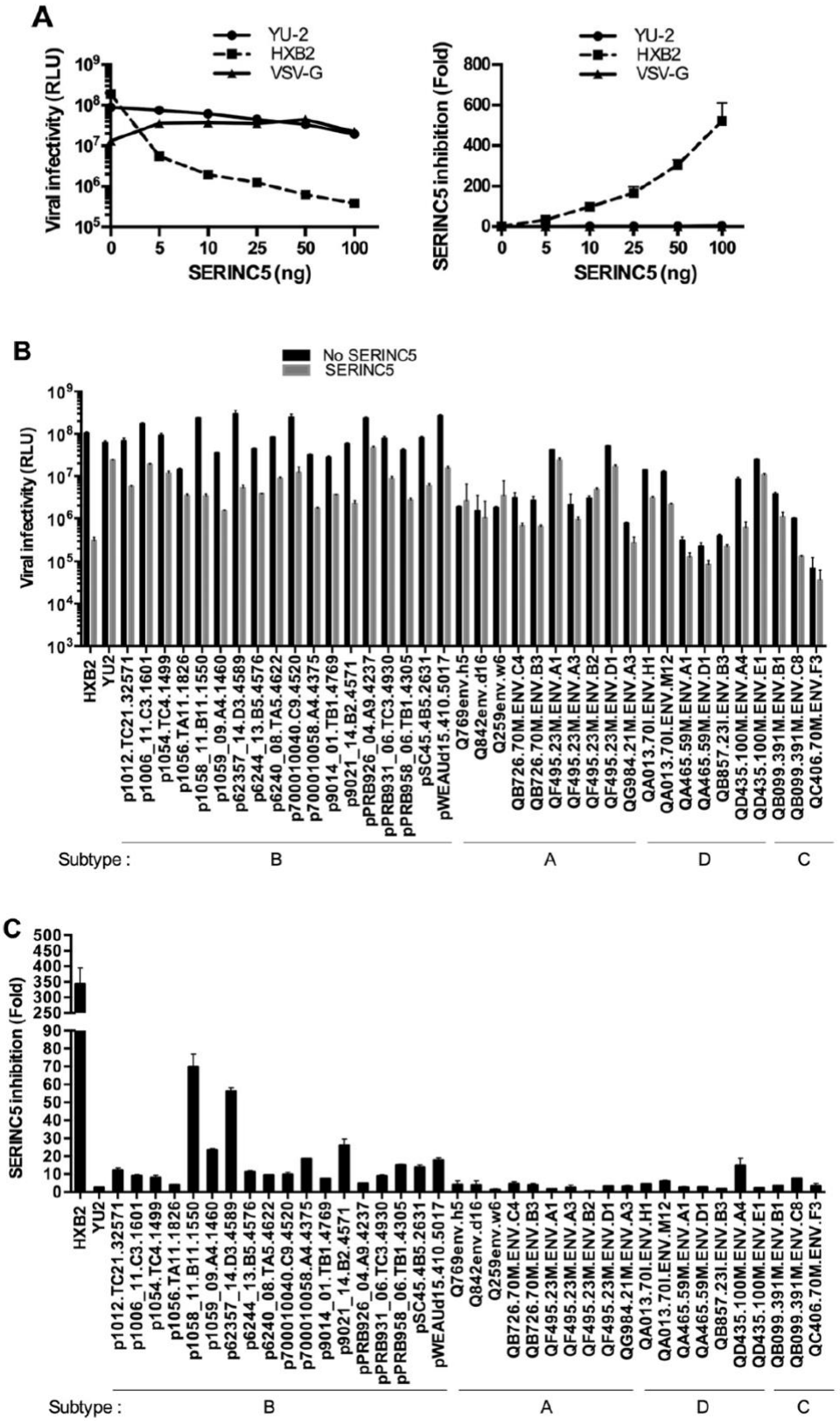

Figure 18. SERINC5 inhibition of HIV-1 bearing Env from different viral strains.
SERINC5 inhibition of HIV-1 bearing Enc from different viral strains (A) Env of YU-2 resists

SERINC5 as effectively as the VSV G protein. ΔEnv/NefG2A NL4-3 DNA was cotransfected with different amounts of SERINC5 DNA and the Env DNA of either HIV-1 strain HXB2 or YU-2 or the VSV G protein. (Left) The infectivity of the viruses was determined by infecting TZM-bl cells. (Right) The fold inhibition by SERINC5 was also calculated. (B) SERINC5 inhibition of HIV-1 carrying Env from different viral strains. HEK293T cells were transfected with ΔEnv/NefG2A NL4-3 DNA, SERINC5 DNA, and Env DNA from different HIV-1 strains of different subtypes. The infectivity of the viruses in the absence or presence of SERINC5 was determined by infecting TZM-bl cells. (C) The fold inhibition by SERINC5 was calculated, and the averages from three experiments are presented.

3.4.4 SERINC5 Sensitizes HIV-1 Env to Inhibition by Maraviroc and Some Neutralizing Antibodies.

Studies have shown that Nef is able to prevent SERINC5 incorporation into HIV-1 particles [239, 240]. To test whether HIV-1 Env employs the same mechanism to overcome SERINC5 inhibition, we cotransfected SERINC5 DNA with NL4-3, NL(AD8V3), or NL(WITOV3) viral DNA and measured the levels of virion-associated SERINC5 by Western blotting. The results showed that both wild-type NL4-3 and the Nef-mutated NL4-3(NefG2A) viruses carried similar levels of SERINC5 (Fig. 19A and B), indicating that Nef is unable to prevent the overexpressed SERINC5 from incorporation into virus particles. This explains the equally strong inhibition of both the wild-type and the Nef-mutated NL4-3 viruses by ectopic SERINC5. Interestingly, the SERINC5-resistant viruses NL(AD8V3) and NL(WITOV3), as well as the VSV G protein-pseudotyped viruses, also contained high levels of SERINC5 (Fig. 19A and B). Therefore, the NL(AD8V3) and NL(WITOV3) Env proteins, as well as the VSV G protein, act by resisting the inhibition of virion-associated SERINC5 rather than by preventing viral incorporation of SERINC5. We next investigated whether some HIV-1 Envs, such as the NL(AD8V3) Env, even though they are resistant to virion-associated SERINC5, might be affected by SERINC5 so that Env becomes vulnerable to some inhibitory pressures. We first measured the responses of SERINC5-free and SERINC5-bearing NL(AD8V3) viruses to the CCR5 inhibitor maraviroc and

the fusion inhibitor enfuvirtide (T20). Much stronger inhibition of SERINC5-bearing NL(AD8V3) than SERINC5-free viruses by maraviroc but not by T20 was observed (Fig. 19C and D), which suggests that virion-associated SERINC5 might have interfered with the HIV-1 Env usage of CCR5 as the coreceptor for entry and, as a result, sensitized HIV-1 to the CCR5 antagonist maraviroc. One possible impact of virion-associated SERINC5 on Env is a change in the Env conformation, which can be detected using antibodies that recognize specific epitopes on Env. We therefore tested a panel of neutralizing antibodies for their ability to inhibit SERINC5-free and SERINC5-bearing viruses (Table 4). Among the antibodies tested, three antibodies, 35O22, 4E10, and 10E8, inhibited SERINC5-bearing NL(AD8V3) or NL(NefG2A/AD8V3) viruses to a greater degree than they inhibited the SERINC5-free virus (Fig. 20A; Table 5). We further tested 4E10 inhibition of the NL(NefG2A/WITOV3), NL(NefG2A/RHPAV3), and NL(NefG2A/YU2V3) viruses and observed stronger inhibition of these viruses when SERINC5 was cotransfected (Fig. 20B; Table 5). In support of these data, viruses that were produced from SERINC3/5-knockout Jurkat cells exhibited greater resistance to the 35O22 antibody than viruses from either the parental Jurkat cells or the SERINC3/5-reconstituted cells (Fig. 20C; Table 6), which demonstrates the negative impact of endogenous SERINC3/5 proteins on the response of HIV-1 to inhibition by neutralizing antibody 35O22. Therefore, the virion-associated SERINC5 may have altered the accessibility of certain epitopes in HIV-1 Env to some neutralizing antibodies, including those targeting.

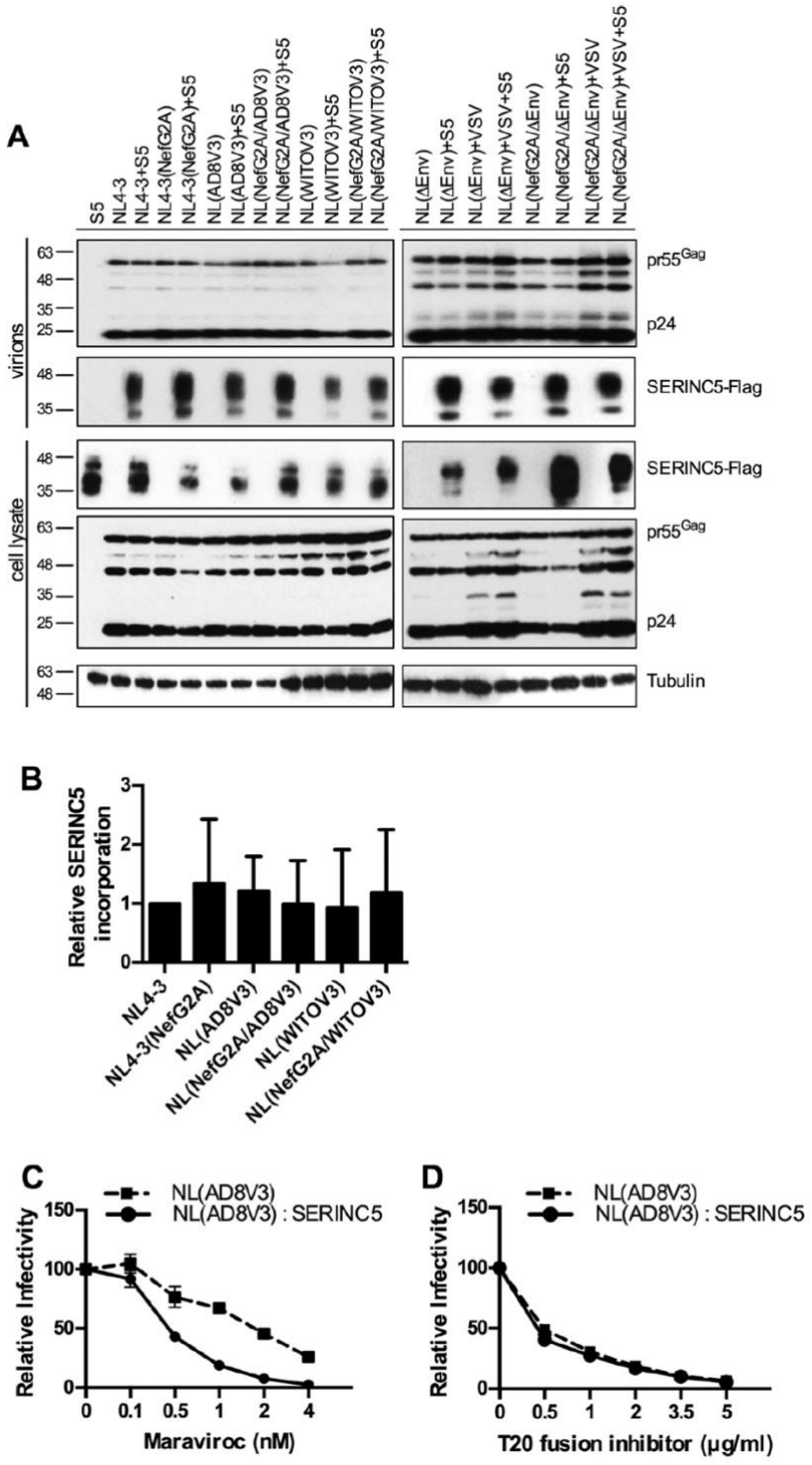

Figure 19. Effect of virion-associated SERINC5 on inhibition of HIV-1 by maraviroc and T20. (A) Incorporation of SERINC5 (S5) into HIV-1 particles. SERINC5 DNA (25 ng) was transfected into HEK293T cells with ΔEnv NL4-3 DNA (500 ng) and VSV G protein DNA

Figure 18. Effect of virion-associated SERINC5 on inhibition of HIV-1 by maraviroc and T20 (continued). (25ng) or with NL4-3 DNA that expressed an Env-bearing V3 loop from strain AD8-1 or WITO. Both wild-type Nef and the NefG2A mutant viruses were investigated. SERINC5 was transfected alone as a control for SERINC5 potentially associated with extracellular vesicles. Viral particles were harvested and subjected to Western blotting to detect virion-associated SERINC5. A representative Western blot is shown. The numbers on the left are molecular masses (in kilodaltons). (B) Relative levels of SERINC5 associated with HIV-1 particles. Protein band intensities were quantified using ImageJ software (NIH). The levels of SERINC5 in different cell lysates were adjusted by the levels of tubulin. The levels of SERINC5 in different virus samples were normalized to the levels of the viral CA protein, followed by further adjustment to the SERINC5 levels in the cell lysates. The final values represent the SERINC5 virion incorporation efficiency. The SERINC5 level in the wild-type NL4-3 virus is arbitrarily set equal to 1. The results shown are the averages from four independent cotransfection experiments. (C) Effect of SERINC5 on the inhibition of HIV-1 by maraviroc. NL(AD8V3) DNA was transfected into HEK293T cells with or without SERINC5 DNA to produce SERINC5-free or SERINC5- bearing virus particles. Viruses with the same amounts of RT activity were used to infect TZM-bl cells that had been pretreated with different concentrations of maraviroc. Viral infection was determined by measuring luciferase activity. The level of viral infectivity without maraviroc treatment was set equal to 100. (D) Effect of SERINC5 on the inhibition of HIV-1 infection by the fusion inhibitor T20. The same amounts of SERINC5-free and SERINC5-bearing NL(AD8V3) viruses were used to infect TZM-bl cells in the presence of different concentrations of T20. Viral infection was determined by measuring luciferase activity. The level of viral infectivity without T20 treatment was set equal to 100. The results shown are the averages from three independent infections.

Table 4. Summary of neutralizing antibodies used in this study.

Neutralizing antibody	Target or Function	Reference
VRC03	CD4 binding site	[357]
17b	Binds to a CD4-induced discontinuous epitope	[358]
7H6	MPER	[353]
447-52D	V3 loop	[359]
PG16	V1/V2 loop	[360]
10-1074	V3 loop	[361]
2F5	gp41 epitope ELDKWA	[354]
Z13e1	MPER	[355]
4E10	gp41 epitope NWFDIT	[352]
10E8	MPER	[353]
35022	gp41/gp120 interface	[356]

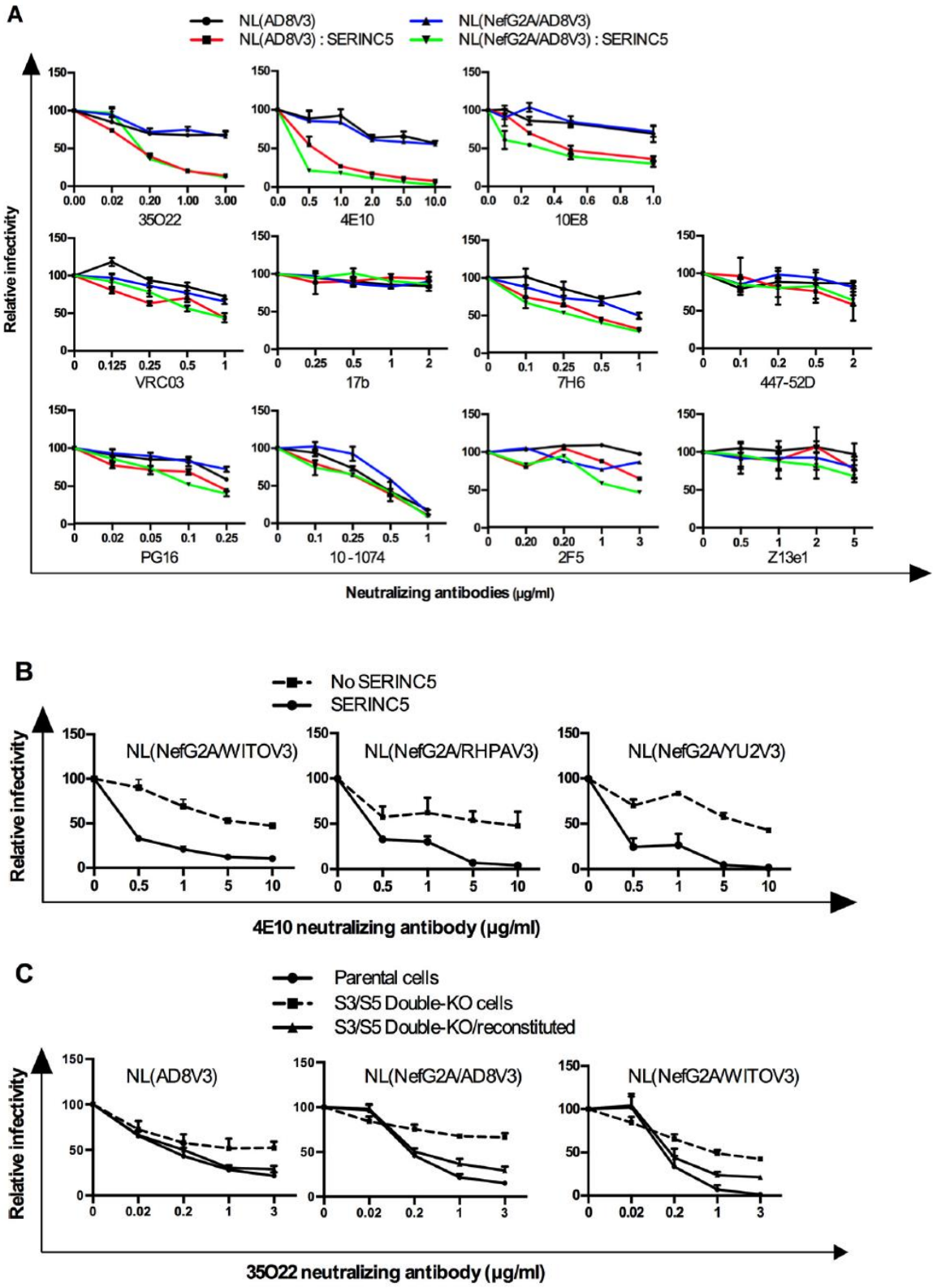

Figure 20. Effect of SERINC5 on inhibition of HIV-1 by neutralizing antibodies.

(A) NL(AD8V3) viruses with either wild-type Nef or the NefG2A mutant were produced by transfecting HEK293T cells with or without SERINC5 DNA. Viruses with the same levels of RT activity were incubated with different concentrations of each neutralizing antibody before they were used to infect TZM-bl cells. Viral infection was determined by measuring luciferase activity. The level of viral infectivity without neutralizing antibody treatment was set equal to 100. Details about the neutralizing antibodies that were used are presented in Table 4. The results shown represent those from three independent infection experiments. The 50% inhibitory concentration values for antibodies 35O22, 4E10, and 10E8 were calculated from three independent experiments, and the results are summarized in Table 5. (B) Responses of NL(NefG2A/WITOV3), NL(NefG2A/RHPAV3), and NL(NefG2A/YU2V3) viruses to inhibition by the 4E10 neutralizing antibody in the absence or presence of SERINC5. The 50% inhibitory concentration values were calculated, and the results are shown in Table 2. (C) The NL(AD8V3), NL(NefG2A/AD8V3), and NL(NefG2A/WITOV3) viruses were produced by infecting Jurkat cells, SERINC3/5 (S3/S5)-knockout Jurkat cells, or SERINC3/5-reconstituted Jurkat cells. Viruses with the same amounts of RT were incubated with the neutralizing antibody 35O22 for 1 h before they were used to infect TZM-bl cells. Viral infection was determined by measuring the luciferase activity in the TZM-bl cell lysates. The 50% inhibitory concentration values were calculated from three independent infection experiments, and the results are summarized in Table 6.

Table 5 IC50s of neutralizing antibodies against HIV-1 containing ectopic SERINC5[a]

	IC50)					
	4E10		**35O22**		**10E8**	
Virus	**-S5**	**+S5**	**-S5**	**+S5**	**-S5**	**+S5**
NL(AD8V3)	>10	0.50.04	>3	0.110.01	>1	0.530.02
NL(NefG2A/AD8V3)	>10	0.060.05	>3	0.150.02	>1	0.250.09
NL(NefG2A/WITOV3)	0.4	0.110.09				
NL(NefG2A/RHPAV3)	0.12	0.220.10				
NL(NefG2A/YU2V3)	0.35	0.180.20				

[a] IC50, 50% inhibitory concentration; _S5, virus not bearing SERINC5; _S5, virus bearing SERINC5

Table 6. IC50 of 35O22 neutralizing antibody against HIV-1 from Jurkat cells with or without SERINC3/5 expression [a].

	IC50)		
Virus	**Parental**	**S3/S5 double KO**	**S3/S5 double KO, reconstituted**
NL(AD8V3)	0.12	>3	0.20
NL(NefG2A/AD8V3)	0.02	>3	0.09
NL(NefG2A/WITOV3)	0.09	2.60.17	0.03

[a]IC50, 50% inhibitory concentration; S3/S5, SERINC3/5.

3.5 Discussion

The results of our study show that neither HIV-1 Nef nor Env is able to prevent high levels of ectopic SERINC5 from incorporation into virus particles. However, some HIV-1 Env proteins, but not Nef, resist the inhibition of virion-associated SERINC5, indicating that Env and Nef counteract SERINC5 by different mechanisms. Since Nef is known to enhance HIV-1 infectivity and can exclude SERINC5 from HIV-1 virions [239, 240], the inability of Nef to counter the ectopic SERINC5 indicates that a much higher level of ectopic SERINC5 than endogenous SERINC5 was used in this study. Nonetheless, experimentation with ectopic SERINC5 led to the finding of the greater ability of HIV-1 Env than Nef to overcome SERINC5, albeit by distinct mechanisms. Given that the envelope glycoproteins of VSV and Ebola virus also resist SERINC5 inhibition [239, 240], it is expected that more viral envelope proteins will be found to be refractory to SERINC5. We mapped the HIV-1 Env determinant of SERINC5 resistance activity to the V3 loop.

We were unable to examine the role of the V1 and V2 loops in the Env resistance of

SERINC5. However, studies from other groups have suggested that the V1 and V2 loops allow Env to counter SERINC5 [238, 239]. Since the V1 and V2 loops fold into a pocket in which the V3 loop resides, it is conceivable that V1, V2, and V3, as an interdependent structural entity modulating Env stability and the Env conformation as well as coreceptor usage, could function together to counter SERINC5.

Since SERINC5 impedes HIV-1 entry, likely through restricting the expansion of the viral fusion pore [239, 240], it is possible that some HIV-1 Env proteins, such as EnvAD8-1 and EnvYU-2, have an entry function strong enough to overcome this SERINC5 restriction, whereas those HIV-1 Env proteins with a relatively weaker entry function are inhibited by SERINC5, which may have led to the wide range of responses of different HIV-1 Env proteins to SERINC5 inhibition. In line with this possibility, when maraviroc was used to diminish the cell surface level of CCR5 that can be engaged by HIV-1 Env and, as a result, delay viral entry, otherwise resistant HIV-1 becomes sensitive to the inhibition of the virion-associated SERINC5.

In spite of the resistance of HIV-1 Env to SERINC5, the inability of Env to prevent SERINC5 from incorporation into virus particles allows the virion-associated SERINC5 to have the opportunity to impact the Env conformation. One consequence of this impact is the increased vulnerability of Env to inhibition by some neutralizing antibodies. This effect of SERINC5 on Env might help explain why the function of Nef to exclude SERINC5 from HIV-1 particles has been conserved across all HIV-1 strains. The Pizzato group previously showed that Nef renders HIV-1 refractory to neutralizing antibodies, including 4E10, which targets the MPER region of gp41, and that this function of Nef is independent of its ability to enhance virion infectivity [362]. In light of our observation that virion-associated SERINC5 sensitizes HIV-1 to

neutralizing antibodies, including 4E10, we suggest that one mechanism by which Nef protects HIV-1 from attack by some anti-gp41 antibodies may involve SERINC5 downregulation.

In summary, the results of our study demonstrate that HIV-1 Env is capable of resisting virion-associated SERINC5. This SERINC5 resistance function is also shared by envelope proteins of other viruses, including VSV. However, the sensitization of HIV-1 Env to some neutralizing antibodies by virion-associated SERINC5 may have pressured the virus to exclude SERINC5 from virion incorporation using Nef.

Chapter 4

Differential Pressures of SERINC5 and IFITM3 on HIV-1 Envelope Glycoprotein Over the Course of HIV-1 Infection

4.1 Preface

This chapter is adapted from the following published research article:

Beitari, S., Pan, Q., Finzi, A., Liang, C**.** Differential pressures of SERINC5 and IFITM3 on HIV-1 envelope glycoprotein over the course of HIV-1 infection. J. Virol, 2020.

DOI: 10.1128/JVI.00514-20

In Chapter 4, we expand our findings on the role of the HIV-1 Env protein against the inhibition by SERINC5. We have thus examined 70 HIV-1 Env clones that were generated from the stages of viral transmission, acute infection and chronic infection. While HIV-1 Env clones from the transmission stage are resistant to both SERINC5 and IFITM3, as infection progresses into the acute and chronic stages, the resistance to IFITM3 but not to SERINC5 is gradually lost. We further discovered a significant correlation between the resistance of HIV-1 Env to soluble CD4 inhibition and the resistance to SERINC5. Importantly, the CD4 mimetic M48U1 sensitizes HIV-1 Env to the inhibition by SERINC5 and moderately to IFITM3. Together, these data indicate that SERINC5 and IFITM3 exert differential inhibitory pressures on HIV-1 Env over different stages of HIV-1 infection.

Author contributions:

C.L. conceived the study. S.B. and Q .P. performed the experiments. S.B. and C.L. analyzed the data. S.B., A.F., and C.L. prepared the manuscript.

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4.2 Introduction

The HIV-1 envelope glycoprotein (Env) is not only under the selection pressure of adaptive immunity, it is also the target of innate immunity. A group of cellular factors, often interferon-induced, have been reported to inhibit HIV-1 Env-mediated virus entry [363]. These include interferon induced transmembrane (IFITM) proteins [213, 219], the 90K protein [275, 290, 291], serine incorporator 5 (SERINC5) [239, 240], membrane-associated RING-CH (MARCH) proteins [299-301], endoplasmic reticulum class 1 α-mannosidase (ERManI) [267], and guanylate binding protein 5 (GBP5) [274, 281, 282]. Among these HIV-1 Env inhibitors, inhibition by IFITM and SERINC5 proteins have been shown to be overcome by Env mutations [215, 303, 305, 307, 330, 364].

SERINC5 was originally discovered as the cellular restriction factor that is antagonized by HIV-1 Nef accessory protein [239, 240]. In the absence of Nef, SERINC5 is incorporated into HIV-1 particles and impairs HIV-1 infectivity by inhibiting the expansion of a viral fusion pore [239, 240, 305]. The ability of Nef to antagonize SERINC5 appears to be important for HIV-1 pathogenesis, since this ability of Nef is lost or severely attenuated in viruses from elite controllers [365]. Nef is not the only mechanism used by HIV-1 to counter SERINC5. Our group and others have found that HIV-1 Env is able to resist SERINC5 restriction [305, 307]. The V3 loop of Env has been further identified as one determinant of this function of Env [307]. Similarly, IFITM3 can also inhibit HIV-1 entry by impairing the hemifusion of the viral membrane and the formation of a viral fusion pore [213, 223, 309]. One difference is that IFITM3 is able to exert its inhibition either in the target cells or in the virus particles, while

SERINC5 only inhibits when present in HIV-1 particles [239, 240, 306]. Nonetheless, IFITM3 is also countered by HIV-1 Env, and the Env determinant of this resistance was mapped to the V3 loop [215].

In spite of these similarities between IFITM3 and SERINC5 in their anti-HIV-1 activity and HIV-1 countering mechanisms, these proteins are structurally and functionally very different. SERINC5 has 10 transmembrane domains, located on cellular membrane and implicated in lipid modification [241, 242], whereas IFITM3 has only 132 amino acids, with one transmembrane domain and an intra-membrane domain and predominantly located in late endosomes [209, 314]. It remains unclear how IFITM3 and SERINC5, once incorporated into HIV-1 particles, act on viral Env and whether Env reacts differently to resist these two inhibitors. It has been reported that transmitted founder (T/F) HIV-1 strains resist IFITM3 inhibition, but this resistance diminishes with the progression of HIV-1 infection, as a result of the need of HIV-1 Env to change and escape from the inhibition by neutralizing antibodies [303]. However, it is not entirely known how the susceptibility of HIV-1 Env to SERINC5 inhibition changes over the course of HIV-1 infection.

To answer these questions, we have examined a panel of primary HIV-1 Env clones for their susceptibility to IFITM3 and SERINC5 inhibition. These Env clones were derived from either T/F HIV-1 strains, acute or chronic infections. While HIV-1 Env becomes more sensitive to IFITM3 inhibition as the infection progresses to the chronic stage, the Env clones of all stages of infection are resistant to SERINC5 restriction.

4.3 Materials and Methods

4.3.1 Plasmid DNA

pNL4-3 DNA was obtained from the NIH AIDS Reagent Program. pNL(AD8) DNA was kindly provided by Eric O Freed [350]. pBJ6-SERINC5-HA was obtained from EURIPRED (Reference number 100107). The pQCXIP retroviral expression vector was purchased from Clontech (catalog number 631516, Clontech). N-terminal Flag tagged QCXIP-IFITM3 was generated as previously described [215]. NL4-3ΔNefΔEnv was generated by inserting stop codons to amino acids positions 31/32 in Nef and amino acid positions 39/40 in Env. HIV-1 Env expressing clones tested in this paper were obtained from NIH AIDS Reagents Program, catalog number 11663 [90], catalog number 11227 [366], catalog number 11326 [367-369], catalog number 11672 [370], catalog number 11673 [370], catalog number 12670 [371], and catalog number 11674 [372]. Of note, each Env clone was isolated from a different individual.

4.3.2 Virus Production

HIV-1 was produced by transfecting human embryonic kidney cell line HEK293T with HIV-1 proviral DNA. Viruses in the supernatants were clarified by centrifugation in a CS-6R centrifuge (Beckman Coulter) at 3,000 rpm for 25 min at 4$_{o}$C. The amounts of viruses were determined by measuring viral reverse transcriptase (RT) activity. To produce NL4-3ΔNefΔEnv viruses carrying different HIV-1 Envs, 200 ng of NL4-3ΔNefΔEnv proviral DNA was co-transfected with 25 ng of HIV-1 Env expressing plasmid DNA.

To investigate the effect of SERINC5 or IFITM3 on HIV-1 infectivity, 200 ng of HIV-1 proviral DNA was co-transfected with 100 ng of SERINC5 DNA, or 100 ng of IFITM3 DNA into HEK293T cells that were seeded in 6-well plates. Viruses thus produced were used to infect the TZM-bl indicator cells as described below.

4.3.3 Measuring Viral Infectivity

Viral infectivity was measured by infecting TZM-bl indicator cells, which contain an HIV-1 LTR-luciferase expression cassette. These cells were obtained from the NIH AIDS Reagent Program (catalog number 8129). TZM-bl cells were first seeded into 24-well plates (40,000 cells per well) before being infected with HIV-1. At 48 h after viral infection, the TZM-bl cells were lysed in passive lysis buffer. Cell lysates were mixed with luciferase substrate and luciferase activity was measured using a luminometer. The levels of luciferase activity were normalized by the relative quantities of viral RT activity, and the results represent the infectivity of the virus particles. The amount of HIV -1 used in TZM -bl infection is in the range 294 of 2000 cpm (count per minute) of viral reverse transcriptase activity.

4.3.4 M48U1 and 17b Inhibition Assay

Viruses were incubated with different concentrations of the CD4 peptide mimetic M48U1 compound [373] or the 17b non-neutralizing antibody [374] for 1 hour at 37$_o$C, and then used to infect TZM-bl cells to assess their infectivity levels. After 48 hours, infected TZM-bl cells were lysed and the levels of luciferase activity were measured.

4.3.5 Correlation Analysis

The data published by Keele et al 2008 [90] were adopted to examine the 303 potential correlation between the SERINC5 or IFITM3 sensitivity and the responses of HIV -1 304 T/F Env clones to the inhibition by CD4 monoclonal antibody (mAb) RPA -T4 (555344, BD 305 PharMingen), sCD4 (514 -CD, R&D Systems), or CCR5 inhibitor TAK779 (NIH AIDS Reagents 306 Program, 4983)

4.3.5 Statistics

P- values were calculated with Student t test. The R values and P values of correlation graphs were calculated using correlation spearman in GraphPad Prism.

4.4 Results

4.4.1 HIV-1 Env Clones of Both Acute and Chronic Infections Manifest Resistance to SERINC5 Inhibition.

We asked to which extent primary HIV-1 Env resist the inhibition by IFITM3 and SERINC5 and whether the level of resistance persists with the progression of HIV-1 infection. To answer these questions, we examined 70 HIV-1 Env clones for inhibition by IFITM3 and by SERINC5. Among these Env clones, 19 isolates were derived from T/F HIV-1, 35 from acute infections, and 16 from chronic infection. We used the Nef-deleted and Env-deleted HIV-1 clone (NL4-3 ΔNefΔEnv) to produce virus particles that were pseudotyped with these primary Env proteins by co-transfecting HEK293T cells. IFITM3 or SERINC5 DNA was included in the co-transfection experiments to test their inhibition of the pseudotyped HIV-1 particles. The Nef-deleted HIV-1 was used in order to accurately measure the susceptibility of Env clones to SERINC5 and IFITM3 inhibition without the interference of Nef which is able to antagonize SERINC5. The results of Figure 21A showed that the lab-adapted HXB2 Env-mediated infection was inhibited by SERINC5 by up to 60-fold, whereas the primary YU-2 Env was resistant, this is in agreement with previous publications [305, 307].

We then tested the primary Env clones (Fig. 21A) and summarized their folds of inhibition by SERINC5 in Figure 1B and Tables 7 to 9. The mean fold of inhibition was 2.5 for T/F Env clones, 2.6 for acute Env clones, and 1.8 for chronic Env clones, which are not statistically different from each other and show similar level of resistance to SERINC5 as the YU-2 Env. Four out of the 19 T/F Env clones showed more than 5-fold inhibition, two out of the 35 acute

clones were inhibited by more than 5-fold, none of the 16 chronic was inhibited by more than 5-fold. Therefore, resistance to SERINC5 is preserved by most of the HIV-1 Env over the course of infection, from transmission until the chronic stage.

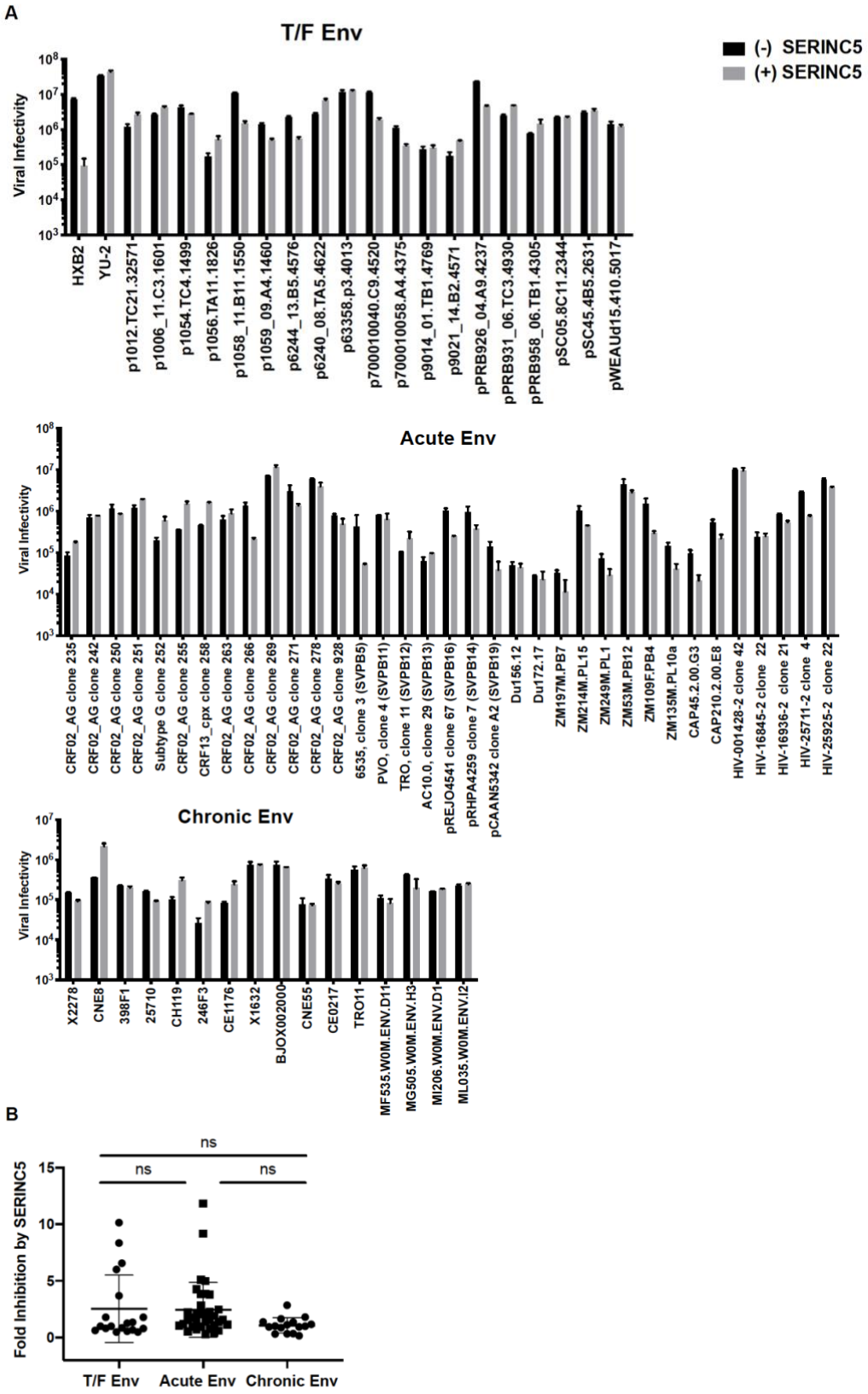

Figure 21. Inhibition of HIV-1 Env clones by SERINC5.
HEK293T cells were cotransfected with NL4-3 ΔNefΔEnv proviral DNA, different HIV-1 Env

clones, and 100 ng of SERINC5 cDNA. 48 hours after transfection, pseudotyped HIV-1 was used to infect TZM-bl cells. (A) Luciferase data of one representative experiment are presented. (B) Fold inhibition by SERINC5 were determined by calculating the ratio of infectivity of the SERINC5-free viruses to those of the SERINC5-bearing viruses. Fold inhibition by SERINC5 for each Env clone from three independent transfections is shown. The data of all Env clones are presented. ns, not significant.

4.4.2 HIV-1 Env Clones Present Distinct Profiles of Susceptibility to the Restriction by SERINC5 and IFITM3.

We next measured IFITM3 inhibition of these primary Env clones. As controls, the HXB2 Env was inhibited by 10-fold, while the YU-2 Env was relatively resistant to IFITM3 inhibition (Fig. 22A). The mean fold of inhibition by IFITM3 was 3.6 for the T/F Env clones, whereas the values increased to 5.8 for the acute Env clones, and to 8.17 for the chronic Env clones, both of which are higher than that of the T/F Env (Fig. 22B, Tables 7 to 9). Therefore, as opposed to the persistent resistance of HIV-1 Env clones to SERINC5 across different stages of infection, the T/F Env clones are relatively resistant to IFITM3 restriction, but this resistance property is lost as the infection advances, which supports the findings by Foster et al. [303]. Importantly, we observed no significant correlations between the restriction by IFITM3 and SERINC5 in either Env groups (Fig. 23), thus indicating that the same Env is not necessarily sensitive or resistant to both IFITM3 and SERINC5.

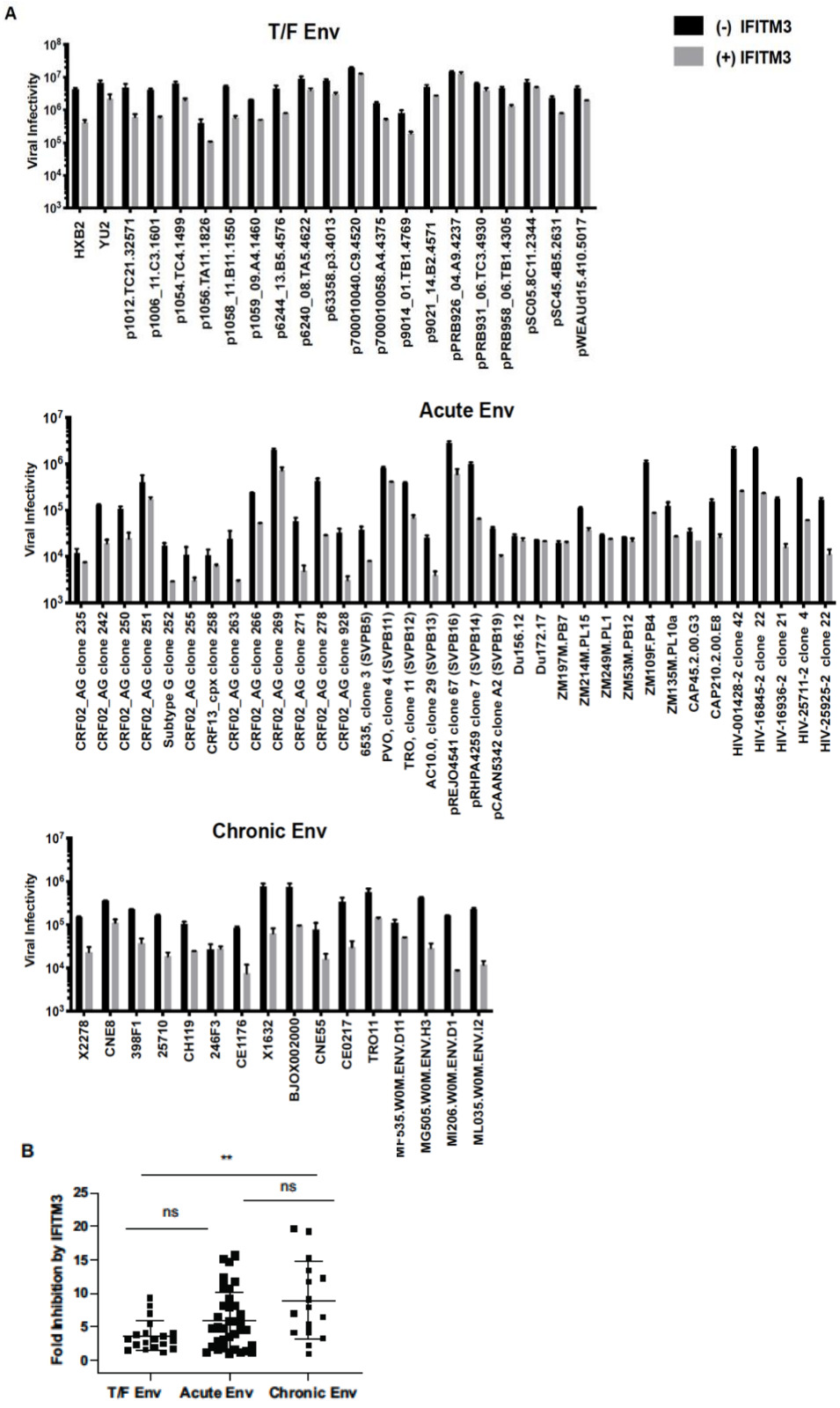

Figure 22. Inhibition of HIV-1 Env clones by IFITM3.

HEK293T cells were cotransfected with NL4-3 ΔNefΔEnv proviral DNA, different HIV-1 Env

clones (as indicated), and 100 ng of IFITM3 DNA. The infectivity of viruses were determined by infecting TZM-bl cells. (A) Luciferase data of one representative experiment are shown. (B) Fold inhibition by IFITM3 were determined by calculating the ratio of infectivity of the IFITM3-free viruses to those of the IFITM3-bearing viruses. Folds inhibition by IFITM3 from three independent transfections are presented. * denotes P <0.05. ns, not significant.

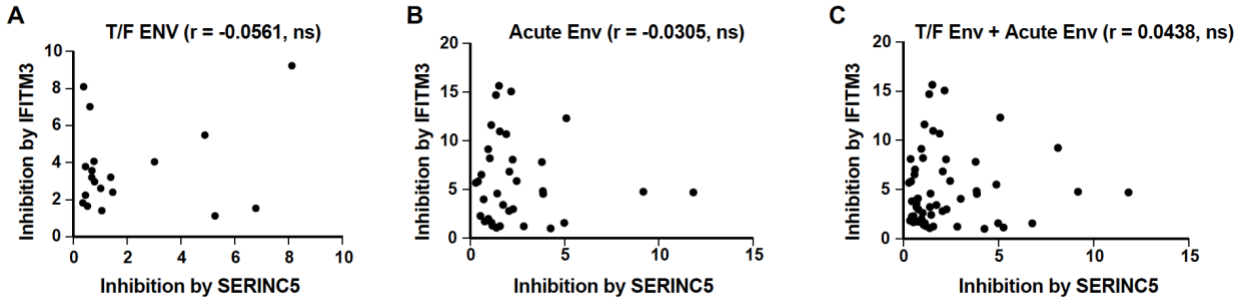

Figure 23. Lack of correlation between SERINC5 and IFITM3 inhibition.

Correlation analysis between SERINC5 inhibition and IFITM3 inhibition for HIV-1 T/F Env (A), HIV-1 acute Env (B), and T/F Env and Acute Env together (C). Correlation was assessed using spearmen rank test. ns, not significant.

Table 7. Response of T/F Env clones to the inhibition by SERINC5, IFITM3, CD4 mAb (RPA-T4), sCD4, and CCR5 antagonist TAK-779.

T/F Env[a]	Subtype	Fold Inhibition by SERINC5[b]	Fold Inhibition by IFITM3[c]	CD4 mAb (µg/ml)[d]	sCD4 (nM)[d]	TAK-779 (µM)[d]
p1012.TC21.32571	B	0.48 ± 0.01	8.09 ± 0.23	0.09	331	0.15
p1006_11.C3.1601	B	0.80 ± 0.20	7.01 ±0.80	0.05	241	0.06
p1054.TC4.1499	B	1.8 ± 0.53	3.21 ±0.02	0.11	113	0.05
p1056.TA11.1826	B	0.6 ± 0.42	3.78 ±0.70	0.06	635	0.4
p1058_11.B11.1550*	B	10.14 ± 1.30	9.22 ±1.30	0.13	298	0.02
p1059_09.A4.1460	B	3.7 ± 0.09	4.05 ± 0.022	0.13	201	0.09
p6244_13.B5.4576	B	6.0 ± 0.06	5.4 ± 1.04	0.05	254	0.09
p6240_08.TA5.4622	B	0.56 ± 0.01	2.2 ± 0.04	0.06	478	0.14
p63358.p3.4013	B	1.25 ± 0.002	2.6 ± 0.04	0.05	538	0.03
p700010040.C9.4520	B	8.34 ± 0.027	1.53 ± 0.01	0.17	97	0.03
p700010058.A4.4375	B	0.85 ± 0.02	3.20 ± 0.37	0.06	413	0.3
p9014_01.TB1.4769	B	0.99 ± 0.32	4.06 ± 0.35	0.06	>1000	0.54
p9021_14.B2.4571	B	0.48 ± 0.20	1.82 ± 0.28	0.05	378	0.04
pPRB926_04.A9.4237	B	6.56 ± 0.77	1.13 ± 0.04	0.18	93	0.03
pPRB931_06.TC3.4930	B	0.67 ± 0.15	1.65 ± 0.16	0.12	>1000	0.16
pPRB958_06.TB1.4305	B	0.81± 0.31	3.57 ± 0.02	0.1	141	0.12
pSC05.8C11.2344	B	1.35 ± 0.33	1.14 ± 0.16	N.D. [e]	N.D.	N.D.
pSC45.4B5.2631	B	0.99 ± 0.10	2.96 ± 0.37	0.09	268	0.28
pWEAUd15.410.5017*	B	1.8 ± 0.06	2.40 ± 0.26	N.D.	N.D.	N.D.

a All the Env clones are R5 tropic except the two clones marked by * which are duel tropic.

b Fold inhibition by SERINC5 was determined by calculating the ratio of infectivity of the SERINC5-free virus to that of the SERINC5-bearing virus.

c Fold inhibition by IFITM3 was determined by calculating the ratio of infectivity of the IFITM3-free virus to that of the IFITM3-bearing virus.

d The IC50 values obtained from the study by Keele et al 2008 [90]

e N.D. indicates "not determined".

Table 8. Response of acute Env clones to the inhibition by SERINC5 and

Acute Env[a]	Subtype	Fold Inhibition by SERINC5[b]	Fold Inhibition by IFITM3[c]
CRF02_AG clone 235	A/G	1.13 ± 0.55	1.57 ± 0.25
CRF02_AG clone 242	A/G	2.08 ± 0.64	6.83 ± 1.19
CRF02_AG clone 250	A/G	9.18 ± 0.32	4.75 ± 1.54
CRF02_AG clone 251	A/G	0.54 ± 0.11	2.28 ± 0.51
Subtype G clone 252	A/G	0.41 ± 0.09	5.8 ± 0.66
CRF02_AG clone 255	A/G	1.74 ± 0.56	3.41 ± 0.88
CRF13_cpx clone 258	A/G	0.77 ± 0.11	1.7 ± 0.46
CRF02_AG clone 263	A/G	2.24 ± 0.66	8.06 ± 2.49
CRF02_AG clone 266	A/G	1.43 ± 0.24	4.57 ± 0.06
CRF02_AG clone 269	A/G	2.06 ± 0.60	2.78 ± 0.19
CRF02_AG clone 271	A/G	1.11 ± 0.18	11.61 ±0.75
CRF02_AG clone 278	A/G	1.37 ± 0.40	14.7 ± 1.84
CRF02_AG clone 928	A/G	1.91 ± 0.07	10.67 ±0.002
6535, clone 3 (SVPB5)	B	11.83 ± 1.98	4.69 ± 0.54
PVO, clone 4 (SVPB11)	B	0.96 ± 0-.04	1.98 ± 0.13
TRO, clone 11 (SVP. B12)	B	0.37 ± 0.03	5.66 ± 0.63
AC10.0, clone 29 (SVPB13)	B	0.60 ± 0.15	6.50 ± 1.51
pREJO4541 clone 67 (SVPB16)	B	3.86 ± 0.56	4.82 ± 0.67
pRHPA4259 clone 7 (SVPB14)	B	2.177 ± 0.49	15.56 ± 1.0
pCAAN5342 clone A2 (SVPB19)	B	0.71 ± 0.018	3.98 ± 0.40
Du156.12	C	1.17 ± 0.33	1.26 ± 0.20
Du172.17	C	1.40 ± 0.55	1.05 ± 0.005
ZM197M.PB7	C	4.26 ± 2.33	0.98 ± 0.09
ZM214M.PL15	C	2.28 ± 0.50	2.97 ± 0.39
ZM249M.PL1	C	2.84 ± 1.30	1.21 ± 0.03
ZM53M.PB12	C	1.58 ± 0.54	1.22 ± 0.14
ZM109F.PB4	C	5.10 ± 1.71	12.30 ± 0.63
ZM135M.PL10a	C	3.87 ± 1.40	4.53 ± 0.64
CAP45.2.00.G3	C	5.00 ± 1.92	1.55 ± 0.17
CAP210.2.00.E8	C	2.46 ± 0.10	5.85 ± 0.14
HIV-001428-2 clone 42	C	1.04 ± 0.06	8.19 ± 0.52
HIV-16845-2 clone 22	C	0.95 ± 0.08	9.13 ± 0.39
HIV-16936-2 clone 21	C	1.57 ± 0.15	10.96 ± 0.70
HIV-25711-2 clone 4	C	3.80 ± 0.27	7.81 ± 0.04
HIV-25925-2 clone 22	C	1.52 ± 0.03	15.64 ± 4.07

a All the Env clones are R5 tropic.

b Fold inhibition by SERINC5 was determined by calculating the ratio of infectivity of the SERINC5-free virus to that of the SERINC5-bearing virus.

c Fold inhibition by IFITM3 was determined by calculating the ratio of infectivity of the IFITM3-free virus to that of the IFITM3-bearing virus.

Table 9. Response of chronic Env clones to the inhibition by SERINC5 and IFITM3.

Chronic Env [a]	Subtype	Fold Inhibition by SERINC5[b]	Fold Inhibition by IFITM3[c]
X2278	B	1.64 ± 0.13	6.89 ± 1.72
CNE8	A/E	0.67 ± 0.02	3.27 ± 0.40
398F1	A	1.159 ± 0.07	6.4 ± 1.42
25710	C	1.8 ± 0.0.07	9.11 ± 1.35
CH119	B/C	0.33 ± 0.03	4.2 ± 0.57
246F3	A/C	0.31 ± 0.08	0.96 ± 0.27
CE1176	C	0.34 ± 0.02	13.368 ± 5.03
X1632	G	0.993 ± 0.10	12.238 ± 1.15
BJOX002000	B/C	1.12 ± 0.17	7.93 ± 1.37
CNE55	A/E	1.02 ± 0.27	5.3 ± 2.7
CE0217	C	1.37 ± 0.33	11.7 ± 1.33
TRO11	B	0.93 ± 0.24	4.13 ± 0.8
MF535.W0M.ENV.D11	D/A	1.35 ± 0.11	2.22 ± 0.2
MG505.W0M.ENV.H3	A	2.8 ± 1.4	15.26 ± 3.5
MI206.W0M.ENV.D1	A	0.86 ± 0.006	19.2 ± 0.7
ML035.W0M.ENV.I2	D/A	0.93 ± 0.08	19.6 ± 2.18

a All the Env clones are R5 tropic.

b Fold inhibition by SERINC5 was determined by calculating the ratio of infectivity of the SERINC5-free virus to that of the SERINC5-bearing virus.

c Fold inhibition by IFITM3 was determined by calculating the ratio of infectivity of the IFITM3-free virus to that of the IFITM3-bearing virus.

4.4.3 SERINC5-Resistant HIV-1 Env Tends to Be Refractory to Soluble CD4 Inhibition.

HIV-1 Env sequentially engages CD4 and CCR5 before triggering the fusion of the viral membrane with the cellular membrane. We thus asked whether the efficiency of using CD4 and/or CCR5 by Env correlates with the susceptibility to restriction by SERINC5 and IFITM3. Answering this question is facilitated by the data that are available for T/F Env clones in regard to their sensitivity to agents that inhibit CD4 or CCR5 (Table 7) [90]. We first ran the correlation analysis between the IC50 values of T/F Env against a CD4 monoclonal antibody (mAb) RPA-

T4 and the folds of inhibition by SERINC5. A significant positive correlation was detected (Fig. 24A), which indicates that the SERINC5-resistant T/F Env clones (with lower fold of inhibition) tend to require greater levels of CD4 (with lower IC50 of CD4 mAb) for entry into target cells. This is likely because these SERINC5-resistant T/F Env clones often have lower affinity for soluble CD4 (sCD4), given the significant negative correlation between the folds of inhibition by SERINC5 and the IC50 values for sCD4 against the T/F Env clones (Fig. 24B). We then examined the correlation of SERINC5 inhibition and the response to the CCR5 inhibitor TAK-779, and observed a significant negative correlation (Fig. 24C). This suggests that SERINC5-resistant Envs tend to be more independent of the CCR5 levels present in the target cell. Interestingly, the T/F Env clones which are more resistant to sCD4 (with higher IC50 of sCD4) tend to be more independent on CCR5 (higher IC50 of TAK-779) (Fig. 24D). However, when we ran the same analysis for the inhibition by IFITM3, no significant correlation was observed with the responses to any of these three agents (Fig. 24, E to G), suggesting that SERINC5 and IFITM3 target Envs sampling different conformations.

Given the dependence of SERINC5-resistance with CD4 binding, we tested whether there is an opportunity to sensitize the SERINC5-resistant HIV-1 Env to SERINC5 inhibition by using the miniprotein CD4 mimetic (CD4mc) M48U1 which binds to the CD4-binding pocket in gp120 [375]. It is possible that the quaternary architecture of primary Envs may resist engagement with proteins such as sCD4 but because of their smaller size, CD4mc might bypass such constraints. We used the NL(AD8) virus which carries the Env sequence of the primary AD8 strain and is completely resistant to both SERINC5 and IFITM3 [215, 307]. At the 10 nM and 25 nM concentrations, M48U1 did not notably inhibit NL(AD8) (Fig. 25A and 25B). However, the NL(AD8) virus became markedly inhibited by SERINC5 upon exposure to M48U1, known to

stabilize the "open" CD4-bound conformation [376] (Fig. 25A and 25B). A moderate inhibition by IFITM3 was also observed (Fig. 25B). We further tested the response of NL(AD8) to the anti-coreceptor binding site 17b antibody, a CD4-induced (CD4i) antibody [377]. The infectivity of NL(AD8) increased by more than 2-folds upon exposure to 1 and 5 μg/ml 17b (Fig. 25C). This phenotype was previously reported and shown to be determined primarily by the configuration of the V1, V2 and V3 variable loops [378-381]. If a conformation of high free energy is required to activate the trimer, the binding by 17b could stabilize a lower-energy conformation that is favorably for viral entry. In the case of 17b, it has been suggested that suboptimal occupation of the binding site (i.e., binding to one subunit of the trimer) might induce conformational changes in the unoccupied subunits facilitating entry [381]. However, occupation of the other subunits abrogates this activity, consistent with the lack of enhancement of viral entry at higher 17b concentrations (10ug/mL, Fig. 25C). Interestingly, the 17b-infectivity enhancing effect was abrogated by SERINC5 or IFITM3 (Fig. 25C), suggesting that these restriction factors stabilize Env in conformation(s) that more readily expose the coreceptor binding site. In agreement with the capacity of SERINC5 and IFITM3 to stabilize more "open" Env conformation(s), the CD4mc M48U1 facilitated neutralization by 17b at low but not high concentrations (Fig. 25D).
Taken together, these data suggest that SERINC5 and IFITM 3 stabilize more "open" Env conformations that result in the exposure of certain CD4i-epitopes such as the coreceptor binding site. This provides an opportunity to sensitize HIV-1 to the inhibition by SERINC5 by targeting the gp120 Phe43 cavity using small CD4mc [382, 383].

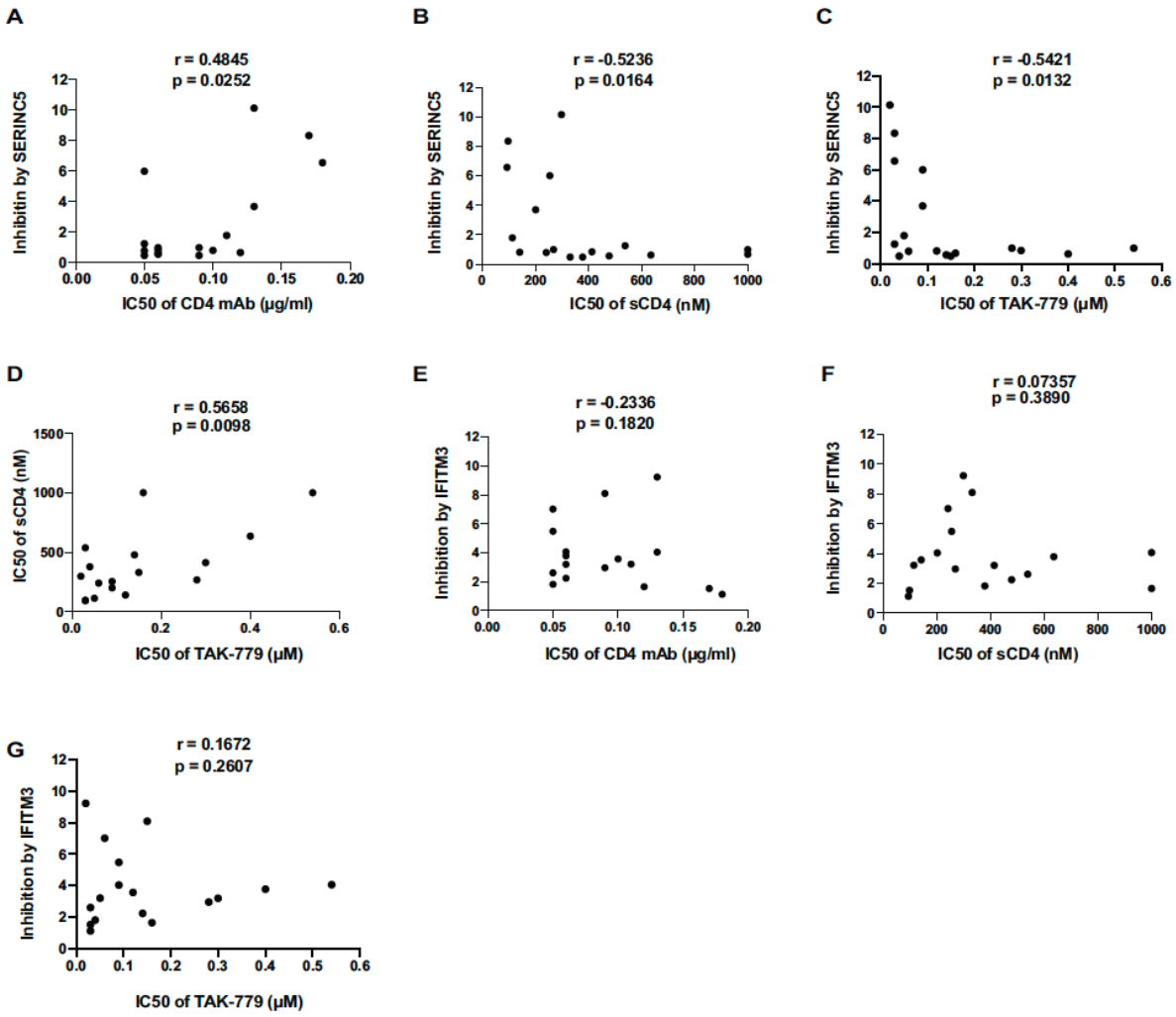

Figure 24. Correlation between the inhibition of HIV-1 Env clones by SERINC5 and IFITM3 with the responses of HIV-1 Env clones to CD4 mAb, sCD4 and CCR5 inhibitor TAK-799.

(A, B, C) Correlation analyses were performed between the folds of SERINC5 inhibition and the IC50 values of T/F Env clones for CD4 mAb (A), sCD4 (B), and TAK-779 (C). The IC50 values of these agents were obtained from the study by Keele et al [90]. (D) Correlation between the IC50 values of sCD4 and TAK-779. (E, F, G) Correlation between the folds of inhibition by IFITM3 and the IC50 values for CD4 mAb (E), sCD4 (F), and TAK-779 (G). Correlation were assessed using spearmen rank test.

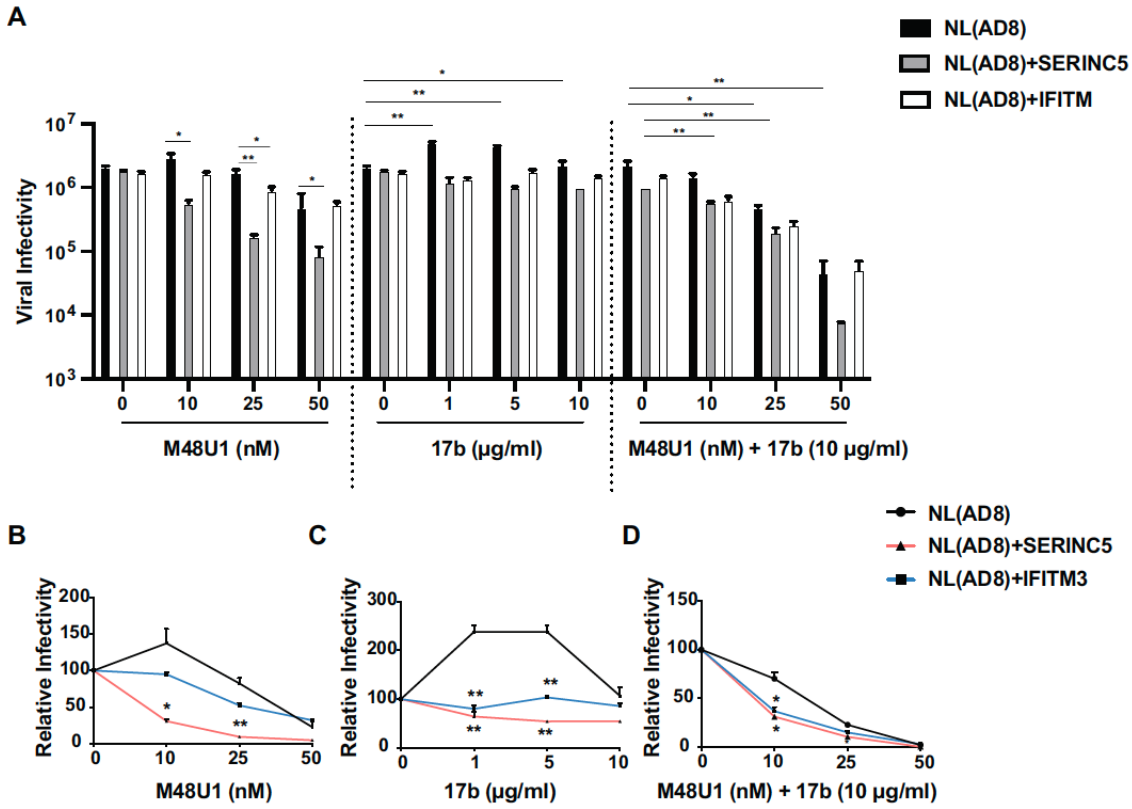

Figure 25. Effect of CD4 mimetic M48U1 and 17b antibody on the inhibition of HIV-1 Env by SERINC5 and IFITM3.

(A) The NL(AD8) viruses that carried either SERINC5 or IFITM3 were incubated with different concentrations of M48U1 or the 17b antibody or the combination of M48U1 and 10 µg/ml 17b antibody before infecting the TZM-bl cells. Luciferase data of one representative experiment are shown. (B-D) Effect of SERINC5 or IFITM3 on the inhibition by M48U1 (B), the 17b antibody (C), and the combination of M48U1 with 17b antibody (10 µg/ml) (D). The levels of viral infectivity without M48U1 or 17b treatment was arbitrarily set as 100. The results were calculated from three independent infection experiments. * indicates $P < 0.05$. ** indicates $P < 0.01$.

4.4.4 The Combination Effect of SERINC5 and IFITM3 on HIV-1 Infection.

Since HIV-1 is exposed to both SERINC5 and IFITM3 during the natural course of infection, we tested whether these two restriction factors together elicit stronger inhibition in combination than alone. We first examined the T/F Env clones. The mean fold of inhibition by SERINC5 and IFITM3 was higher than that by either SERINC5 or IFITM3, but only statistically significant for IFITM3 (Fig. 26A). This might be because the mean fold of inhibition by SERINC5 is already

higher than that by IFITM3. We next examined the chronic Env clones and did not observe significantly stronger inhibition by the combination of SERINC5 and IFITM3 compared to IFITM3 alone (Fig. 26B). Taken together, the data suggest that the combination of IFITM3 and SERINC5 does not tend to inhibit HIV-1 more than the stronger inhibitor between IFITM3 and SERINC5.

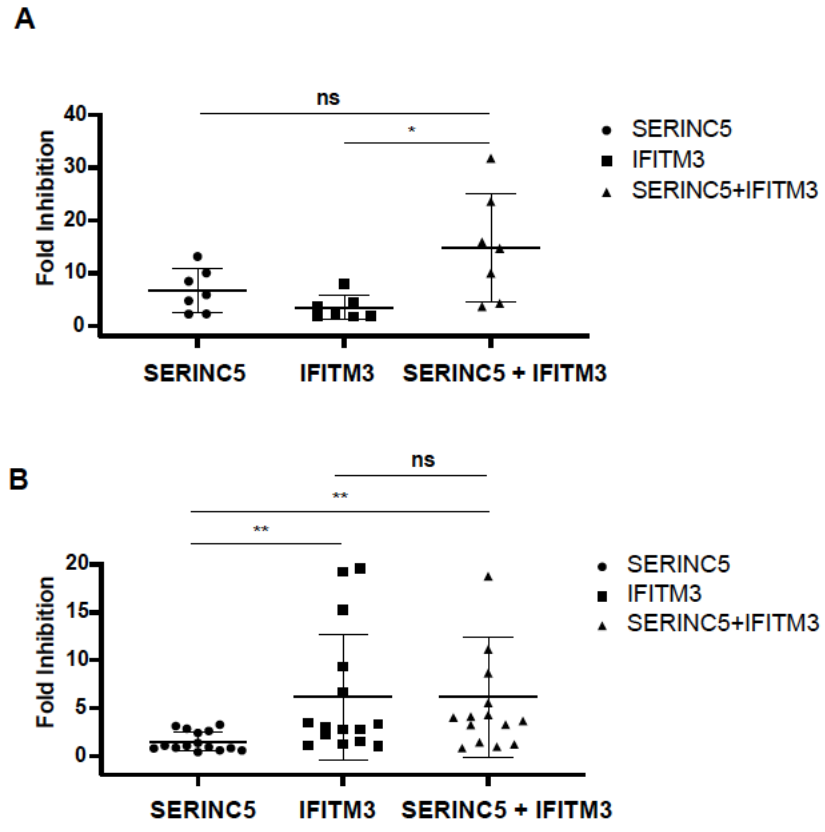

Figure 26. Inhibition of HIV-1 Env by SERINC5 and IFITM3 together.

(A) The NL4-3ΔNefΔEnv viruses were pseudotyped with the T/F Env clones. Viruses that carried either SERINC5, IFITM3 or both of these two proteins were used to infect TZM-bl cells. Folds of inhibition were calculated from the data of three independent experiments. (B) Inhibition of chronic Env clones by either SERINC5 or IFITM3 alone or by these two proteins together. Folds of inhibition were calculated from data of three independent experiments. * denotes $P < 0.05$. ns, not significant.

4.5 Discussion

In this study, we report the preserved resistance to SERINC5 by HIV-1 Env across different stages of HIV-1 infection, from HIV-1 transmission to acute infection and subsequent chronic infection. In contrast, while resisting IFITM3 inhibition during transmission, HIV-1 Env gradually loses this resistance property as the infection progresses to the chronic stage, which is in agreement with the findings by Foster et al [303]. These different responses to SERINC5 and IFITM3 over the course of HIV-1 infection may be attributed to the fact that expression of IFITM3 is induced by interferon, while SERINC5 is constitutively expressed [246]. Therefore, SERINC5 poses a constant inhibitory pressure on HIV-1. In contrast, as interferon response tapers off, IFITM3 level also goes down, in particular as HIV-1 infection advances to the chronic stage. With the need to evade the inhibition by neutralizing antibodies, Env constantly changes and thus may lose the resistance to IFITM3 [303].

While Env protein is able to overcome the inhibition by SERINC5, it does not prevent incorporation of SERINC5 into HIV-1 particles [307], which gives the opportunity of SERINC5 to act on Env and, as a result, sensitize the virus to neutralizing antibodies and Env-targeting compounds [305, 307]. This property of SERINC5 necessitates its removal from HIV-1 particles by Nef. We noticed that the chronic Env clones tend to be more resistant to SERINC5 than the T/F and acute Env clones. This may partly result from the accumulated polymorphisms in viral Nef protein over the long course of chronic infection, which impair the ability of Nef to counter SERINC5 [384]. Partial loss of SERINC5 antagonism by Nef could lay more pressure on HIV-1 Env to resist SERINC5. Our observation that chronic Env becomes sensitive to IFITM3 while maintaining resistance to SERINC5 suggests that HIV-1 Env has different strategies to evade the inhibition by these two restriction factors.

Our data suggest a correlation between the susceptibility of HIV-1 Env to SERINC5 and the affinity of Env to CD4. We observed that SERINC5-resistant Envs are more resistant to sCD4 inhibition (Fig. 24), which suggests a low affinity of SERINC5-resistant Envs for CD4. Our results also suggest that SERINC5 and IFITM3 stabilize Env in more "open" conformation(s) resulting in the exposure of the coreceptor binding site and potential neutralization by otherwise non-neutralizing antibodies. To avoid this from happening primary HIV-1 Envs assume a "closed" conformation, thus effectively concealing epitopes recognized by non-neutralizing antibodies as well as antibodies that mediate antibody dependent cellular toxicity (ADCC) [253, 374]. We speculate that by doing so, HIV-1 Env also happens to gain resistance to SERINC5. In support of this speculation, cell surface expression of CD4 renders the SERINC5-resistant HIV-1 Env prone to SERINC5 inhibition, through induction of an "open" conformation of Env as a result of interaction with CD4 [385]. Furthermore, our study showed that CD4 mimetic transforms the SERINC5-resistant Env to a sensitive one, likely through its ability to "open up" Env trimers, which has been shown to enhance antibody access and consequently promotes ADCC [363, 373, 376]. It is thus not surprising that HIV-1 has evolved multiple strategies to downregulate CD4 in the infected cells, including Vpu and Nef [231, 345, 386], because premature interaction of CD4 with Env trimer exposes Env not only to antibodies [387] but also to restriction factors including SERINC5. At the same time, agents like CD4mc are expected to sensitize HIV-1 Env to the attack by both antibodies and SERINC5, which might have therapeutic potential.

Conclusions of this study are based on the analysis of a relatively large group of HIV-1 Env clones from different stages of infection, thus may not apply to each and every Env clone that has been tested here or remains to be tested. For example, some Env clones can have low IC50

values of sCD4, i.e. high affinity to CD4, yet exhibit resistance to SERINC5. This variation among the Env proteins of different HIV-1 strains indicates that more factors than the affinity to CD4 modulate Env susceptibility to SERINC5 restriction.

In summary, our results indicate a constant inhibitory pressure on HIV-1 Env imposed by SERINC5 over the course of HIV-1 infection. Assuming a "closed" Env conformation may have allowed HIV-1 not only to evade the humoral response but also the restriction by SERINC5, even though this mechanism may not be effective against IFITM3. Along this line, "opening up" Env trimers with CD4mc could expose HIV-1 to the attack by both antibodies and SERINC5 restriction.

Chapter 5

Discussion

5.1 Summary of Findings and Contribution to General Knowledge

In 2015, two groups independently discovered SERINC5 as a novel restriction factor inhibiting HIV-1 infectivity, and published their findings in *Nature* [239, 240]. This breakthrough discovery solved a two-decades-old mystery: how does the Nef protein of HIV-1 increase viral infectivity? Findings from these two groups show that Nef increases HIV-1 infectivity by antagonizing SERINC5. Since 2015, many studies focused on elucidating the underlying mechanism of Nef counteracting exogenous SERINC5. It is now known that Nef downregulates SERINC5 from the plasma membrane using the endosome/lysosome degradation system. However, recent study shows that Nef may not necessarily block the association of endogenous SERINC5 with the virions [388]. This finding argues for additional mechanism by which HIV-1 counters SERINC5.

Findings from this thesis have identified a novel role for the HIV-1 Env protein to antagonize SERINC5. For the first time, I found that in addition to Nef , HIV-1 also uses the Env protein to overcome SERINC5 inhibition. This finding expands our understanding of the arms race between HIV-1 and SERINC5, also may inspire the development of novel therapeutic interventions, as will be further discussed in this chapter.

In Chapter 3, I showed that Env determines HIV-1 susceptibility to SERINC5. Some HIV-1 strains, including primary isolates and T/F strains, carry SERINC5-resistant Env. I further showed that even in the absence of Nef, Env counters SERINC5 without excluding SERINC5 from the HIV-1 particles. Importantly, by mutagenesis studies, I mapped the SERINC5 resistant phenotype of Env to the V3 loop region of Env.

Next, I looked into the effect of SERINC5 incorporation into the HIV-1 virions. I found that HIV-1 carrying SERINC5 is more susceptible to neutralizing antibodies targeting MPER of the

Env protein. This indicates that SERINC5 incorporation into the HIV-1 particles may modulate the conformation of Env by exposing the epitopes to neutralizing antibodies.

In Chapter 4, I further explored the role of HIV-1 Env in antagonizing SERINC5 inhibition. I examined 70 HIV-1 Env isolates for the inhibition by SERINC5. These HIV-1 Env clones are derived from different stages of infection including transmission, acute stage, and chronic stage. I found that over the course of infection, in the absence of Nef, HIV-1 Env remains resistant to SERINC5 inhibition. Unlike the SERINC5-resistant phenotype of HIV-1 Env clones throughout the course of infection, Env isolates show different susceptibility profiles to IFITM3 inhibition. Viruses carrying T/F Env are resistant to IFITM3. On the contrary, HIV-1 carrying Env isolates from chronic infection are sensitive to IFITM3. This distinct susceptibility profile of HIV-1 Env clones against SERINC5 and IFITM3 can be attributed to the fact that unlike the expression of IFITM3 which is interferon-inducible, SERINC5 is constitutively expressed. Therefore, to avoid the constant inhibition by SERINC5, HIV-1 Env is forced to remain resistant during the course of infection.

Finally, I investigated the properties of SERINC5-resistant HIV-1 Env by running correlational analyses between the sensitivity of the Env to SERINC5 and the responses of the Env to the entry inhibitors such as CD4 mAb, sCD4, and CCR5 antagonist TAK-779. These correlational analyses revealed that SERINC5-resistant Env has lower affinity for the CD4 receptor, while having a higher affinity for CCR5 coreceptor. This suggests that SERINC5- resistant Env has a "closed" conformation with less exposed epitope which allows HIV-1 to evade the adaptive immunity response as well as the restriction by SERINC5. Treating HIV-1 with CD4 mimetic peptide which binds to the gp120 binding pocket of Env renders SERINC5-resistant Env

sensitive. This suggests that inducing an "open" conformation makes Env more sensitive to SERINC5 inhibition.

5.2 Outstanding Questions

In this thesis, I presented a novel function for HIV-1 Env protein in counteracting SERINC5 inhibition. However, there are still many questions that remain unanswered and require further investigations. In this section I will discuss the remaining outstanding questions and future work for this research.

5.2.1 How Does the V3 Loop Determine the Sensitivity of HIV-1 to SERINC5 Inhibition?

In Chapter 3, I showed that by exchanging the V3 loop of a SERINC5-sensitive HIV-1 with the V3 loop of a SERINC5-resistant virus, HIV-1 becomes resistant to SERINC5 inhibition. The V3 loop region locates in gp120 of Env and consists of 35 amino acids (residues 296 to 331 of gp120) which are connected through a disulfide bridge [389]. The V3 loop region is essential for HIV-1 infectivity since it binds to the coreceptors, CXCR4 or CCR5, and mediates viral entry. This region contains high variability which allows the virus to escape from neutralizing antibodies [390, 391].

It is still unclear how the V3 loop of Env determines the HIV-1 sensitivity to SERINC5. It remains unknown whether a particular amino acid on the V3 loop or the combination of the amino acids are required to modulate the response of HIV-1 to SERINC5. It is shown that chimeric viruses carrying the V3 loop from SERINC5-resistant Env maintain higher stability of the Env trimer, this suggests that the V3 loop may confer resistant to SERINC5 by stabilizing the Env trimer [215]. This is in agreement with recent findings showing that HIV-1 Env carrying an "open" conformation such as NL4-3 is more affected by SERINC5 [385]. To this end, the study also shows that SERINC5 expression in the virus producing cells interferes with the clustering of

the Env trimer and dissociates Env trimeric complex. Hence, HIV-1 carrying a "closed" conformation Env with a higher stability of the Env trimer, tends to be more resistant to SERINC5 inhibition [385]. To understand how the HIV-1 V3 loop contributes to the SERINC5-resisatnt phenotype, I performed single site amino acid mutagenesis assay in the V3 loop of the SERINC5-resistant NL(AD8V3) proviral DNA (data not shown). Five different single site amino acid mutations were induced in the V3 loop of the NL(AD8V3) construct including H306A, I321A, D324A, I325A, and D320A. The sensitivity of this mutants were examined against SERINC5 inhibition. None of the mutated V3 loops became sensitive to SERINC5-inhibition. This suggests that mutation in one amino acid of the V3 loop does not change the sensitivity of HIV-1 to SERINC5. Further studies are required to investigate whether other regions of Env including V1 or V2 also contribute to the resistant phenotype of the Env to SERINC5 inhibition.

5.2.2 Why Did HIV-1 Develop Two Distinct Mechanisms to Counter SERINC5 Inhibition?

Throughout my thesis in Chapters 3 and 4, I explored why HIV-1 developed two distinct mechanisms to overcome the antiviral activity of SERINC5. However, a definite answer to this question remains to be elucidated. In Chapter 3, I showed that regardless of the type of the Env protein that HIV-1 is carrying, SERINC5- associated HIV-1 is sensitive to neutralizing antibodies targeting MPER region. This provides evidence that HIV-1 requires Nef to remove SERINC5 from the plasma membrane and to prevent SERINC5 incorporation into the virus particles. Thus, HIV-1 remains immune to the neutralizing antibodies. The necessity role of Nef to remove SERINC5 from the plasma membrane shows that due to the pressure from the adaptive immunity, HIV-1 needs to employ more than one mechanism to counter SERINC5.

In Chapter 4, I showed that HIV-1 Env from different stages of infection remain resistant to SERINC5 inhibition. This finding is in agreement with previous report showing that Nef isolates

from chronic stage are impaired in countering SERINC5 [384]. The impaired ability of Nef isolates from chronic stage to counter SERINC5 is due to accumulations of Nef polymorphisms over the course of infection. [384] Therefore, Env is pressured to remain resistant to the inhibition by SERINC5 during the course of infection. The exact interplay between Nef and Env to counter SERINC5 inhibition remains to be investigated. It is still unknown whether under in vivo conditions, Nef and Env counter SERINC5 through fully independent mechanisms or there is a potential cross-talk between their anti-SERINC5 activity.

Lastly, It is shown that Nef clones from EC are less efficient in downregulating SERINC5 from the cellular membrane [365]. It will be of great interest to examine the SERINC5-antagonism function of Env clones from EC. Future studies are required to compare the response of Env clones from EC to those from HIV-1 progressors for their anti-SERINC5 activities. As discussed in Chapter 1, research on how ECs manage HIV infection is of great interest because it opens new avenues for novel vaccine strategies.

5.2.3 What is The Impact of SERINC5 or IFITM3 on the Env?

In Chapter 3, I tested SERINC5-bearing HIV-1 against a panel of neutralizing antibodies, and I reported that SERINC5 sensitizes HIV-1 to MPER targeting neutralizing antibodies. It is speculated that SERINC5 interferes with the conformation of the Env protein, which leads to the exposure of the Env epitopes. Later on, BiFC studies from another group showed that SERINC5 interacts with HIV-1 Env [385]; which further supports our speculation that SERINC5 interacts with HIV-1 Env and interferes with the Env conformation. However, it is still not known how SERINC5 is able to expose MPER epitopes of HIV-1 Env to neutralizing antibodies. Recent structural study showed that SERINC5 is fully embedded in plasma membrane. Therefore, membrane-embedded SERINC5 is in close proximity of the MPER epitope of HIV-1 Env which

is near the viral membrane [242]. This study further supports that the interaction between SERINC5 and the Env leads to the exposure of HIV-1 MPER epitope.

Earlier studies reported that Nef determines the response of the HIV-1 Env protein to neutralizing antibodies. However, a new study using flow virometry found that Nef does not change the Env response to neutralizing antibodies [392]. Hence, the mechanistic interplay among Nef, Env, and SERINC5 in response to neutralizing antibodies remains to be further elucidated. More studies are required to use endogenous SERINC5 and primary HIV-1 isolates to decipher the interplay between SERINC5, Nef, and Env. Understanding the strategies that HIV-1 uses to escape from neutralizing antibodies will help us to advance HIV-1 vaccine research.

5.2.4 The Interplay Among CD4, Nef, Env, and SERINC5

It is established that HIV-1 employs two distinct mechanisms to downregulate the CD4 receptor from the cellular surface. Nef induces CD4 downregulation via the lysosomal/endosomal pathway [345], while Vpu induces CD4 downregulation through the ERAD pathway [393]. There is a growing body of studies demonstrating that CD4 downregulation by HIV-1 is beneficial for HIV-1 replication and infectivity [394]. For example, downregulation of the CD4 receptor by Nef and Vpu protects the infected cells from ADCC response [395]. Moreover, CD4 downregulation prevents superinfection of the infected cells [396].

HIV-1 entry consists of sequential steps, and HIV-1 Env undergoes dynamic conformational changes. The interaction between the HIV-1 Env Phe43 cavity close to the CD4 binding site and the CD4 receptor leads to an irreversible conformational changes on Env. This conformational changes induces Env to CD4-bound state [397]. The shift from unliganded state or "closed state"

to the CD4 bound state or "open state" releases the energy required to mediate the HIV-1 fusion to the target cell.

It has been over a decade since the development of CD4mc compound as a novel therapeutic approach to prevent and control HIV-1 infection [376] . CD4mc compound is a small peptide that binds to the Phe43 cavity in gp120 and induces Env to an "open" conformation state. The thermodynamic changes on Env induced by CD4mc compound are similar to those induced by the CD4 receptor or sCD4 [376].

In Chapter 4, I showed that there is a negative correlation between the sensitivity of HIV-1 to sCD4 and the response of HIV-1 Env to SERINC5 inhibition. This indicates that SERINC5-resistant Env has lower affinity for the CD4 receptor. This finding is in agreement with the data showing that opening the Env using CD4mc peptides such as M48U1 renders Env more sensitive to SERINC5. In agreement with my findings from Chapter 4, recent study has shown that expression of CD4 in virus producing cells also render HIV-1 sensitive to SERINC5 inhibition [385].

Recent study from Strapoli et al.[392] investigates the effect of SERINC5 and CD4 on Env accessibility to neutralizing antibodies, which is known as Env profile. This study shows that expression of SERINC5 and CD4 in virus producing cells independently or additively change the Env profile and reduce HIV-1 infectivity, regardless of Nef expression. By using flow virometry analysis, this study was able to shed light on the complex interplay between CD4, Nef, and Env. However, they demonstrate that the effect of CD4 and SERINC5 on Env profile varies with different HIV-1 viral strains [392].

Altogether, a growing body of evidence suggests that CD4 interaction with Env induces an "open" conformation state on Env. This "open" state is more sensitive to humoral response as

well as SERINC5 inhibition. Investigating different approaches to induce an "open" state on HIV-1 Env is a potential avenue to develop novel vaccine strategies.

5.2.5 Is there any Interplay Between the Antiviral Activities of SERINC5 and IFITM3?

In Chapter4, I looked into the the combination effect of SERINC5 and IFITM3 when expressed in virus producing cells. I showed that upon the expression of both SERINC5 and IFITM3 in virus producing cells, no synergistic antiviral inhibition is observed. This indicates that the combination of SERINC5 and IFITM3 does not inhibit HIV-1 more strongly compare to SERINC5 or IFITM3 alone. This study is one of the few studies in the field to investigate the interplay between restriction factors. Another study looking into the interplay between restriction factor was done by Liu's group showing that SERINC5 expression potentiates TIM-1 mediated inhibition of HIV-1 release by stabilizing TIM-1 protein [398]. Future studies are required to investigate the effect of SERINC5-associated virions on IFITM3 antiviral activity when expressed on target cells. Studying the interplay among restriction factors and the potential cross-talk between the antiviral host proteins are of high importance and physiologically relevant, since in vivo and upon viral infection, all these restriction factors are expressed together.

5.2.6 Does SERINC5 inhibit other family of viruses?

As discussed earlier, the antiviral activity of SERINC5 was discovered in 2015. Ever since, different retroviruses have been reported to employ different mechanisms to antagonize SERINC5. S2 from equine infectious anemia virus (EIAV) [247] and glycosylated gag from murine leukemia virus (MLV) [248] use similar strategies as HIV-1 Nef to antagonize SERINC5 inhibition. This is done through targeting SERINC5 into the endosomes. It will be of great interest to investigate whether the antiviral activity of SERINC5 is specific to retroviruses or it has the ability to inhibit other viruses as well. To investigate the inhibitory effect of SERINC5 on

other enveloped viruses beyond retroviruses, I tested the ability of SERINC5 to restrict ZIKA virus (data not shown). ZIKA virus belongs to the family of flaviviruses. I have shown that SERINC5 has modest antiviral effect on the infectivity of ZIKA virus. However, further experiments are required to understand how SERINC5 inhibits the infectivity of the ZIKA virus and whether ZIKA virus has employed any strategies to counter SERINC5.

Lastly, due to the potency of SERINC5 antiviral activity, it is important to investigate whether SERINC5 has any role in cross-species transmission of virus to humans. Studies found that the anti-SERINC5 potency of Nef correlates with SIV prevalence in the ape and monkey species [244]. This suggests that SERINC5 is a potential determinant of HIV spread. However, at the genetic levels there is lack of evidence for evolutionary arms race for SERINC5 [245]. Thus, the role of SERINC5 in cross species transmission and whether it has a universal antiviral activity remain to be determined.

5.3 SERINC5 as The Potential Drug Target

Due to the emergence of HIV-1 multi-drug resistance and patients failing their current antiviral regimens, there is a constant demand for developing novel therapies to manage the infection. Development of novel antiretroviral therapies can be done by understanding the virus-host interaction. One potential avenue of investigation in the field of SERINC5 and HIV-1 interaction is to target the strategies that HIV-1 employs to counter SERINC5 inhibition. Understanding the antiviral activity of SERINC5 and targeting the mechanisms that HIV-1 employs to counter SERINC5 pave the path for development of novel therapies.

SERINC5 is a potent antiviral host protein which, in the absence of Nef, inhibits HIV-1 by almost 100 fold and renders viruses more sensitive to neutralizing antibodies. By targeting mechanisms that Nef uses to downregulate SERINC5 to ensure SERINC5 incorporates into the

virions; the SERINC5-associated HIV-1 particles become sensitive to the inhibitory pressures such as neutralizing antibodies from adaptive immunity. Subsequently, by allowing SERINC5 association into the virions, the infectivity of HIV-1 will be reduced.

Findings from this thesis show that HIV-1-assocatiated SERINC5 renders viruses more sensitive to neutralizing antibodies and entry inhibitors such as maraviroc and CD4mc. At the same time, compounds like CD4mc are expected to sensitize HIV-1 Env to the attack by SERINC5 and antibodies, which might have therapeutic potentials. Thus, getting a mechanistic understanding on how HIV-1-associated SERINC5 modulate the response of Env to the inhibitory pressures, new entry inhibitor compounds can be developed. These new entry inhibitors may mimick the effect of SERINC5 on HIV-1 Env. Thus, HIV-1 becomes sensitive to the humoral response.

5.4 Conclusions

In this thesis, I contribute to the field of host-virus interactions by studying the antiviral protein SERINC5. I present a novel function for HIV-1 Env in counteracting SERINC5 antiviral inhibition, and shed light on why HIV-1 has developed two distinct mechanisms to counter SERINC5 inhibition using Nef and Env proteins. HIV-1 Env resists SERINC5 inhibition without preventing SERINC5 from incorporating into the HIV-1 particles. And SERINC5-associated virions are sensitive to neutralizing antibodies. This finding highlights the necessity role of Nef to downregulate SERINC5 from the plasma membrane and thus prevent SERINC5 incorporation into the virions. Furthermore, throughout this thesis, I highlighted the link between the response from the innate immunity including SERINC5 and the adaptive immunity to control HIV-1 infection.

It is important to understand how HIV-1 Env respond to the inhibition by SERINC5 throughout the course of infection. Thus, I examined the susceptibility of a panel of HIV-1 Env isolates at

different stages of viral infection, transmission, acute, and chronic, against SERINC5 inhibition. I also expanded my screening study to measure the inhibition by IFITM3. I showed that while HIV-1 Env clones from the transmission stage are resistant to both SERINC5 and IFITM3, as infection progresses into the acute and chronic stages, the resistance to IFITM3 but not to SERINC5 is gradually lost. This indicate that SERINC5 and IFITM3 exert differential inhibitory pressures on HIV-1 Env over different stages of HIV-1 progression. Finally, I showed that virion-associated SERINC5 render HIV-1 sensitive to CD4mc M48U1. This provides an additional support that SERINC5 incorporation induces an "open" conformation state on Env. Understanding the impact of SERINC5 on HIV-1 Env encourages the development of novel therapies by sensitizing HIV-1 to humoral response and impairing viral entry. Altogether, my research unravels a novel and important role for HIV-1 Env to antagonize SERINC5 inhibition, and the findings open new avenues to develop novel HIV-1 therapeutics.

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