UNIVERSITY OF KWAZULU-NATAL

THE ROLE OF NEF-MEDIATED SERINC5 DOWN-REGULATION ON HIV-1 DISEASE PROGRESSION

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Preface.

This project represents original work done by the author and others whose contribution has been acknowledged in the text. All experimental work mentioned in this dissertation was accomplished in the P2+ Laboratory at HIV Pathogenesis Programme in the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of Kwa-Zulu Natal, Durban, South Africa, from July 2019 to May 2021, under the supervision of Dr Jaclyn Mann.

10 May 2021

Marshlin Delon Naicker (Student)   Date

7 May 2021

Dr. Jaclyn Mann (Supervisor)   Date

Declaration.

I, Marshlin Delon Naicker, declare that:

(i)   The research reported in this study, except otherwise indicated, is my original work.

(ii)   This study has not been submitted for any degree or examination at any other university.

(iii)   This study does not contain other person's data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

(iv)   This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a) Their words have been re-written, but the general information attributed to them has been referenced.

    b) Where their exact words have been used, their writing has been placed inside quotation marks and referenced.

(v)   This dissertation does not contain text, graphics or tables copied and pasted from the internet unless specifically acknowledged, and the source being detailed in the dissertation and in the references section.

Signed: Date: 10 May 2021

Acknowledgements.

I would like to thank:

List of Abbreviations

ADCC– Antibody-dependent cellular cytotoxicity

AIDS – Acquired immunodeficiency syndrome

AP-1 - Activator protein 1

AP-2 - Activator protein 2

APC - Antigen-presenting cells

ART – Antiretroviral therapy

BLT – Bone marrow liver thymic

bp – Base pair

BREC - Biomedical Research Ethics Committee

CA – Capsid protein

CART – Combined antiretroviral therapy

CCR5 - Chemokine receptor type 5

CD1 – Cluster of differentiation 1

CD28 – Cluster of differentiation 28

CD4 – Cluster of differentiation 4

CD74 – Cluster of differentiation 74

CD8 – Cluster of differentiation 8

CD86 - Cluster of differentiation 86

CME - Clathrin-mediated endocytosis

CPSF6 - Cleavage and polyadenylation specificity factor 6

CRF – Circulating recombinant forms

CTL - Cytotoxic T lymphocyte

CXCR4 – Chemokine receptor type 4

DMSO – Dimethylsulfoxide

DNA – Deoxyribonucleic acid

EIAV - Equine infectious anemia virus

ESCRT – Endosomal complexes required for transport

EST - Expressed sequence tag

FBS - Fetal bovine serum

GFP – Green fluorescent protein

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV – Human immunodeficiency virus

HLA-I – Human leukocyte antigens class I

HPP – HIV pathogenesis programme

ICL4 - Intracellular loop 4

IN - Integrase

kDa – Kilodalton

LAMP1 - Lysosomal-associated membrane protein 1

Lck - Lymphocyte-specific protein tyrosine kinase

LEDGF – Lens epithelium-derived growth factor

LTR – Long terminal repeat

MA – Matrix protein

MLV – Murine leukemia viruses

mRNA – Messenger ribonucleic acid

MSM – Men having sex with men

NC – Nucleocapsid protein

Nef - Negative regulation factor

nm – Nanometer

NMR – Nuclear magnetic resonance

PACS - P1-derived artificial chromosome

PBMC – Peripheral blood mononuclear cell

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

PIP2 – Phosphatidylinositol 4,5-bisphosphate

PR - Protease

Rev - RNA splicing protein

RNA – Ribonucleic acid

RT – Reverse transcriptase

RT-PCR - Reverse transcription polymerase chain reaction

SERINC – Serine incorporator

SERINC1 - Serine incorporator 1

SERINC2 - Serine incorporator 2

SERINC3 - Serine incorporator 3

SERINC4 - Serine incorporator 4

SERINC5 - Serine incorporator 5

SFK – Src kinase family

SIV – Simian immunodeficiency virus

SU -Gp 120

Tat - Transactivator protein

TCR– T-cell receptor

TDG - Tumour differentially expressed genes

TGN – Trans-Golgi network

TM – Gp 41

TRAPS - Tenofovir Gel Research for AIDS Prevention Science

Vif - Viral infectivity factor

Vpr - Virus protein R

Vpu - Virus protein U

WASP – The Wiskott–Aldrich Syndrome protein

Abstract.

HIV-1 Nef is a small accessory protein that plays a vital role in enhancing HIV-1 pathogenesis, evidenced by a strongly attenuated disease course following infection with a virus with gross Nef defects. Nef has multiple cellular effects, which enhance HIV-1 replication and immune evasion. Major activities of Nef include CD4 down-regulation, HLA-I down-regulation, and CD4-independent enhancement of virion infectivity. Recent studies have uncovered Nef-mediated down-regulation of the host restriction factor SERINC5 as an important mechanism by which Nef enhances virion infectivity. However, there is a lack of studies defining the role of this function in HIV-1 pathogenesis. Previous studies indicated that Nef-mediated CD4 down-regulation and enhancement of infectivity are likely the major contributors to Nef's effect of enhancing pathogenicity; the relative significance of each Nef function for HIV-1 disease progression remains incompletely understood.

Given the key role of Nef-mediated SERINC5 down-regulation in enhancing virion infectivity, the primary aim of the present study was to determine if this Nef activity contributes significantly to disease progression in individuals infected with HIV-1 subtype C, which is the dominant HIV-1 subtype worldwide. To investigate this, SERINC5 down-regulation activity of 106 Nef clones derived from patients with early HIV-1 subtype C infection were evaluated in a CD4$^+$ T cell line using a flow cytometry-based assay and subsequently related to viral load set point and to the rate of CD4$^+$ T cell decline using linear regression analysis. The second aim of this study was to assess the overall contribution of SERINC5 down-regulation to Nef function, using linear regression analysis with E values as a proxy for overall Nef function *in vivo*.

The third aim of the study was to identify amino acid variants that significantly alter Nef-mediated SERINC5 down-regulation using a codon-by-codon sequence-function analysis tool available online.

No significant relationship was found between each Nef function and viral set point (SERINC5 down-regulation, p=0.28) or rate of CD4$^+$ T cell decline (SERINC5 down-regulation, p=0.48). CD4 down-regulation (p=0.02) and SERINC5 down-regulation (p=0.003) were significant determinants of the E value in univariate analyses, and SERINC5 down-regulation remained significant in the multivariate analysis (p=0.003). We found several amino acids that were significantly associated with increased (10I, 11V, 38D, 51T, 65D, 101V, 188H and, 191H) or decreased (10K, 38E, 65E, 135F, 173T, 176T and, 191R) SERINC5 down-regulation activity.

In conclusion, none of the Nef functions in our study, including SERINC5 down-regulation, were found to be significant individual contributors to disease progression. However, interestingly we found CD4 down-regulation and SERINC5 down-regulation to be the largest contributors, of the Nef functions considered here, to overall Nef function and that the contribution of SERINC5 down-regulation was the most significant. Taken together, this could be explained by multiple Nef functions acting together to facilitate the enhancement of viral spread and immune evasion *in vivo* that ultimately enhance disease progression. We found several amino acid variants that either increased or decreased Nef's ability to down-regulate SERINC5; however, further studies in the form of site-directed mutagenesis are warranted to further understand their effect on SERINC5 down-regulation activity. In summary, the results suggest that SERINC5 down-regulation is a strong contributor to overall Nef function and identifies potential genetic determinants of this Nef function that may have relevance for vaccines or therapeutics.

CHAPTER

ONE:

INTRODUCTION

1.1 Background.

In 1981, several young men having sex with men (MSM) began dying of unusual opportunistic infections and cancers. These infections would become the first reported cases of acquired immune deficiency syndrome (AIDS) (Fauci 2008). AIDS is an illness that develops because of progressive immune dysfunction, allowing opportunistic infections and cancers to thrive, resulting in death. The causative agent for AIDS was established in 1984 to be Human Immunodeficiency Virus (HIV), a retrovirus that belongs to the *Retroviridae* family and the *Lentivirus* genus (Fauci 2008). HIV infects cells associated with the human immune system, such as T helper cells, dendritic cells, and macrophages that express the cluster of differentiation 4 (CD4) protein (Cunningham et al. 2010).

1.1.1 Origin and epidemiology.

HIV shares similar characteristics to simian immunodeficiency viruses (SIV), which is a primate virus. Several zoonotic transmissions of SIV have resulted in infection in humans together with the emergence of different HIV lineages (Hemelaar 2012). Two main types of HIV exist, namely HIV-1, which was first identified in 1984 (Fauci 2008) and HIV-2, which was first identified in 1986 (Esbjörnsson et al. 2019). The predominant strain of HIV globally is HIV-1. It is classified into four groups: group M, group N, group O, and group P. Group M represents the pandemic form of HIV-1 (Hemelaar 2012).

In addition to this, there are several circulating recombinant forms (CRF) of the virus. Groups N, O, and P are less prevalent and represent a small percentage of global HIV-1 infections (Hemelaar 2012). HIV-2 is relatively less prevalent and appears to be confined to West Africa (Campbell-Yesufu and Gandhi 2011).

The HIV pandemic remains one of the biggest challenges facing global health care. Since HIV was discovered in 1984 to cause AIDS, approximately 35 million people have died of HIV, and more than 70 million have been infected with the virus (Global 2019). Anti-retroviral therapy (ART) and combined antiretroviral therapy (CART), although effective at suppressing viremia and reducing morbidity, do not eliminate the virus, and therefore lifelong treatment is required. This is unsustainable in the long-term, especially in resource-poor countries (Obiako and Muktar 2010).

With no cure currently available, an emphasis is placed on prevention rather than treatment to combat the HIV-1 epidemic globally. Tools that are available for prevention include the use of condoms (Steiner and Cates Jr 2006), pre-exposure prophylaxis (Baeten et al. 2013) (including microbicides (Nuttall 2010) such as tenofovir gel (Karim et al. 2010)), and post-exposure prophylaxis (Sultan, Benn, and Waters 2014). Despite several vaccine trials, an effective vaccine remains elusive, with the most promising trial being the RV144 vaccine tested in Thailand. The trial, which was completed in 2009, has thus far shown the best, albeit modest, efficacy of approximately 31% (Tomaras and Plotkin 2017).

The extraordinary genetic diversity of HIV, the many characteristics of the Env glycoprotein that protect the virus from antibody-mediated neutralization, the rapid nature of viral replication, and latency have so far proven insurmountable challenges for vaccine design (Fauci et al. 2008).

1.1.2 HIV-1 structure and genome.

An HIV-1 virion is spherically shaped and approximately between 100-120 nanometres (nm) in diameter (Sierra, Kupfer, and Kaiser 2005). Surrounding the virus particle is a lipid-bilayer membrane that encapsulates a conical capsid. Within this conical capsid are two un-spliced, noncovalently linked, identical positive-sense single-stranded ribonucleic acid (RNA) molecules (Sierra, Kupfer, and Kaiser 2005) together with the enzymes reverse transcriptase, integrase, and polymerase (Turner and Summers 1999) (Figure 1.1).

The HIV-1 genome is 9719 base pairs (bp) in length (Sierra, Kupfer, and Kaiser 2005) and is flanked at either end by two identical long terminal repeat (LTR) regions. HIV-1 contains nine genes that encode twelve proteins and three enzymes (Li and De Clercq 2016) (Figure 1.1).
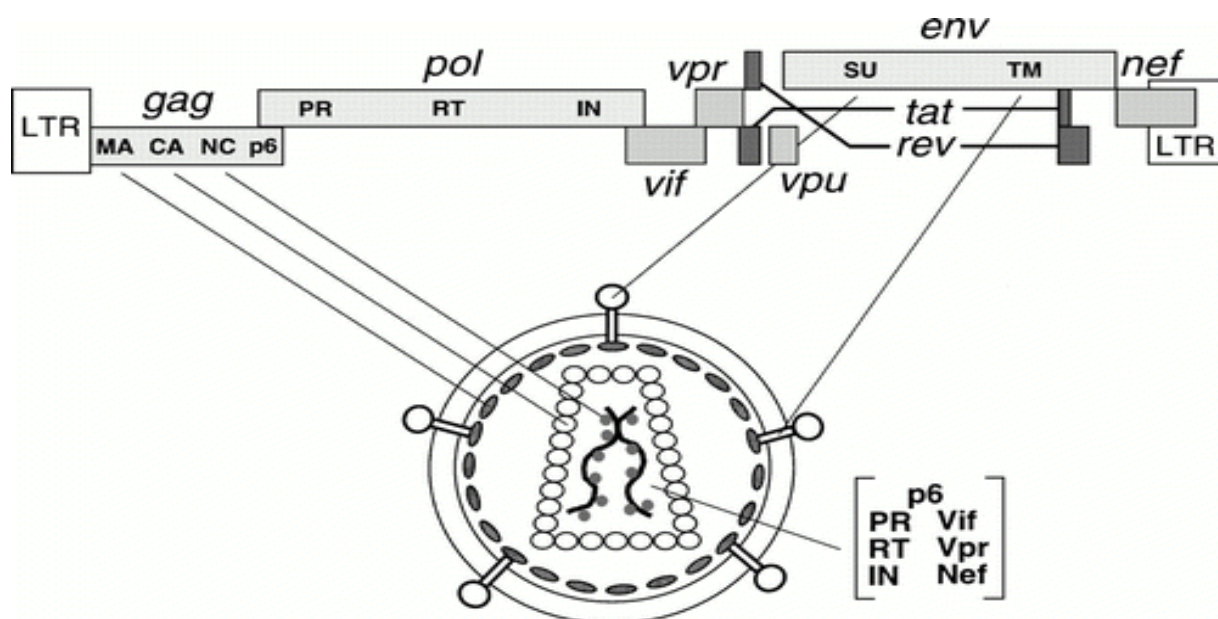
Figure 1.1: The structure of an HIV virion and the genome.

Figure 1.1 illustrates an HIV-1 virion is spherically shaped and within this conical capsid is two identical positive-sense single-stranded RNA molecules. The HIV-1 genome contains nine genes flanked by two long terminal repeats (LTRs). These nine genes are *Pol*, *Gag*, *Env*, *Tat*, *Rev*, *Vpr*, *Vpu*, *Vif* and *Nef.* These nine genes code for 12 proteins and three enzymes. *Gag* codes for matrix protein (MA), capsid protein (CA), nucleocapsid protein (NC) and a p6 protein. *Pol* codes for the three enzymes protease (PR), reverse transcriptase (RT), and integrase (IN). *Env* codes for surface protein or gp120 (SU) and transmembrane protein or gp41 (TM). Transactivator protein (Tat) and RNA splicing protein (Rev) are the regulatory proteins and negative regulation factor (Nef), virus protein R (Vpr), viral infectivity factor (Vif) and virus protein U (Vpu) are the accessory proteins. The image was sourced from (Frankel and Young 1998).

The *gag* gene encodes for the matrix protein (MA), the capsid protein (CP), the nucleocapsid protein (NC), and a p6 protein (Li and De Clercq 2016). The *pol* reading frame follows the *gag* reading frame and encodes the enzymes protease, reverse transcriptase, ribonuclease H (RNase H), and integrase (Li and De Clercq 2016). The *env* reading frame encodes for the two envelope glycoproteins, surface protein (SU) gp120 and transmembrane protein (TM) gp41 (Li and De Clercq 2016). The HIV genome, in addition to the structural proteins, also codes for regulatory proteins, namely transactivator protein (Tat) and RNA splicing protein (Rev) (Li and De Clercq 2016). In addition to the structural and regulatory proteins, four accessory proteins are also encoded by the HIV-1 genome: negative regulation factor (Nef), virus protein R (Vpr), viral infectivity factor (Vif) and, virus protein U (Vpu) (Li and De Clercq 2016).

1.1.3 HIV-1 replication cycle.

HIV-1 infection involves a series of steps that are crucial for viral replication, as shown in (Figure 1.2). The stages of the replication cycle are viral entry, capsid uncoating, reverse transcription, nucleus import, integration of pro-viral DNA into the host genome, transcription of the integrated genome, virus protein synthesis, virus assembly, budding and finally virion maturation (Sierra, Kupfer, and Kaiser 2005).
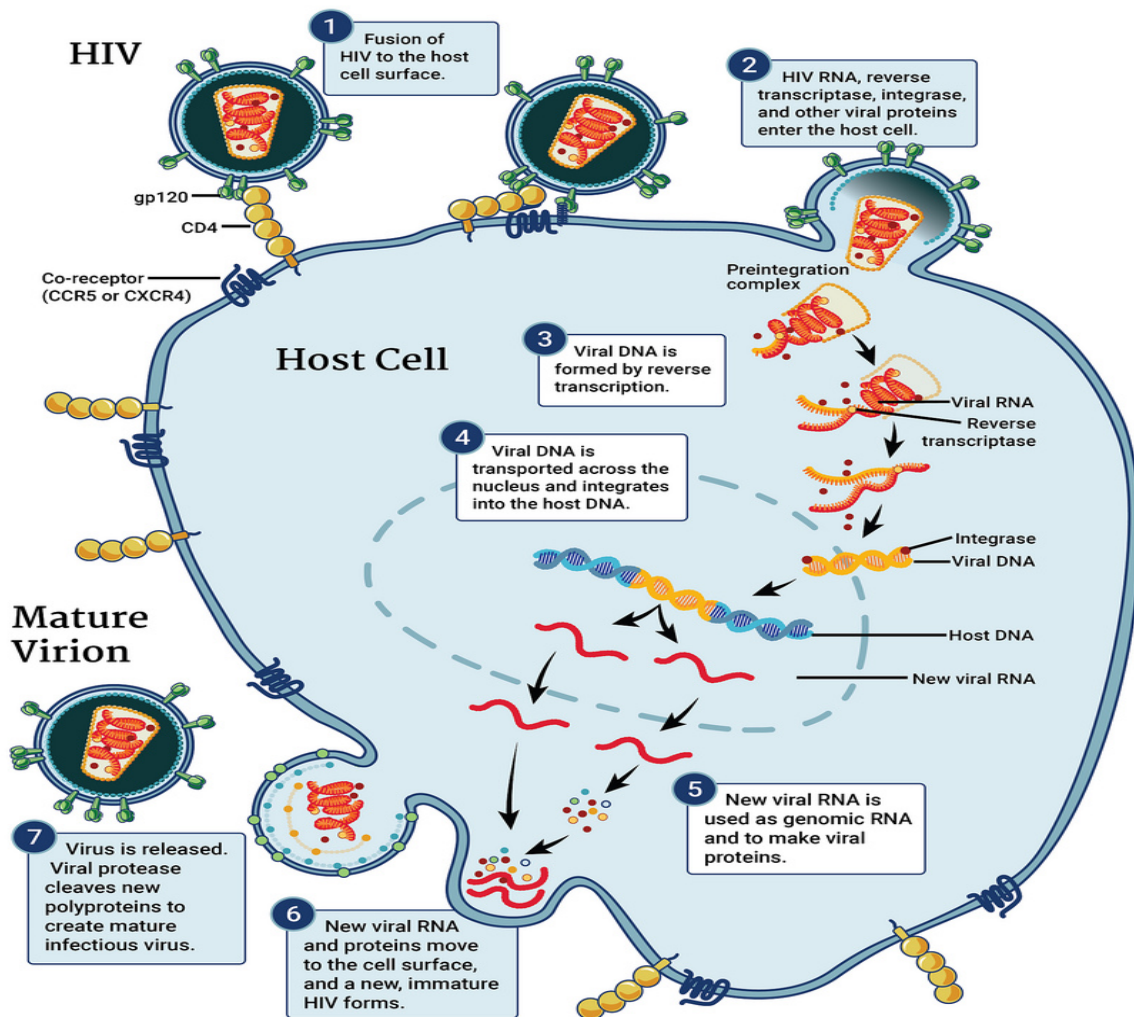
Figure 1.2: The HIV-1 replication cycle.

Figure 1.2 illustrates the HIV-1 replication cycle. The initial step of the HIV-1 replication cycle is the entry of the virus into the host cell. Following this, the process of reverse transcription occurs to convert HIV RNA to pro-viral DNA. The HIV DNA is then integrated into the host genome by the enzyme integrase, and viral DNA is transcribed and translated to produce viral proteins. These newly formed proteins migrate to the surface of the cell, and new HIV-1 virions are formed. Once the enzyme protease releases the proteins, the HIV-1 virions are mature. The image was sourced from (National Institute of Allergy and Infectious Diseases).

1.1.3.1 Viral entry.

The first step of the HIV-1 replication cycle is viral attachment. Following conformational changes in the Env trimer, the C-C chemokine receptor type 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4) co-receptors interact with gp120. As a result of the interaction with the co-receptors, gp41 initiates direct contact between the target host cell and the virus (Kirchhoff 2013).

1.1.3.2 Uncoating, reverse transcription and integration.

Following the successful fusion of the viral and host cellular membrane, the viral capsid enters the cytoplasm. A pre-integration complex (PIC) is formed by viral and host proteins (Bowerman et al. 1989). Translocation of the PIC to the host nucleus and integration into the host genome is mediated by viral integrase and the host protein lens epithelium-derived growth factor (LEDGF/p75), follows (Cherepanov et al. 2003). A recent study by (Burdick et al. 2020) discovered that intact (or nearly intact) viral cores enter the nucleus through a mechanism involving interactions with host protein cleavage and polyadenylation specificity factor 6 (CPSF6), complete reverse transcription in the nucleus before uncoating, and un-coat before integration near their genomic integration sites.

1.1.3.3 Transcription and nuclear export.

The newly integrated HIV-1 DNA in the host genome then undergoes transcriptional activation by the HIV-1 regulatory protein Tat and cellular transcriptional factors. Both singly and multiply spliced RNA molecules are synthesized and exported, using HIV Rev, to the cytoplasm from the nucleus (Sorin and Kalpana 2006). Once in the cytoplasm, the RNA molecules are translated into proteins using the host cell machinery.

1.1.3.4 Protein synthesis and assembly.

The assembly process involves virion protein synthesis and the packaging of components into the capsid. This occurs in specialized membrane microdomains that are located at the plasma membrane, and the Env glycoproteins are transported to these domains. Virion assembly is controlled by the Gag protein by forming protein-protein interactions in the plasma membrane to facilitate the production of a particle that is spherical in shape and packages genomic RNA. The Gag matrix binds to Env, the Gag capsid domain which controls the protein-protein interactions that result in the formation of a conical shell and the Gag nucleocapsid binds the viral genome (un-spliced RNA) (Sundquist and Kräusslich 2012).

1.1.3.5 Budding and maturation.

During the budding process, the assembled virus moves across the producer cell, acquiring a lipid envelope together with Env spikes. The host endosomal sorting complexes required for transport machinery (ESCRT) controls the budding process (Sundquist and Kräusslich 2012), which occurs within membrane rafts (Chazal and Gerlier 2003).

The occurrence of maturation is when the virions undergo structural changes and then proceed to infect other cells. The cleavage of Gag into proteins by protease results in changes in conformation (Sundquist and Kräusslich 2012), and following this, the virions now become infectious.

1.1.4 HIV-1 pathogenesis.

1.1.4.1 Transmission.

HIV-1 transmission may be percutaneously through damaged tissue or viral exposure directly at mucosal surfaces (Moumneh 2017). The source of the virus is in the breast milk, blood, semen, as well as pre-seminal, vaginal, and rectal fluids of infected individuals; therefore, the most common ways of transmission are through unprotected sexual intercourse (both anal and vaginal), the sharing of needles during intravenous drug use, organ transplants, and blood transfusions (Shaw and Hunter 2012). It is worth mentioning that other body fluids such as sweat, tears and saliva do not contain HIV-1 (Moumneh 2017).

1.1.4.2 Disease progression.

Following infection, there are four important stages of progression: the eclipse phase, the acute phase, the chronic phase, and AIDS, as reviewed in (Coffin and Swanstrom 2013). The eclipse phase is between 1-2 weeks post initial infection. During this phase, the virus replicates locally. The viremia during this phase is undetectable, and there is a lack of detectable immune response and symptoms.

The acute phase of HIV-1 infection is approximately 2-4 weeks post-infection. It is associated with relatively elevated levels of viremia due to widespread dissemination of the virus throughout the body and a large proportion of infected $CD4^+$ T cells in the lymph nodes and blood. Symptoms coincide with the acute phase. At the time of peak viremia, the first antibodies against HIV-1 proteins become detectable, and they are non-neutralising, with neutralising antibodies taking longer to develop. The peak in $CD8^+$ T cell responses to antagonize HIV-1-infected cells is also only reached after the peak viremia and coincides with the viral decline from the peak viremia. During the acute phase, there is an impermanent decline in the number of $CD4^+$ T cells present in the blood (there is a partial recovery). Also associated with this phase is an impermanent decline in the number of $CD4^+$ T cells present in the blood.

The chronic phase of infection lasts between 1-20 years. It is associated with the initial stabilisation of the viral load after the acute phase (known as viral load set point), followed by a slowly elevating level of viremia and a gradual decline in the level of $CD4^+$ T cells. Patients are mainly asymptomatic during this phase of the infection; however, $CD4^+$ T cells continue to be infected by the virus and die.

The final phase of HIV-1 infection results in a serve depletion of $CD4^+$ T cells to a level below 200 cells/mm^3, following which opportunistic infections begin to emerge and thrive (Lackner, Lederman, and Rodriguez 2012). The level of viremia elevates during this phase and culminates in the death of the infected individual (Coffin and Swanstrom 2013).

1.2. HIV-1 Nef.

HIV-1 effectively evades the host immune system and promotes replication within the endo-membrane system by efficiently manipulating cell machinery and the control of protein trafficking (Pereira and daSilva 2016). The virus accomplishes this by encoding accessory proteins that create cellular conditions that optimize viral replication and dissemination *in vivo* (Aldrovandi and Zack 1996). The accessory protein Nef has been extensively studied and confirmed to be the most prominent viral factor that is associated with pathogenesis (Arien and Verhasselt 2008; Das and Jameel 2005; Pereira and daSilva 2016).

Nef is a 27-32 kilodalton (kDa) non-enzymatic protein that consists of approximately 206 amino acids (Geyer and Peterlin 2001) and is translated from messenger RNA (mRNA) that is multiply spliced (Basmaciogullari and Pizzato 2014). Nef is an N-terminally myristoylated protein (Arien and Verhasselt 2008). Nuclear magnetic resonance (NMR) (Grzesiek et al. 1996; Grzesiek et al. 1997) and crystal structures (Arold et al. 1997; Grzesiek et al. 1997; Lee et al. 1996) show that Nef is composed of a folded core containing residues 79 to 203 (at the centre of which is a dileucine motif-containing loop (149-179), an N-terminal flexible loop containing the residues 1 to 78, and a C-terminal loop containing residues 204 to 206 (Buffalo et al. 2019) (Figure 1.3).
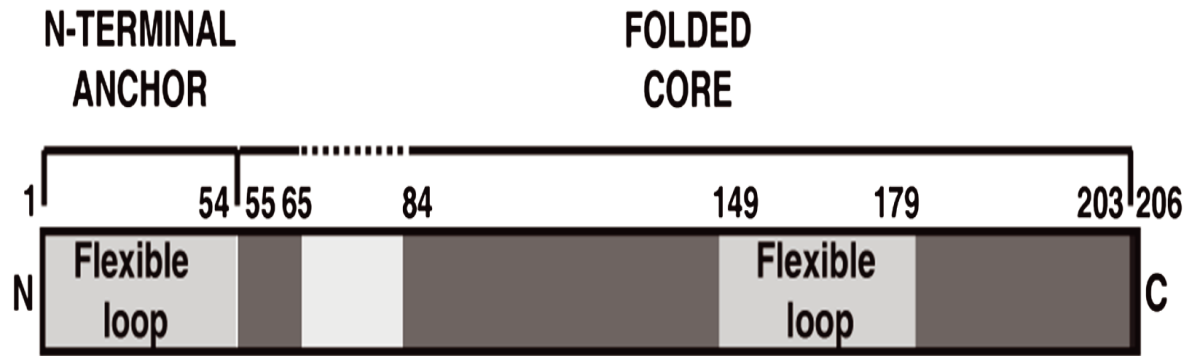
Figure 1.3: The schematic depiction of structural domains of HIV-1 Nef.

Figure 1.3 illustrates the structural domains present in the HIV-1 accessory protein Nef. The image was sourced from (Pereira and daSilva 2016).

The crucial residues required for the interaction with the several host factors are all found in these regions (Basmaciogullari and Pizzato 2014). The region that constitutes the largest surface of Nef is the flexible, and less structured region. This region can undergo conformational changes and allows for easy accessibility concerning interacting with other proteins (Pereira and daSilva 2016).

1.2.1 Nef activities.

The multi-functional characteristic of Nef is perhaps its most remarkable feature. Nef has multiple cellular effects, which enhance HIV-1 replication and immune evasion. Examples of the major activities of Nef include CD4 down-regulation, human leukocyte antigen–I (HLA-I) down-regulation, and CD4-independent enhancement of virion infectivity (Collins et al. 1998; Das and Jameel 2005; Schwartz et al. 1995).

Several Nef activities include modulation of receptors at the infected cell surface. For example, Nef down-regulates entry receptors – including CD4 (Atkins et al. 2008), chemokine receptor 4 (CXCR4) (Venzke et al. 2006) and chemokine receptor 5 (CCR5) (Michel et al. 2005)– from the infected cell surface to prevent superinfection and enhance replication. Further, Nef down-regulates cluster of differentiation 28 (CD28) (Swigut, Shohdy, and Skowronski 2001), cluster of differentiation 8 (CD8) (Stove et al. 2005), cluster of differentiation 80 (CD80) (Chaudhry et al. 2005), and cluster of differentiation 1 (CD1) (Chen et al. 2006).

Other mechanisms of evading host immune responses include the Nef-mediated down-regulation of HLA-I (Mann et al. 2014) as well as upregulation of immature HLA-II (CD74) (Mahiti et al. 2015). CD4-independent enhancement of virion infectivity was also recently shown to involve Nef modulation of cell surface receptors – serine incorporator 3 (SERINC3) and serine incorporator 5 (SERINC5) (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015). Aside from the modulation of receptor levels in the infected cell, Nef induces apoptosis in both uninfected and infected immune effector cells (Das and Jameel 2005), enhancing HIV-1 pathogenesis.

Nef also enhances infection in the host by stimulating the release of a lymphocyte-stimulating factor by macrophages, thereby increasing the pool of infectable lymphocytes (without additional stimuli) (Das and Jameel 2005; Manninen, Renkema, and Saksela 2000). A more detailed review of the Nef activities analysed in the present study follows.

1.2.1.1 Nef-mediated CD4 down-regulation.

The ability of Nef to interact with several host proteins allows it to exert numerous cellular functions while containing no enzymatic activity (Pereira and daSilva 2016). Several proteins found on the surface of cells play a crucial role in the viral life cycle and host immunity and the expression of these proteins is controlled by the cellular machinery, which is hijacked by Nef to create an optimal environment for viral replication (Das and Jameel 2005).

CD4 is an important 55 kDa (Das and Jameel 2005) transmembrane type 1 glycoprotein (Buffalo et al. 2019). CD4 is located on the surface of important cells related to the immune system, such as monocytes, T-helper cells and macrophages, and plays a crucial role in the maintenance and development of immune function (Lama 2003). CD4 attaches onto HLA-II and functions significantly in signal transduction via the T cell receptor (TCR) (Weiss and Littman 1994).

CD4 plays a significant role in HIV-1 infection as it is the main cellular receptor the virus attaches to (Buffalo et al. 2019). Nef down-regulates CD4 from the surface of cells using AP-2 and clathrin-dependent endocytosis and directs CD4 through the endosome to the lysosome for degradation (Gondim et al. 2015). At the plasma membrane, Nef binds directly to AP-2, which is directly interacting with CD4, and this results in the formation of a tripartite complex CD4-Nef-AP2 (Chaudhuri et al. 2009). AP-2 and CD4 interact via a dileucine motif in the CD4 tail (Pitcher et al. 1999). Nef has its own dileucine motif 164LL165, through which it interacts with AP-2 (Garcia and Miller 1991; Kelly et al. 2008; Janvier et al. 2003).

An additional two motifs located within Nef's dileucine loop are also vital in mediating the AP-2 and Nef interaction. These include an acidic 174DD175 motif and a hydrophobic motif M168/L170 that binds to the µ1 basic patch and the σ2 subunits of AP-2, respectively (Chaudhuri et al. 2009). The Nef mediated downregulation of CD4 enhances the dissemination of HIV-1 envelope expression virions that are fully infectious (Argañaraz et al. 2003; Lundquist et al. 2002; Mann et al. 2013; Ross, Oran, and Cullen 1999).

A study by (Veillette et al. 2014) showed that Nef-mediated CD4 down-regulation might also allow for evasion of antibody-dependent cell-mediated cytotoxicity (ADCC) as the Env interaction with CD4 exposes the epitopes targeted by ADCC. Further, the downregulation of CD4 prevents premature death as a result of "superinfection" of the host cell (Aiken et al. 1994; Lama 2003).

1.2.1.2 Nef-mediated HLA-I down-regulation.

The response mediated by adaptive immunity is dependent on the recognition of cells that have been infected. This recognition happens before the elimination of the infected cells by cytotoxic T-lymphocytes (CTLs) (Das and Jameel 2005). HLA-I on virus-infected cells presents viral epitopes to CD8^{+} antigen-specific cytotoxic T-lymphocytes (CTLs) (Elliott and Hoyne 2015). HIV-1 Nef down-regulates HLA-I by rerouting it from the cell surface to lysosomes for degradation. Nef does this by promoting the interaction between the clathrin adaptor complex AP-1 and HLA-I (Buffalo et al. 2019; Wonderlich, Williams, and Collins 2008). Specifically, the acidic Nef sequence 62EEEE65 attaches to µ1 basic patch of AP-1 by an electrostatic association with the positively charged residues (Jia et al. 2012). Nef also binds to the HLA-I cytoplasmic tail.

This is accomplished via polyproline (68PxxP78) repeats, and it results in a stable HLA-1-AP1 μ1 complex (Collins and Collins 2014). Further HLA-1 D327 and Nef D123 together bind to a μ1 basic patch, and thus Nef D123 is also crucial in AP-1 binding (Buffalo et al. 2019).

1.2.1.3 Nef-mediated alteration of TCR signalling.

The activation of T-lymphocytes affects their development, proliferation, and immune function (Smith-Garvin, Koretzky, and Jordan 2009). As reviewed by (Abraham and Fackler 2012), together with co-stimulatory signals, T-cell activation results when the T-cell antigen receptor (TCR) forms a complex with peptides that are bound to the HLA-1 in antigen-presenting cells (APC). The resulting interactions are referred to as an immunological synapse and trigger a broad spectrum of downstream signalling events. The initiation of TCR signalling occurs at the plasma membrane, and lymphocyte-specific protein tyrosine kinases (Lck) is a crucial part of this process. Nef initiates the alteration of TCR signalling by the re-targeting of Lck away from the plasma membrane and into the early and recycling endosomes as well as the trans-Golgi network (TGN). Nef also alters TCR signalling by inhibiting the Wiskott-Aldrich syndrome protein (WASP), which plays an important role in TCR signalling by regulating the internalization of TCR complexes (Haller et al. 2006).

Altogether, Nef blocks signalling from the plasma membrane and redirects signalling to a narrow TCR downstream response, resulting in the avoidance of activation-induced apoptosis and the promotion of HIV-1 replication in infected cells (Abraham and Fackler 2012). Importantly, the effect of Nef on TCR signalling depends on the activation status of the infected cell, such that Nef fine-tunes the activation state (Neri et al. 2011).

1.2.1.4 Nef-mediated enhancement of infectivity.

Over the years, one of the more mysterious characteristics of Nef has been the mechanism behind the enhancement of infectivity. The dependence of HIV-1 viral infectivity on Nef was first discovered in a study by (Chowers et al. 1994), where the researchers sought to determine the replication competence of HIV-1 genomes that contained mutations in the open reading frame of Nef. The study showed the positive contributory effect Nef had towards viral particle infectivity. Since then, the phenomenon of Nef-mediated enhancement of viral infectivity was consistently validated across numerous different subtypes and cell lines; however, the extent of enhancement of viral infectivity would vary between 3 to 40 fold (Basmaciogullari and Pizzato 2014).

Until 2015, there remained an elusive question of what the mechanism was driving Nef's enhancement of infectivity. This was answered by (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015), who identified Nef-mediated down-regulation of SERINC5 to be the contributing factor to the enhancement of infectivity as reviewed in greater detail below (Section 1.3).

1.3 Nef-mediated down-regulation of SERINC proteins.
1.3.1 The SERINC family

The serine incorporator (SERINC) family are transmembrane proteins (Gonzalez-Enriquez et al. 2017), classified as type III integral membrane proteins that are highly conserved in eukaryotic cells (Inuzuka, Hayakawa, and Ingi 2005).

The physiological function of the SERINC family remains largely unknown; however, it is known that these proteins participate in the transport of the non-essential amino acid serine through the lipid bilayer of the cell membrane and that SERINC proteins are involved in the biosynthesis of sphingolipids and phosphatidylserine by incorporating serine into membrane lipids (Inuzuka, Hayakawa, and Ingi 2005). Due to cysteine-rich regions in the SERINC proteins, their high hydrophobicity, and their tendency to cross-link and aggregate, these protein are complex to study. This could explain the length of time it required to identify them as host restriction factors following the initial discovery of Nef's ability to affect infectivity (Firrito et al. 2018).

The family consists of five members, namely SERINC1, SERINC2, SERINC3, SERINC4, and SERINC5 (Gonzalez-Enriquez et al. 2017) (Figure 1.4). These five proteins share more than seventeen percent amino acid identity, together with a predicted membrane topology that is similar (Rosa et al. 2015). Structural characterization of the five SERINC proteins reveals 10-11 transmembrane domains (Gonzalez-Enriquez et al. 2017; Zhang et al. 2017).
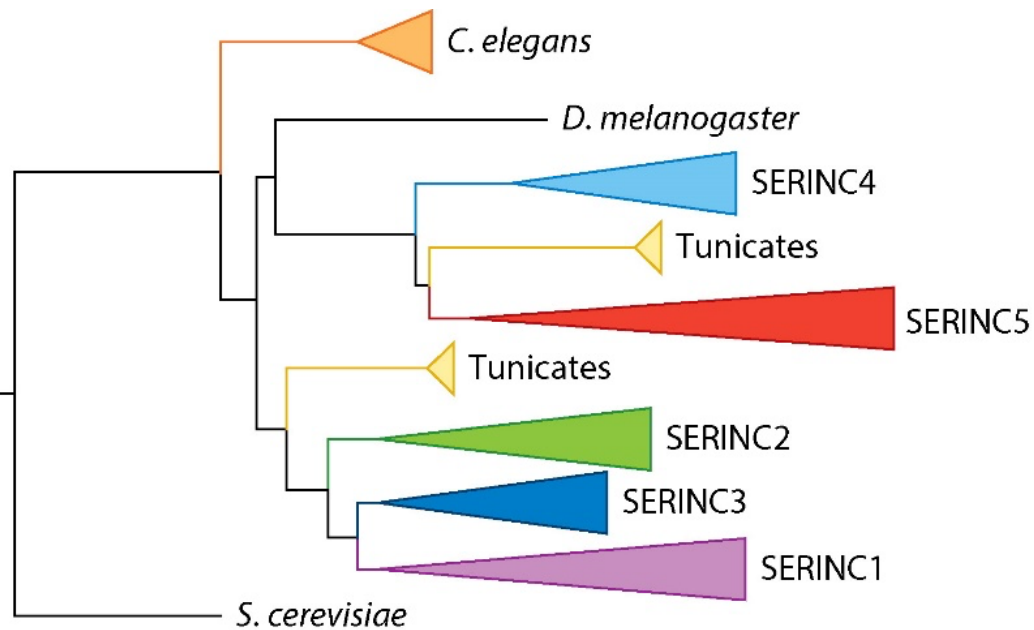
Figure 1.4: The SERINC family of genes and proteins

Figure 1.4 illustrates the phylogenetic relationship that exists among the family of SERINC proteins. Saccharomyces cerevisiae, Caenorhabditis elegans, and Drosophila melanogaster contain a single copy of the SERINC gene family. Mammals contain five copies that are divided in two clusters that are derived by a duplication that occurred in an early ancestor. This has given rise to a cluster that includes SERINC1, SERINC2, SERINC3, and another cluster that includes SERINC4 and SERINC5. The different colours are indicative of the differing branches of SERINC genes. The triangle lengths share a proportionality to the number of nucleotide substitutions that have occurred in a particular branch. The image was taken from (Firrito et al. 2018).

SERINC3 was the first protein from the SERINC family to be identified. It was discovered in testicular tumours overexpressed in polyomavirus large T-antigen transgenic mice (Lebel and Mes-Masson 1994), leading to the initial naming of the gene family as tumour differentially expressed genes (TDG). SERINC3 does not contain any spliced isoforms (Gonzalez-Enriquez et al. 2017).

SERINC1 was first retrieved from the mouse expressed sequence tag (EST) database and in human liver and lung cancers (Lebel and Mes-Masson 1994; Player et al. 2003). SERINC5 is a host restriction factor that was first identified in cultured rat pro-oligodendrocytes, differentiated into myelinating oligodendrocytes (Firrito et al. 2018). It contains five alternatively spliced isoforms (Zhang et al. 2017); however, isoform SERINC5_001 is the most relevant as it has stable expression and is localized to the plasma membrane, while this is not the case for the other isoforms (Zhang et al. 2017). In recent years, a growing body of evidence suggests SERINC family proteins harbour anti-viral abilities that make them host restriction factors (Firrito et al. 2018; Gonzalez-Enriquez et al. 2017; Zhang et al. 2017).

1.3.2 The anti-viral activity of the SERINC proteins.

Host proteins that interfere at different stages of the viral cycle are known as "viral restriction factors". SERINC proteins can act as a restriction factor (Firrito et al. 2018; Gonzalez-Enriquez et al. 2017; Zhang et al. 2017). SERINC3 and SERINC5 have been shown to restrict several retroviruses, namely HIV-1 (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015), murine leukemia viruses (MLVs) (Ahi et al. 2016) and equine infectious anemia virus (EIAV) (Chande et al. 2016), although SERINC5 is more potently antiretroviral than SERINC3 (Rosa et al. 2015; Schulte et al. 2018; Usami, Wu, and Göttlinger 2015).

From the SERINC family of proteins, only SERINC2 displays no ability to inhibit HIV-1 infectivity, although SERINC1 also shows very little anti-HIV activity (Schulte et al. 2018). SERINC3 modestly inhibits HIV-1 infectivity, while SERINC5 and the closely related SERINC4 show the most potent anti-HIV-1 activity (Schulte et al. 2018).

However, the relevance of the SERINC4 activity requires further investigation as mRNA expression of this protein was not detected in tissues (Firrito et al. 2018).

Studies using various cell types infected by HIV-1 clearly show that SERINC3 and SERINC5 restrict HIV-1 virion infectivity. A study by (Usami, Wu, and Göttlinger 2015) provided evidence of elevated expression levels of SERINC3 and SERINC5 mRNA in monocyte-derived macrophages. Silencing these genes results in the enhanced infectivity of HIV-1 virions produced from these cells (Usami, Wu, and Göttlinger 2015). Similarly, the knockout of SERINC5 in peripheral blood mononuclear cells (PBMCs) dramatically enhanced HIV-1 infectivity (Rosa et al. 2015). Notably, the SERINC5 restriction of HIV-1 was demonstrated *in vivo* for the first time recently (Timilsina et al. 2020).

The anti-viral activity of SERINC5 appears to have been evolutionarily conserved. In a study by (Heigele et al. 2016), the researchers analysed seven different orthologs of SERINC5 from humans, rhesus macaques/sooty mangabeys, chimpanzees/gorillas, African green monkeys, tamarins, common marmosets, and mice (Dai et al. 2018; Heigele et al. 2016). All of them reduced infectivity of HIV-1 (Nef-defective), which was consistent with their high level of sequence conservation. However, not all isoforms of SERINC5 display anti-HIV activity. As previously mentioned, there are five alternatively spliced isoforms of SERINC5, with the SERINC5-001 isoform being the most stable and abundantly expressed.

Consistent with this, a study done by (Zhang et al. 2017) highlighted that SERINC5-001 exhibited the most potent inhibition of HIV-1 infectivity while the other isoforms that did not contain the tenth transmembrane domain and were not localized in the plasma membrane showed inferior activity.

The tenth transmembrane domain (Zhang et al. 2017) and localization to the plasma membrane were crucial for anti-viral activity (Pye et al. 2020; Zhang et al. 2017). A study by (Schulte et al. 2018) revealed SERINC5 exhibited superior viral association when compared to SERINC2, and unlike SERINC2, SERINC5 was identified to localize preferentially into domains of the plasma membrane that are detergent-resistant where virus budding could occur (Brügger et al. 2006).

1.3.3 Potential mechanisms of SERINC-mediated inhibition of HIV-1 infectivity.

It was initially hypothesized that SERINC anti-viral activity was linked to their involvement in the biosynthesis of sphingolipids and phosphatidylserine by incorporating serine into membrane lipids (Inuzuka, Hayakawa, and Ingi 2005); however, a study investigating this hypothesis showed that there is no alteration by SERINC5 of the lipid composition of progeny HIV-1 virions or alteration of the exposure on their surface of phosphatidylserine (Trautz et al. 2016).

A hallmark feature of SERINC5 is its ability to associate with virions, and the incorporation of SERINC5 into virion particles is linearly related to HIV-1 inhibition by SERINC5 (Firrito et al. 2018). SERINC5 prevents detection of the viral core in the cytoplasm shortly post-infection and thus inhibits a step prior to this (Schulte et al. 2018).

Three main mechanisms of SERINC5-mediated inhibition of HIV-1 infectivity are currently proposed, relating to (i) inhibition of virion fusion with the target cell via the fusion pore, (ii) increasing susceptibility of virions to neutralising antibodies, and (iii) altering the structure or distribution of Env (Beitari et al. 2017) (Figure 1.5). These are discussed in more detail below.
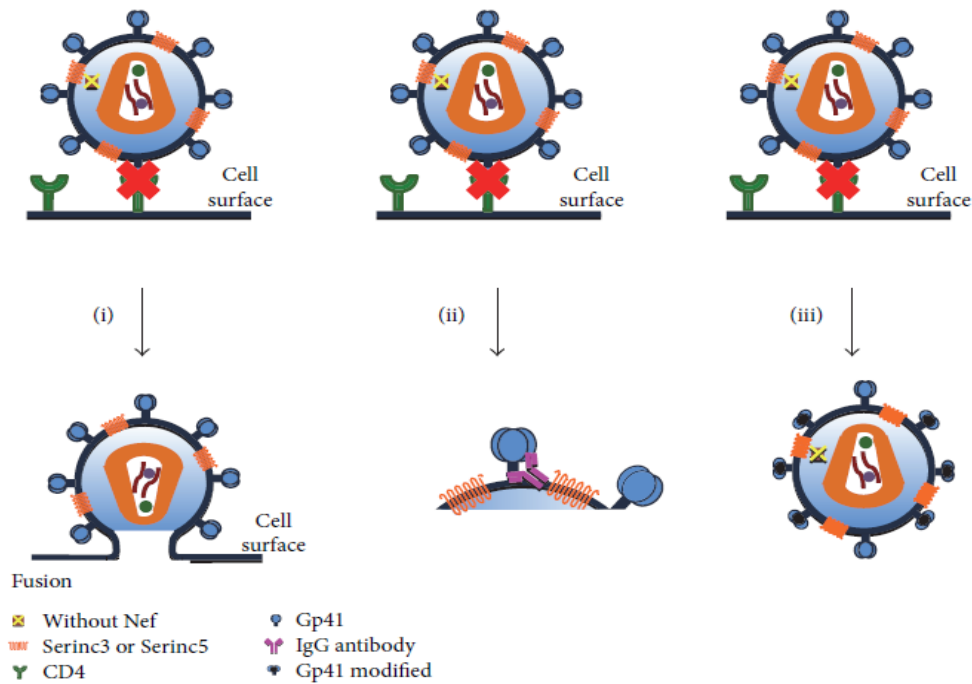
Figure 1.5: The schematic representation of possible mechanisms used by SERINC5 to inhibit HIV-1 activity.

Figure 1.5 illustrates the three possible mechanisms of SERINC5-mediated inhibition of HIV-1 infectivity. i) SERINC5 causes an alteration in the enlargement of the fusion pore thus decreasing the capability of virions to fuse with target cells. ii) As a result of the slowed fusion, gp41 would possess an open conformation. This would remain the case for a long time leaving it suspectable to a neutralizing antibody. iii) SERINC5 could possibly promote structural changes in Env thus preventing entry of the virus. The figure is sourced from (Gonzalez-Enriquez et al. 2017).

SERINC5 was shown to inhibit small fusion pore formation (Figure 1.5 i) (Sood et al. 2017). It was suggested by (Firrito et al. 2018) that envelope rigidity, because of the crowding on virions by SERINC5 molecules, could lead to the interference of fusion or the delivery of the viral capsid into the cytoplasm.

They also hypothesise that SERINC5 could increase the energy barrier that is needed for adequate fusion, and it could impair pore expansion and/or the pore formation itself.

The increased rigidity of the viral membrane due to SERINC5 incorporation would slow fusion, thus promoting Env to adopt an open conformation for a longer time, therefore increasing the opportunity for neutralizing antibodies to bind (Figure 1.5 ii) (Sood et al. 2017; Tedbury and Sarafianos 2017). In a study by (Sood et al. 2017), the researchers showed that the incorporation of SERINC5 resulted in an increased sensitivity of the 4E10 antibody that targets the membrane-proximal region of gp41. The researchers concluded that HIV-1 is sensitized by SERINC5 to inhibitory peptides and neutralizing antibodies that recognize the conserved domains on gp41 (Sood et al. 2017).

Alternatively, SERINC5 may affect the Env structure (Figure 1.5 iii), although the two proteins have not been shown to directly interact (Sood et al. 2017). SERINC5 *in vitro* is known to initiate conformational changes in Env and expose conserved regions, increasing susceptibility to neutralizing antibodies (Beitari et al. 2017). Further, SERINC5, like the HIV-1 Env glycoprotein, is localized in lipid rafts and there exists a possibility that SERINC5 could interfere with functional Env clustering at virus assembly sites on the cell surface or on the virion itself (Firrito et al. 2018).

Counteracting the anti-viral activity of the SERINC family of proteins is a crucial part of the ability for HIV-1 to enhance infectivity and the protein responsible for this is Nef (Jin et al. 2019; Rosa et al. 2015; Sudderuddin et al. 2020; Usami, Wu, and Göttlinger 2015).

1.3.4 Nef-mediated SERINC5 down-regulation.

1.3.4.1 Mechanism of SERINC5 down-regulation.

The enhancement of HIV-1 infectivity by Nef was recently shown to be a result of its ability to down-regulate members of the SERINC family of proteins, more specifically SERINC3 and SERINC5 (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015). This was further demonstrated in a study by (Jin et al. 2019) using subtype B patient-derived Nef sequences from elite controllers which exhibited a lower ability to down-regulate SERINC5 than those from chronic progressors, suggesting that ability to down-regulate SERINC5 may contribute to differences in clinical outcome. The mechanism of Nef-mediated SERINC5 down-regulation shares similarities with CD4 down-regulation (Buffalo et al. 2019; Shi et al. 2018) and is illustrated in (Figure 1.6).
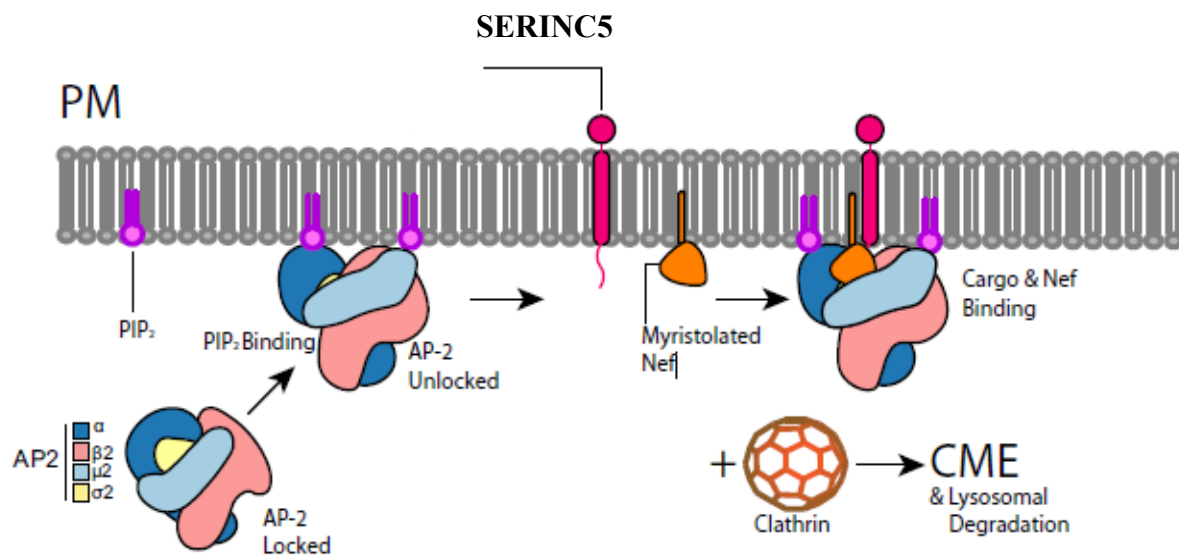
Figure 1.6: The mechanism of Nef-mediated SERINC5 down-regulation.

Figure 1.6 illustrates the mechanism of Nef-mediated down-regulation of SERINC5. At the plasma membrane, AP-2 which is in a locked conformation interacts with phosphatidylinositol 4,5-bisphosphate (PIP2). It then unlocks, leaving the dileucine and tyrosine cargo binding sites exposed. Nef now can interact with AP-2 to initiate the process of down-regulation. This then initiates clathrin-mediated endocytosis (CME) followed directing SERINC5 into the lysosomal degradation pathway. Image taken from (Buffalo et al. 2019).

The mechanism of SERINC5 down-regulation, like CD4 down-regulation, is dependent on the interaction between Nef and AP-2 (clathrin adaptor complex) (Buffalo et al. 2019; Pizzato et al. 2007; Rosa et al. 2015; Shi et al. 2018). As described in (Shi et al. 2018), Nef initiates rapid SERINC5 down-regulation via receptor-mediated endocytosis and re-localizes SERINC5 to the early (Rab5$^+$), late (Rab7$^+$) and recycling (Rab11$^+$) endosomes. Moreover, polyubiquitination is not facilitated by Nef; however, SERINC5 down-regulation is dependent on the ubiquitination pathway where both specific ubiquitin linkages K48- and K64 are a requirement for its down-regulation. Finally, the promotion of SERINC5 colocalization with the lysosomal-associated membrane protein 1 (LAMP1) by Nef ultimately leads to its trafficking to the lysosome for degradation.

1.3.4.2 SERINC5 residues mediating sensitivity to Nef.

SERINC5's sensitivity to Nef is in the intracellular loop 4 (ICL4) of the protein (Figure 1.7). In SERINC5, the surface of the loop that connects helices seven and eight are left exposed (Usami, Wu, and Göttlinger 2015), and it is suggested that the loop that connects helices eight and nine is in the fourth but also the longest intracellular loop (Dai et al. 2018).

There are two hydrophobic residues in ICL4, namely L350 and I353 (Dai et al. 2018; Firrito et al. 2018) that are fundamental for sensitivity to counteraction by Nef. Mutations of L350 and I353 residues rendered SERINC5 resistant to Nef; however, interestingly, it did not inhibit the ability of SERINC5 to exhibit anti-HIV-1 activity (Firrito et al. 2018).
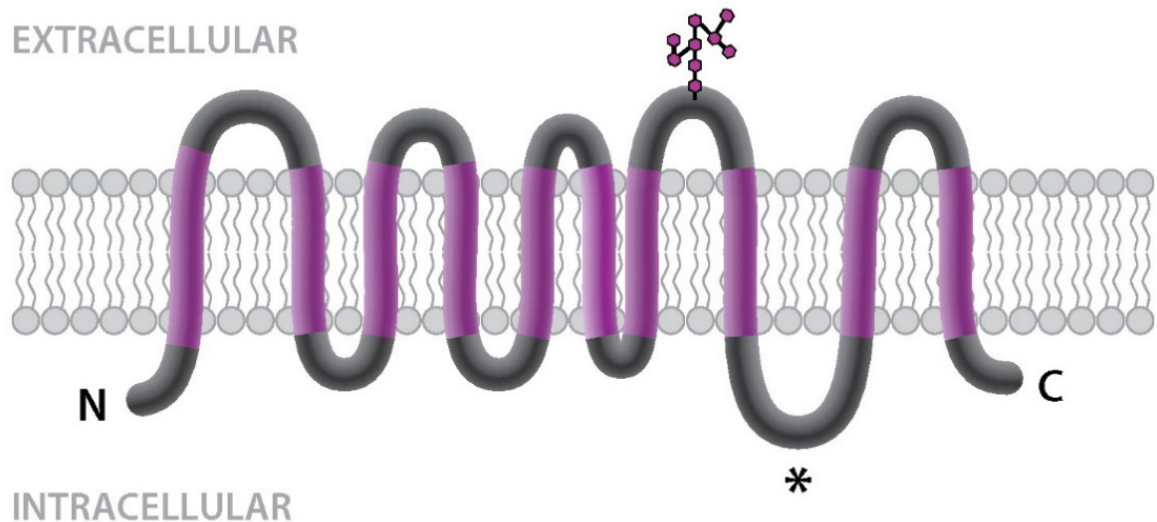
Figure 1.7: The topology and sensitivity of SERINC5 to Nef.

Figure 1.7 illustrates the structure and topology of SERINC5. SERINC5 contains an N terminal, a C terminal and ten transmembrane domains. The asterisk denotes the intracellular loop 4 which is the region in protein that is sensitive to antagonism to Nef. Figure taken from (Firrito et al. 2018).

1.3.4.3 Nef residues involved in SERINC5 down-regulation.

As previously mentioned, Nef's ability to interact with AP-2 remains a crucial feature of the accessory protein's ability to down-regulate SERINC5. Residues that are important for the interaction between Nef and AP-2, and by extension SERINC5 down-regulation, are shown in the (Figure 1.8) and (Table 1.1) below.
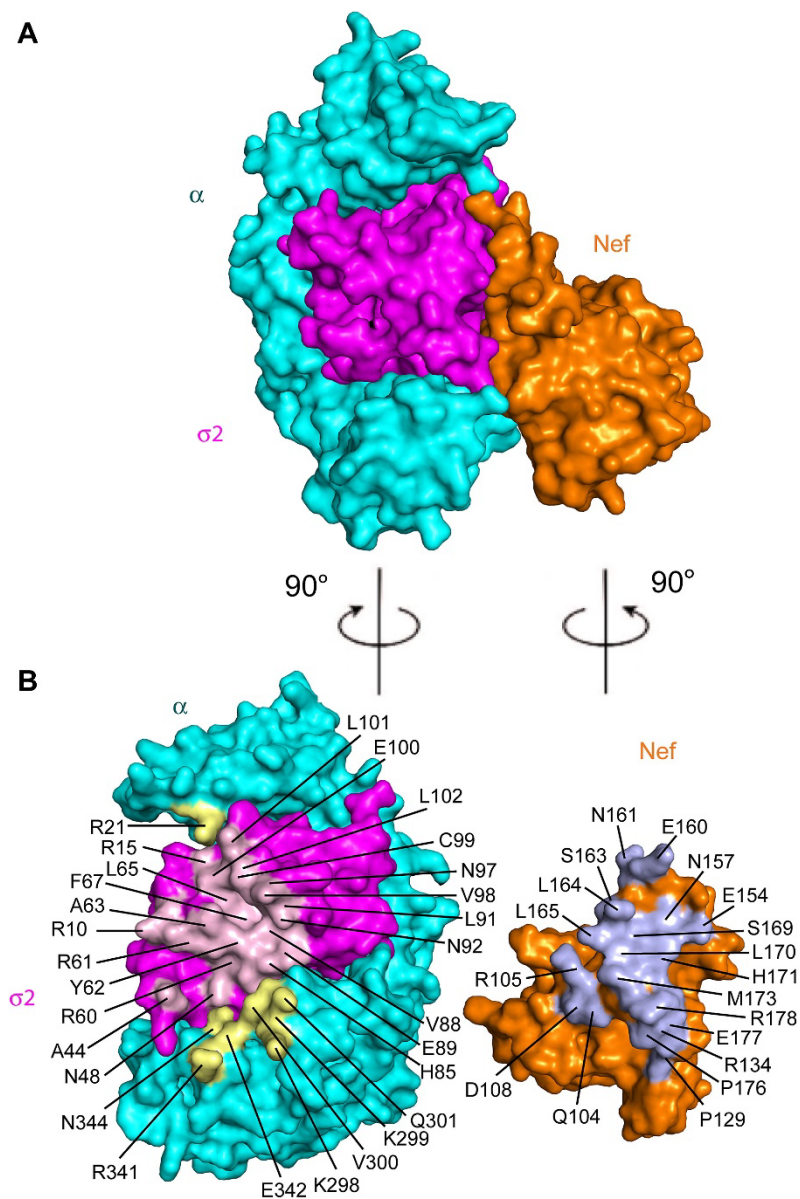
Figure 1.8: The structural mapping of residues that are important to the AP2-Nef interaction.

Figure 1.8 illustrates the structural mapping of residues that are crucial in the process of AP-2 and Nef binding. (A) The surface representation depicting the contact between Nef and AP-2 α−σ2. (B) Nef and AP-2 α−σ2 interfaces are rotated by 90° which exposes their interaction surfaces directly to view. Interacting residues in HIV-1 Nef are shown in light blue, residues in AP-2 α are shown in yellow and residues in AP-2 σ2 are shown in pink. The image was taken from (Ren et al. 2014).

Table 1.1: Nef residues that are crucial for the Nef-AP2 interaction.

Nef residue	References	Interacting residues	Interactions
Q104	This study	α K298*, K299	Hydrogen bond
D108*	This study	α K298*, K299	Salt bridge
P129	This study	α R341*	Van der Waals
R134*	This study	Nef D175*	Nef core-to-loop internal salt bridge
E154	(Lindwasser et al., 2008)	σ2 R61, R10	Salt bridge
N157	(Lindwasser et al., 2008)	σ2 A63	Van der Waals
E160	(Lindwasser et al., 2008)	σ2 R15, α R21	Salt bridge
N161	(Lindwasser et al., 2008)	σ2 C99, L101	Van der Waals
S163	(Lindwasser et al., 2008)	σ2 N97	Weak hydrogen bonds
L164*	(Janvier et al., 2003; Lindwasser et al., 2008)	σ2 Y62, A63, L65, F67, V88, L91, V98, L103	Hydrophobic
L165*	(Janvier et al., 2003; Lindwasser et al., 2008)	σ2 Y62, H85, V88, E89, N92	Hydrophobic
S169	(Lindwasser et al., 2008)	σ2 A63	Van der Waals
L170*	(Lindwasser et al., 2008; Jin et al., 2013)	σ2 Y62	Hydrophobic
H171	This study; (Lindwasser et al., 2008)	σ2 A63	Hydrogen bond to main chain carbonyl
M173	(Lindwasser et al., 2008)	α Q301, V300; σ2 R60, Y62, H85	Hydrophobic and nitrogen-sulfur hydrogen bond
D174*	(Lindwasser et al., 2008)	Nef Q104	Nef core-to-loop internal hydrogen bond to main chain amide
D175*	(Lindwasser et al., 2008)	Nef R134*	Nef core-to-loop internal salt bridge
P176	(Lindwasser et al., 2008)	α R341*, E342	Van der Waals
E177*	This study; (Lindwasser et al., 2008)	α R341*	Salt bridge
R178	(Lindwasser et al., 2008)	α E342, σ2 N48	Strong hydrogen bond, weak salt bridge

This table illustrates the Nef residues that are crucial for the interaction with AP-2. This table was taken from (Ren et al. 2014).

In a study by (Shi et al. 2018), the researchers showed the motifs for SERINC5 down-regulation that overlap with CD4 down-regulation, and those are RR (105-106), LL(164-165), ED (174-175), while mutation of the 62EEEE65 and 72PxxP75 motifs had no observed effect. In a study by (Trautz et al. 2016), the authors showed that the deletion of the CAW (57-59) motif abrogated SERINC5 down-regulation by Nef. Additionally, the direct interaction between Nef and SERINC5 is facilitated by the G2 Nef residue, which confirms the requirement of Nef's association with the inner face of the plasma membrane. Four hydrophobic residues (I109, L112, W115, and F121) in the core region required for homodimerization were also required for Nef's interaction with SERINC5.

These hydrophobic residues required for interaction with SERINC5 were not the same as those needed for interaction with HLA-I, CD4, AP2, P1-derived artificial chromosome (PACS) and Src kinase family (SFK) (Shi et al. 2018). There was also speculation that the down-regulation of SERINC5 involved dynamin 2. In a study by (Buffalo et al. 2019; Rosa et al. 2015), the researchers showed that SERINC5 down-regulation was diminished by the Nef D123 mutation, suggesting the involvement of dynamin 2.

Nearly all the above residues (or key residues in motifs) described to affect SERINC5 down-regulation on mutation are very seldom mutated in natural sequences – they are highly conserved. Natural polymorphisms and their ability to either improve or diminish Nef's ability to down-regulate SERINC 5 remains mostly unknown. However, a study by (Jin et al. 2019) did shed some insight on the matter. The study identified 18 Nef polymorphisms using codon by codon analysis associated with differential function, including two CTL escape mutations, K94E, which was driven by HLA-B*08 and H116N, which was driven by the protective allele HLA-B*57 that contributed to lowering SERINC5 downregulation activity. In addition, a study by (Toyoda et al. 2020) showed that the naturally occurring HLA-B*51:01-associated Y120F and Q125H mutations had a significant association with the reduction of plasma viral load. Further biochemical analysis showed the double mutation Y120F/Q125H, and not the single individual mutations, affected Nef-mediated SERINC5 down-regulation and resulted in decreased viral replication and virion infectivity in primary lymphocytes.

1.3.5 The resistance of Env to SERINC5 activity

The impairment of the infectivity of virus particles is a mechanism SERINC5 adopts to restrict HIV-1 infection. Numerous studies show that this is achieved by the Nef-mediated down-regulation of SERINC5 from the cell surface (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015). However, the Env protein found in some HIV-1 strains may also overcome SERINC5 inhibition.

Contributing factors to SERINC5 resistance are the V1 and V2 loops of Env (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015). This was confirmed in a study by (Beitari et al. 2017) where the researchers showed that viruses that contained all three V loops resulted in resistance to over-expressed SERINC5. The researchers went on to identify the V3 loop as a crucial determinant in Env that facilitates resistance to both endogenous and ectopically expressed SERINC5. The V3 loop resides in a pocket that results from the folding of V1 and V2.

The resistance of Env to SERINC5 does not appear to be linked to Nef because while Nef prevents SERINC5 incorporation into virions, Env can resist SERINC5 in the presence of Nef. Even though Env is somewhat resistant to SERINC5 *in vitro* when SERINC5 is incorporated into virions, the Nef-mediated down-regulation of SERINC5 is still conserved suggesting it is required *in vivo*, perhaps to render the Env less susceptible to neutralising antibodies *in vivo* (Beitari et al. 2017).

1.4 The role of Nef in HIV-1 pathogenesis.

The evidence of Nef's role in retroviral pathogenesis was demonstrated initially in a study by (Kestier III et al. 1991), where the researchers showed that in Rhesus monkeys infected with SIV, the presence of an intact Nef gene was critical for the maintenance of a high viral load and progression to AIDS. Thereafter, studies of long-term non-progressor individuals who were infected with HIV-1 strains with Nef deletions supported the important role of Nef in HIV-1 pathogenesis (Deacon et al. 1995; Kirchhoff et al. 1995).

In a study by (Watkins, Foster, and Garcia 2015), to better characterize the pathogenic effects of Nef, they contrasted Nef (+), and Nef (-) infection of marrow/liver/thymus (BLT) humanized mice. This was the first-time host-specific HIV-1 viral replication suppression was observed in a small animal model. The results of the study highlighted the need for Nef expression for both substantial CD4$^+$ T cell loss from tissue and blood together with systemic T-cell activation. The conclusion drawn from the study supports the idea that Nef strongly drives viral pathogenesis.

The relative importance of various Nef activities for HIV-1 disease progression is not completely known. Previous studies using animal models indicated that Nef-mediated CD4 down-regulation (Iafrate et al. 2000; Watkins et al. 2013), as well as enhancement of infectivity (Iafrate et al. 2000), are likely major contributors to Nef's effect of enhancing pathogenicity, but that there are also one or more unknown functions not dependent on the SH3 domain that are important (Watkins et al. 2013).

Few studies have correlated one or more Nef functions with disease progression in natural HIV-1 infection, and most have focussed on subtype B. In subtype B infection, several Nef activities (namely, CD4 down-regulation, HLA-1 down-regulation, enhancement of replication capacity, and upregulation of CD74) are limited in elite controllers or long-term non-progressors compared to progressors (Corró et al. 2012; Mwimanzi et al. 2011; Mwimanzi et al. 2013), but in progressors, of the Nef functions studied, only Nef-driven virion infectivity correlated with markers of disease progression (Mwimanzi et al. 2013).

There are a limited number of studies that have investigated the relationship between natural variation in different Nef functions and disease outcome in HIV-1 subtype C infection, which is the leading subtype of the pandemic and the most prevalent subtype in the heavily burdened region of Southern Africa (Hemelaar 2012). In one such study performed by (Mann et al. 2014), the data revealed a modest correlation between Nef-mediated down-regulation of CD4 and a higher viral load set point. Further, Nef mediated down-regulation of HLA-I showed a significant association with the rate of CD4$^+$ T cell decline (Mann et al. 2014). In a follow-up study done by (Naidoo et al. 2019) on the same individuals, it was shown that Nef-mediated alteration of TCR signalling did not correlate with markers of disease progression in subtype C infected individuals. To what extent natural variations of other Nef functions influence clinical outcome in HIV-1 subtype C infection remains unknown.

1.5 The role of Nef in HIV-1 therapeutics and vaccines.

Modern anti-HIV drugs predominantly target structural proteins or enzymes crucial to viral integration and replication, namely reverse transcriptase, integrase, and protease (Arts and Hazuda 2012). However, Nef, which is regarded as an accessory protein, fulfils many important functions in its interactions with several motif sequences of host factors (Peterlin and Trono 2003) and is promising as a drug target. In a study done by (Emert-Sedlak et al. 2016), the researchers revealed non-azo group diphenylpyrazolodiazene, which are a novel class of compounds that inhibit Nef-dependent HIV-1 replication.

There is growing evidence that identifies the Src family of kinases (SFKs) as important molecular target sites for Nef (Trible et al. 2013). Several small Nef-interacting peptides and compounds were identified and revealed to target the binding surface on SH3 and disrupt its interaction with Hck (Atkins et al. 2008; Dikeakos et al. 2010; Hung et al. 2007; Mujib et al. 2017; Trible et al. 2013). In a study done by (Liu et al. 2019), the researchers performed a high throughput screening of drugs approved by the Food and Drug Administration (FDA), and among those drugs, they identified a statin drug – lovastatin - with potential. The data from the study showed that lovastatin had the ability to significantly antagonize Nef's ability to down-regulate CD4, HLA-I, and SERINC5.

Despite the advancements in the field of vaccine development and medical research, there is still no effective vaccine available against HIV-1 (Goh et al. 2019). The primary reason for this is the difficulty in designing a vaccine that can elicit an immune response not evaded by the mutable and diverse HIV-1 (Kwong, Mascola, and Nabel 2012).

The Nef protein is highly immunogenic (Radebe et al. 2011) and an important virulence factor (Kestier III et al. 1991; Kirchhoff et al. 1995) and is thus an attractive target site for vaccine development. There is a growing body of evidence that certain naturally occurring Nef mutations may significantly compromise Nef function (Jin et al. 2019; Kuang et al. 2014; Mann et al. 2014; Shahid et al. 2015; Ueno et al. 2008), and that immune responses to specific Nef epitopes may contribute to viral control (Adland et al. 2013; Budde et al. 2012; Mudd et al. 2012). Therefore, although the Nef protein is highly variable and is either absent or a minor component of conserved vaccine constructs in a testing (Borthwick et al. 2014; Mothe et al. 2015), specific Nef regions could be worthy of inclusion in HIV attenuation-based vaccines. The goal of attenuation-based vaccine design is to focus on CD8$^+$ T cell immune responses on the viral epitopes where CD8$^+$ T cell escape mutations compromise viral protein function (Chopera et al. 2011). Studies of Nef sequence and function (Kuang et al. 2014; Mann et al. 2014; Shahid et al. 2015; Ueno et al. 2008), as well as computational models of the Nef viral fitness landscape that predict the functional consequences of single mutations and mutation combinations in Nef, can support the identification of mutations in Nef that compromise its pathogenic functions (Barton et al. 2019).

1.6 Study rationale

Given the key role of Nef-mediated SERINC5 down-regulation in enhancing virion infectivity (section 1.3), the primary aim of the present study is to determine whether this Nef activity contributes significantly to disease progression in individuals infected with HIV-1 subtype C, the dominant HIV-1 subtype world-wide.

To investigate this, SERINC5 down-regulation activity of Nef clones derived from early HIV-1 subtype C infection will be evaluated in a T cell line using a flow cytometry-based assay and related to subsequent rate of CD4$^+$ T cell decline and viral load set point, which are significant markers of HIV-1 disease progression.

The relative contribution of each Nef activity to overall Nef function is unknown (section 1.4). CD4 down-regulation, HLA-I down-regulation and alteration of TCR signalling have previously been measured for the subtype C Nef clones used in this study (Mann et al. 2014; Naidoo et al. 2019), and an E value, which represents overall Nef fitness *in vivo*, has been predicted by computational modelling for each of these patient-derived Nef clones (Barton et al. 2019). Thus, a secondary aim of this study is to assess the overall contribution of SERINC5 down-regulation to Nef function, using E values as a proxy for overall Nef function *in vivo*.

It is also largely unknown how naturally occurring polymorphisms in Nef affect SERINC5 down-regulation activity, particularly for HIV-1 subtype C (section 1.3.3.3). The identification of amino acid variants that Nef requires for optimal function, and immune-driven mutations in Nef that compromise its function, could reveal antiviral targets, and contribute to attenuation-based HIV-1 vaccine design (section 1.5). Therefore, an additional aim of the present study is to identify amino acid variants, which include immune driven mutations that significantly alter Nef-mediated SERINC5 down-regulation.

Aims

- To determine whether Nef-mediated SERINC5 down-regulation ability contributes significantly to HIV-1 subtype C disease progression.

- To assess the relative contribution of SERINC5 down-regulation ability to overall Nef function.

- To identify amino acid variants that are required for optimal SERINC5 down-regulation activity.

Objectives:

- Measure the ability of 106 Nef clones, derived from patients with early HIV-1 subtype C infection, to down-regulate SERINC5 using a flow cytometry-based assay.

- Relate Nef-mediated ability to down-regulate SERINC5 in early infection to subsequent viral set point and rate of $CD4^+$ T cell decline.

- Assess which Nef activities align the closest with E values, a proxy for overall Nef function *in vivo*.

- Use statistical analysis of sequences and functional data to identify amino acid variants associated with increased or decreased Nef-mediated ability to down-regulate SERINC5.

CHAPTER

TWO:

METHODS & MATERIALS

2.1 Ethical considerations.

Ethical approval was obtained from the Biomedical Research Ethics Committee (BREC) of the University of Kwa-Zulu Natal Nelson R Mandela School of Medicine (Ref: BREC/00001225/2020).

2.2 Patient information.

A total of (n=106) antiretroviral naïve patients with acute/early HIV-1 subtype C infection were included in this study. The three cohorts from which these patients represented were the Tshedimoso study in Botswana (Novitsky et al. 2009), the HIV Pathogenesis Programme (HPP) Acute Infection Cohort in Durban, South Africa (Radebe et al. 2011; Wright et al. 2011), and the Tenofovir Gel Research for AIDS Prevention Science (TRAPS) Cohort in KwaZulu-Natal, South Africa (specifically including the participants who seroconverted in the CAPRISA 004 trial and who were from the placebo arm). The age, gender, viral load, CD4$^+$ T cell count, and high-resolution HLA class I allele information was available (Table 2.1). The viral load set point was calculated as the average viral load from 3 to 12 months post-infection. Simple linear regression was used to compute the rate of CD4$^+$ T cell decline for every participant over the treatment-free follow-up period, where the rate of decline was defined as the estimated slope of the fitted regression line (Wright et al. 2011).

Table 2.1: The clinical and demographic characteristics of individuals with acute/early infection.

Characteristics.	Median (inter-quartile range).
Gender	86 females, 20 males
Age	25 (21 – 28) years
Viral load	4.77 (3.90 – 5.29) $\log_{10}$copies/ml
Viral load set point	4.36 (3.63 – 5.03) $\log_{10}$copies/ml
CD4$^+$ T cell count	468 (341 – 626) cells/mm^3
CD4$^+$ T cell decline	-3.99 (-9.37 – 0.30) cells/mm^3

2.3 Preparation of Nef clones.

In the present study, Nef-mediated SERINC5 down-regulation was evaluated for 106 patient-derived Nef clones that were previously prepared (Mann et al. 2014) from the patients described in Section 2.1, and for which HLA-I and CD4 down-regulation, as well as alteration of TCR signalling, were previously measured (Mann et al. 2014; Naidoo et al. 2019). The preparation of the Nef clones was previously described in (Mann et al. 2013) and is summarised in (Figure 2.1).
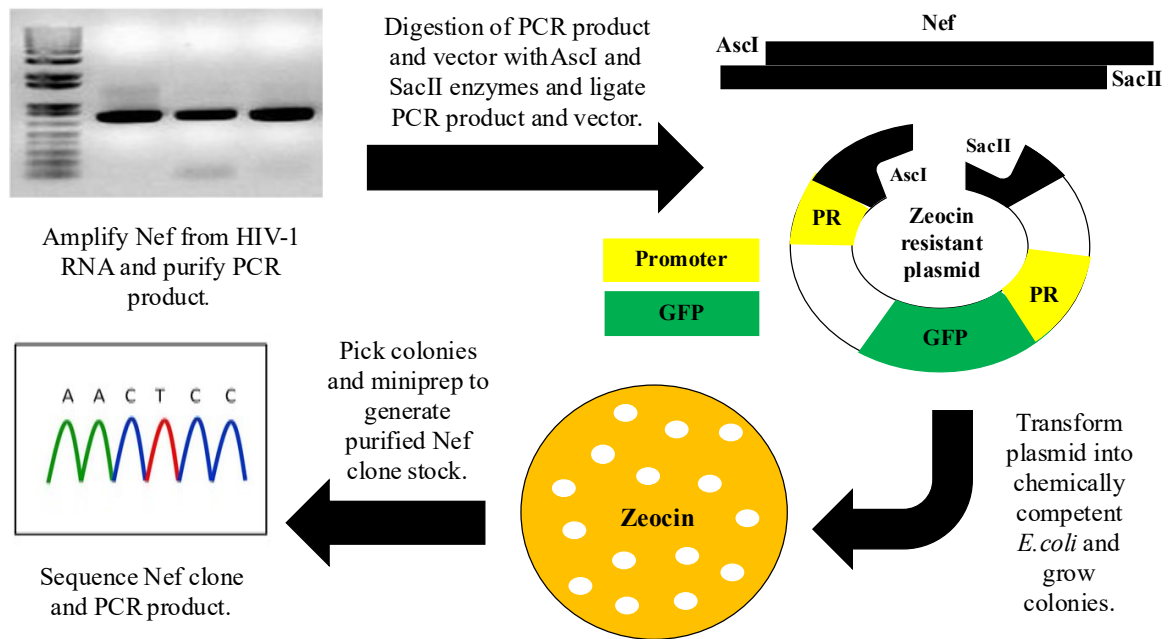
Figure 2.1: Preparation of patient derived Nef clones.

The viral RNA was extracted from the primary blood samples. Nef was amplified using reverse transcriptase PCR (RT-PCR) and a second round of PCR was then performed. The amplicons were then sequenced and purified. The digestion of plasmid was performed using AscI and SacII as the restriction enzymes. AscI and SacII sites were also introduced on either end of the Nef amplicon. Electrophoresis was used to confirm digestion of the plasmid. Gel purification of the digested plasmid was performed. Digested Nef sequences were ligated into the digested pSELECT plasmid. The Nef insert was then transformed into chemically competent *E. coli*. Plating, culturing, and selection followed. The colonies were then sequenced to confirm that the Nef clonal sequences were representative of the bulk amplicon Nef sequences. Diagram was adapted from a presentation by J. Mann.

Briefly, HIV-1 Nef was amplified using reverse transcriptase polymerase chain reaction (RT-PCR) from the HIV-1 RNA extracted from plasma. A second-round polymerase chain reaction (PCR) was then performed. This was necessary to introduce restriction sites on either side of the Nef sequence. The primers used in the second-round PCR contained AscI (forward primer) and SacII (reverse primer) restriction sites for cloning. The amplicons were then sequenced and purified. The digestion of pSELECT-GFPzeo plasmid (which had a modified linker encoding AscI and SacII restriction sites) was performed using AscI and SacII as the restriction enzymes.

To confirm the digestion of the plasmid, electrophoresis was performed, followed by gel purification of the digested plasmid. Digested Nef sequences were ligated into the digested pSELECT-GFPzeo plasmid by T4 ligase and then transformed into competent cells (OneShot TOP 10). The colonies with transformed plasmids were selected on zeocin plates as the plasmid is zeocin-resistant. The colonies were then sequenced to confirm that the Nef clonal sequences were representative of the bulk amplicon Nef sequences, and a single representative Nef clone per patient was selected for functional analysis.

The pSELECT-GFPzeo plasmid containing the patient-derived Nef facilitates functional measurement as it has a promoter allowing for Nef expression and expression of green fluorescent protein (GFP). These Nef clones were subsequently transfected into AO2-GXR cells to allow for Nef expression and measurement of Nef-mediated SERINC5 down-regulation.

Protein expression was previously confirmed for a subset of these Nef clones (Mann et al. 2014). Nef clones that were previously poorly functional for both CD4 and HLA downregulation and for which Nef protein expression could not be confirmed by Western blot, were not included in this study. The Nef clones included in this study expressed Nef, by virtue of displaying CD4 or HLA down-regulation activity and/or confirmation of expression by Western blot.

2.4 Mini-prep and maxi-prep of Nef clones and plasmids.

A mini-prep procedure was carried out to generate stocks of purified Nef clones. Briefly, the selected colony containing the Nef clone (or 3µl of the previously prepared glycerol stock of the Nef clone) was added to 3ml LB broth with zeocin and incubated for 20 hours in the Ecotron shaking incubator (Infors-HT, Basel, Switzerland) at 250 rpm and 37°C. The mini-prep was carried out as per manufacturing instructions provided in the Qiagen FlexiGene DNA kit. Similarly, to generate stocks of purified HA-tagged SERINC5 expression plasmid, 100 ml LB broth was inoculated with 5µl glycerol stock of the plasmid and the maxiprep procedure was carried out as per manufacturing instructions provided in the Qiagen maxi-prep kit. The HA-tagged SERINC5 expression plasmid was kindly provided by Prof. Mark Brockman, Simon Fraser University, Canada.

2.5. Maintenance of the A02-GXR cell line.

The A02-GXR cell line (obtained from Prof. Mark Brockman, Simon Fraser University, Canada) was stored in liquid nitrogen (Mann et al. 2013). When required for use, a vial of cells was taken from the cryo-freezer and thawed at 37°C in a Precisterm water bath (JP Selecta, Barcelona, Spain).

Following the thawing process, 1ml of cells was transferred to 4ml of warmed R10 medium. The R10 medium consisted of RPMI-1640 (Sigma, St Louis, USA) supplemented with 50ml of 10% foetal bovine serum (FBS; Sigma), 5ml of N-2- hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Gibco, New York, USA), 5ml of L-glutamine (Sigma), and 2.5ml of penicillin-streptomycin (Gibco). The cells were then incubated overnight at 37°C and 5% CO_2 in a humidified incubator (NuAire, Minnesota, USA).

Following incubation overnight, the cells were centrifuged in a Heraeus Multifuge 35R+ centrifuge (Thermo Scientific, Massachusetts, USA) at 1500 rpm for 10 minutes at 23°C to remove the dimethyl sulfoxide (DMSO, Sigma) that was contained in the R10 medium used for storing the cells in liquid nitrogen. Following this, the supernatant was discarded, and the cell pellet resuspended in 10ml of fresh warmed R10 medium. Two days later, the cells were counted using a TC10 automated cell counter (Bio-Rad, California, USA) and thereafter maintained (with the estimation that they double every 24 hours) at a concentration of between 0,25 to 1,5 million cells/ml for use in assays to measure SERINC5 down-regulation activity.

2.6 The measurement of the ability of Nef clones to down-regulate SERINC5.

The Nef-mediated down-regulation of SERINC5 was measured as previously described (Jin et al. 2019). The methods are described next and summarised in (Figure 2.2).
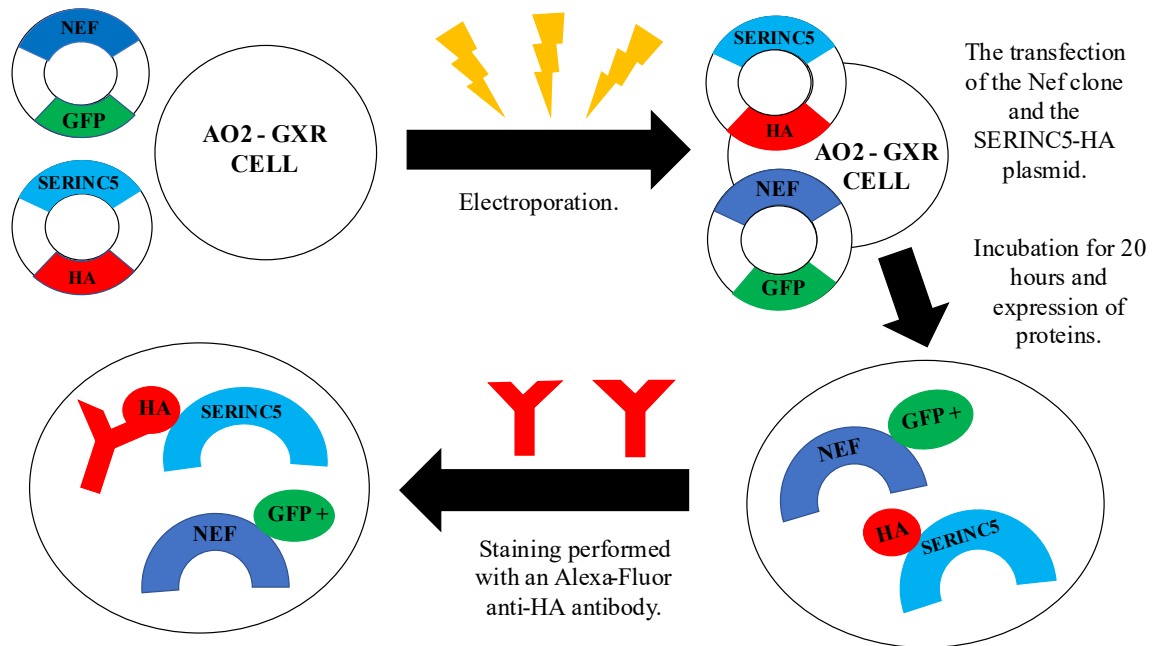
Figure 2.2: Transfection of the Nef clones and SERINC5-HA plasmid into cells and antibody staining.

To measure SERINC5 down-regulation activity, one million AO2-GXR cells were transfected with 2µg of patient-driven Nef clone and 5µg of the SERINC5 expression plasmid with the HA tag. The cells were electroporated and incubated for 20 hours at 37°C. Following the 20-hour incubation period, the cells were stained with a monoclonal Alexa Fluor anti-HA.11 Epitope Tag Antibody. The plasmid used to make the Nef clone encodes promoters allowing for Nef expression and expression of green fluorescent protein (GFP).

2.6.1 Transfection of the Nef clone and SERINC5-Ha plasmids into AO2-GXR cells.

Cells were counted, followed by centrifugation at 1500 rpm for 10 minutes at 23°C of 1 million cells per Nef clone used in the experiment and resuspension of the pelleted cells in fresh R10 at a final concentration of 10 million cells/ml. Next, 2µg of the Nef clone (Nef in the pSELECT-GFPzeo plasmid) and 5µg HA-tagged SERINC5 expression plasmid were mixed with OptiMEM Megacell (Sigma) in a total volume of 300µl. 100µl of cells (1 million cells) and 300µl of the DNA-Megacell mixture were added to a Gene Pulser 4 mm cuvette (Bio-Rad) and mixed gently with the pipette approximately 5 to 7 times while avoiding air bubbles. This mixture was then electroporated using a Gene Pulser Xcell electroporator (Bio-Rad) under the following conditions: voltage: 250V, capacitance: 950µF, resistance: ∞ and cuvette size: 4 mm.

Following electroporation, the samples were then allowed to rest for 10 minutes. 500µl of warm R10 medium was added to the cuvette and gently mixed a maximum of 2-3 times. Following this, 430µl of the electroporated mixture was added to two sterile cluster tubes already containing 250µl warm R10 solution, thereby constituting two replicates for antibody staining. The final volume in both replicates was 680µl. Cells were then incubated for 20 hours to allow for the expression of Nef and SERINC5.

2.6.2 Antibody staining.

Following the 20-hour incubation period, the cells were centrifuged at 1500 rpm for 10 minutes at 23°C, followed by aspiration of the supernatant without disturbing the pellet. A master mix containing 0.75µl of a monoclonal Alexa Fluor anti-HA.11 Epitope Tag Antibody (Biolegend Way, California, USA) and 49.25µl of phosphate buffered-saline (PBS; Gibco) per tube of cells to be stained, was prepared, and 50µl was added to each cluster tube containing the transfected cells. Each tube was then vortexed using a REAX-1 vortex (Heidolph, Schwabach, Germany) and incubated at 4°C for 30 minutes.

Following the incubation period, 750µl of PBS was added to each tube. The cells were then centrifuged at 1500 rpm for 10 minutes at 23°C. The supernatant was aspirated off without disturbing the pellet. To the cluster tubes containing the cells, 300µl of 2% paraformaldehyde (PFA) fixative was added. Briefly, the 2% PFA solution was prepared prior to the experiment by adding 20 grams of PFA power (Merck, New Jersey, USA) to 900ml of PBS. A water bath was warmed to 55°C, and the solution was heated and stirred in the water bath for 30 minutes. Following this, sodium hydroxide (NaOH; Sigma) was added to the solution until the PFA had completely dissolved in PBS. 100 ml of PBS was added, and a pH of 7 was confirmed. The 2% PFA solution was aliquoted and stored frozen at -20°C. Thawed aliquots were maintained in the fridge for a maximum of two weeks. Following the fixative step, the cells in the cluster tubes were incubated at room temperature in the dark for 45 minutes, followed by flow cytometry analysis.

2.6.3 Flow cytometry analysis.

The pSELECT plasmid used expressed GFP. Cells that express GFP were those transfected with the Nef clones and were deemed GFP positive cells. The FACS Calibur (BC Bioscience, San Jose, USA) was used to measure GFP positive Nef-transfected cells and the level of SERINC5-HA expression (through detection of the Alexa Fluor-labelled anti-HA antibody) in the Nef-transfected cells (Figure 2.3).

Figure 2.3: Flow cytometry analysis to measure SERINC5 down-regulation activity.

The plasmid used expressed green florescence protein (GFP). Cells that express GFP were those transfected with the Nef clones and were deemed GFP positive cells. The FACS Calibur was used to measure GFP positive Nef-transfected cells and the level of SERINC5-HA expression (through detection of the Alexa Fluor-labelled anti-HA antibody) in the Nef-transfected cells.
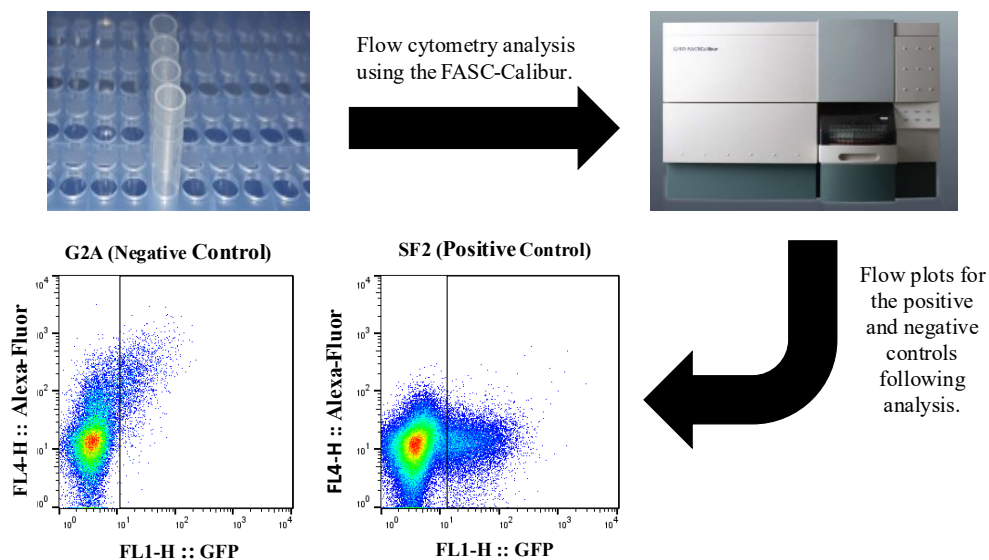
The negative control used to measure the SERINC5 down-regulation activity was (G2A), which had a normalized value of 0%, which indicates no down-regulation activity. The positive control used to measure SERINC5 down-regulation activity was SF2 Nef and represented 100% down-regulation activity.

The cluster tubes containing the samples were vortexed and placed in reusable FACS tubes (BD Biosciences). The instrument settings used for the analysis were: forward scatter (E-1V), side scatter (385V) to determine the live cells, and GFP FL1 (450V) and Alexa Fluor FL4 (360V) to determine the Nef-transfected cells and level of SERINC5-HA expression, respectively. Data was collected from 50000 events (live cells) per sample. Live cells were distinguished from dead cells, and GFP positive cells were distinguished from GFP negative cells by gating the cells using FlowJo software, version 10.1. For each Nef clone, the percentage of GFP-positive cells expressing HA-tagged SERINC5 was normalized to that of the negative control (G2A Nef cloned into the pSELECT-GFPzeo plasmid) and the positive control (SF2 Nef cloned into the pSELECT-GFPzeo plasmid) to determine the relative SERINC5 down-regulation activity:

$$(negative\ control - Nef\ clone)/(negative\ control - positive\ control)\ \ x\ 100$$

The negative control represented 0% down-regulation activity. G2A Nef is the negative control which is essentially SF2 Nef that contains a point mutation at position 2 from G to A. The resultant mutation renders Nef unable to bind to the plasma membrane, and as a result, G2A is non-functional.

The SF2 Nef positive control was previously demonstrated to be highly functional for SERINC5 down-regulation (Usami, Wu, and Göttlinger 2015) and represented 100% down-regulation activity. The analysis was performed in duplicate, and an average of the results was taken.

2.7 Data analysis.

A univariable and multivariable linear regression was used to assess the relationship between SERINC5 down-regulation, CD4 down-regulation, HLA-I down-regulation and alteration of TCR signalling and viral load set point together with rate of $CD4^+$ T cell decline while controlling for baseline CD4 count and length of follow-up. A variable was defined as a potential confounder and included in the multivariate analysis if its inclusion (in the bivariate analysis) resulted in a 10% or greater change in the co-efficient of the Nef function of interest.

A univariable and multivariable linear regression was then used to assess the relationship between SERINC5 down-regulation, CD4 down-regulation, HLA-I down-regulation and alteration of TCR signalling and overall Nef function (E value). An E value, which represents overall Nef fitness, has been predicted by computational modelling for each patient-derived Nef clone (Barton et al. 2019).

The dE0 value was derived from the Ising model where consensus versus mutant (all non-consensus amino acids are counted as the same mutant type) at each codon was modelled, and the dE90 value was derived from the Potts model where all amino acid variants at each codon are considered.

Subsequent analyses involving both dE0 values and dE90 values were performed using quantile (median) regression, which is more robust than standard linear regression. All Nef functions were standardized (by subtracting the mean and dividing by the standard deviation) so that coefficients were comparable. All analyses were performed using Stata 15.0.

Specific Nef amino acids (present at a frequency of $n \geq 5$ in our dataset) significantly associated with increased or decreased Nef activities were assessed using codon-by-codon Mann-Whitney U tests, available online (http://brockman-srv.mbb.sfu.ca/~B_Team_iMac/ Codon_by_codon/). Multiple comparisons were addressed using q-values, the p-value analog of the false-discovery rate (Storey and Tibshirani 2003) and associations with $p<0.05$ and $q\leq0.4$ was considered significant. Differences in the frequency of Nef amino acid variants were compared between cohorts using VESPA (available at https://www.lanl.gov/) and Fisher's exact test with q-values to test for significance ($p<0.05$ and $q<0.2$).

CHAPTER

THREE:

RESULTS

3.1 Natural variation in SERINC5 down-regulation ability of HIV-1 subtype C Nef clones.

The Nef-mediated SERINC5 down-regulation ability was determined for 106 patient-derived Nef clones from early/acute HIV-1 subtype C infection to assess the effect of this Nef function on subsequent disease progression as well as its contribution to overall Nef function. Further we were also able to identify possible amino acid variants that either increase or decrease Nef's ability to down-regulate SERINC5. The Nef clones used for this study were prepared in a previous study by (Mann et al. 2014), which confirmed both subtype C lineage and Nef protein expression.

The down-regulation of SERINC5 was measured by the co-transfection of the patient-derived Nef clones and a SERINC5 expression plasmid into a T cell line, followed by detection of cell-surface SERINC5 using a fluorescently labelled antibody and flow cytometry. Each Nef clone's ability to down-regulate SERINC5 was expressed as a percentage of the controls. The SERINC5 down-regulation assay was performed at least twice, and an average value was taken. The assay showed good reproducibility with a significant correlation between the duplicate measurements (Pearson's correlation; r=0.93 and p<0.0001) (Figure 3.1). Overall, Nef clones varied widely in SERINC5 down-regulation ability: the median SERINC5 down-regulation activity was 84.66% (interquartile range [IQR], 59.53%-93.87%) and ranged from 12.39% to 98.95%. Nef protein expression by Western blot was available for 57 clones and did not correlate significantly with SERINC5 down-regulation ability (Spearman's correlation; r=0.06 and p=0.65) (data not shown), which supports that variability in Nef protein expression did not significantly confound results.
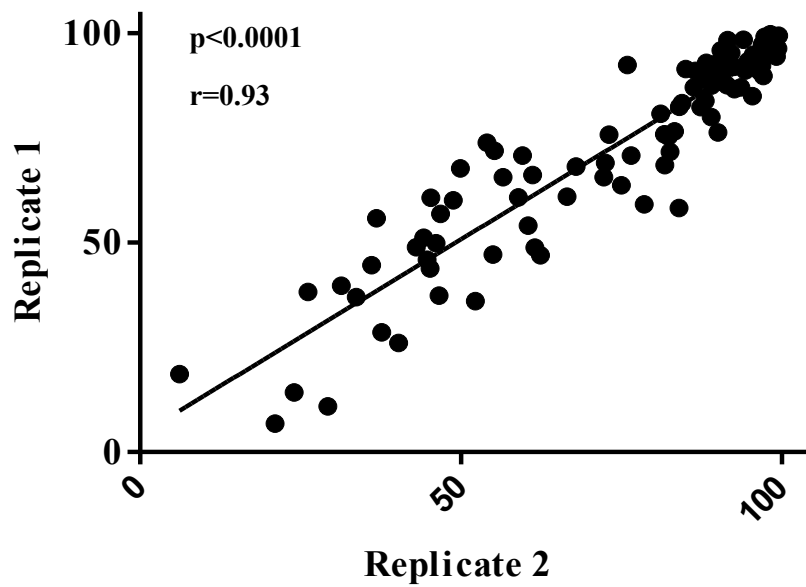
Figure 3.1: The reproducibility and range of Nef-mediated SERINC5 down-regulation measurements.

Figure 3.1 illustrates a scatter plot of the Nef-mediated SERINC5 down-regulation activities of Nef clones derived from early/acute HIV-1 subtype C infection. Nef-mediated SERINC5 down-regulation is expressed as a normalized value of a positive control (SF2 Nef), which represents 100% activity, and negative control (defective Nef with a G2A mutation), which represents 0% activity. The plot shows a strong correlation between the first and second replicates, and the Pearson's correlation test was used.

3.2. SERINC5 down-regulation activity differs by cohort.

The 106-patient derived Nef clones from subtype C early/acute infected individuals were from three different cohorts, two of which are in South Africa, namely the HPP acute infection cohort (n=32) and TRAPS cohort (n=47), and one of which is in Botswana, namely the Tshedimoso cohort (n=27). Before combining and analysing the data from the three cohorts, we assessed whether the SERINC5 down-regulation activity or sequences differed significantly by cohort.

The median values for the Nef-mediated SERINC5 down-regulation activity were 65.50% (IQR=45.61%-90.52%), 86.46% (IQR=68.03%-91.62%) and 94.46% (IQR=73.65%-97.38%) for HPP, TRAPs and Tshedimoso cohorts, respectively (Figure 3.2). Overall, there was a significant difference in SERINC5 down-regulation activity between cohorts (Kruskal-Wallis value p=0.0014). Post-hoc tests showed that the only significant difference was between the Tshedimoso and HPP cohorts, where Nef clones from the Tshedimoso cohort had a significantly greater SERINC5 down-regulation activity overall than those from the HPP cohort (p<0.0001).
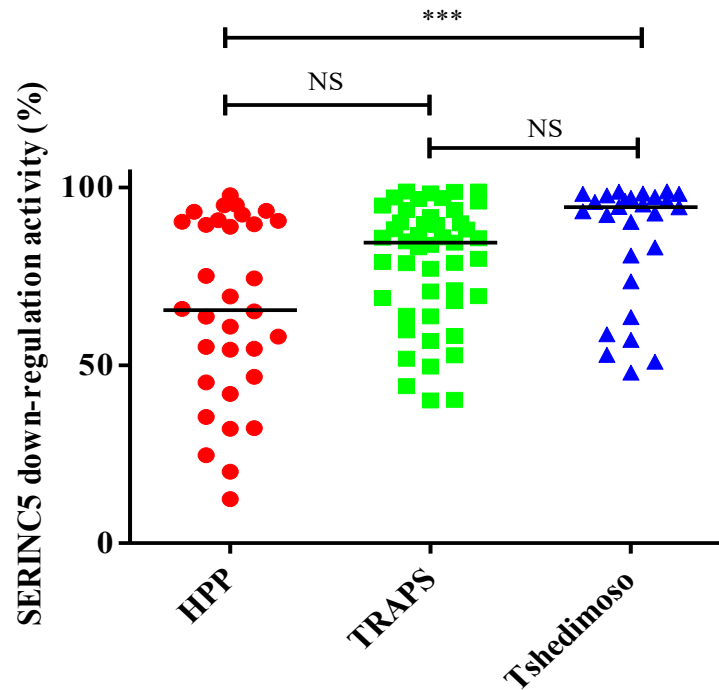
Figure 3.2: Comparison of Nef-mediated SERINC5 down-regulation activity between cohorts.

There was a significant difference in SERINC5 down-regulation ability of Nef clones from different cohorts. The Kruskal-Wallis p-value is shown. The number of asterisks denotes the level of significance in the post-hoc tests, namely, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***). Lines represent the median. NS; no significance.

Although there were observed functional differences in Nef between the cohorts, the Nef sequences obtained from the different cohorts were shown to intermingle in the phylogenetic tree (Figure 3.3), indicating no major genetic distinctions in Nef between the cohorts.
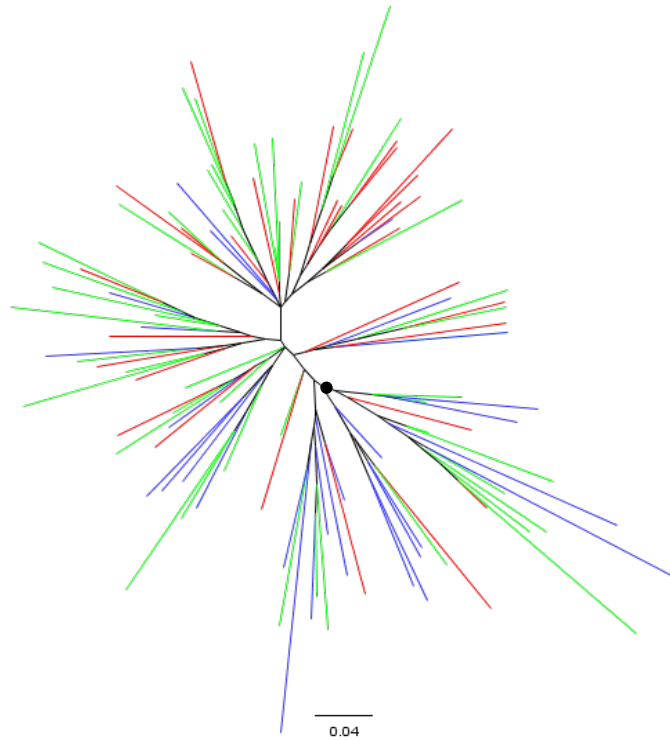
Figure 3.3: Phylogenetic tree of Nef sequences derived from three different cohorts.

A good intermixing of sequences was observed from the maximum likelihood phylogenetic tree, which suggests no major difference in the sequences of the Nef clones derived from the three different cohorts. HPP is displayed in red, TRAPS is displayed in green and Tshedimoso is displayed in blue. The black dot is the reference 2004 consensus C Nef sequence from the Los Alamos HIV sequence database. The tree was constructed using Phyml, an online tool available on the Los Alamos HIV sequence database (https://www.lanl.gov/) (Guindon et al. 2010).

However, since there were functional differences between the cohorts in the ability of Nef to down-regulate SERINC5, cohort was included as a variable in the analysis to investigate the relationship between SERINC5 down-regulation activity and markers of disease progression.

3.3 The relationship between SERINC5 down-regulation activity and other Nef functions.

Previous studies have shown that a moderate correlation exists between CD4 down-regulation and HLA-I down-regulation activity (Mann et al. 2014) and between alteration of TCR signalling and HLA-I down-regulation activity (Naidoo et al. 2019). We sought to determine the overlap between SERINC5 down-regulation activity and other Nef functions.

We observed a statistically significant correlation between SERINC5 down-regulation activity and CD4 down-regulation activity (Spearman's correlation; r=0.63; p<0.0001). This is likely explained by Nef's similar mechanism to down-regulate both surface proteins (Shi et al. 2018). On the other hand, there was no correlation observed between SERINC5 down-regulation activity and HLA-I down-regulation activity (Spearman's correlation; r=0.13 and p=0.16) and neither between SERINC5 down-regulation activity and alteration of TCR signalling (Spearman's correlation; r=-0.04 and p=0.66) (Figure 3.4).
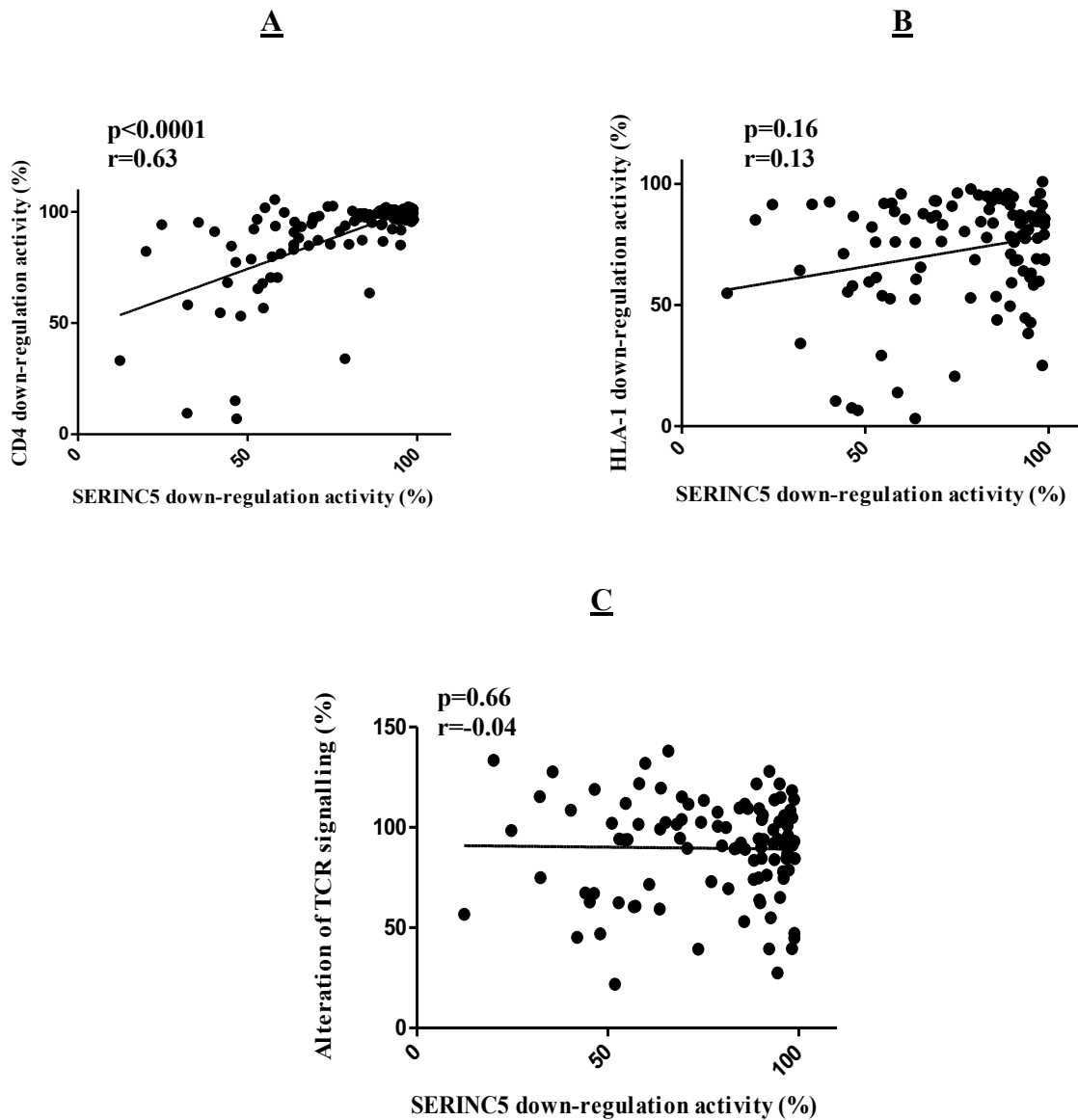
Figure 3.4: The relationship between SERINC5 down-regulation activity and the other Nef functions.

A statistically significant correlation was observed between SERINC5 down-regulation and CD4 down-regulation (A). There was no correlation observed between SERINC5 down-regulation activity and HLA-I down-regulation activity (B) or CD4 down-regulation activity (C). Spearman's correlation test was used to test for significant relationships.

3.4 The relationship between SERINC5 down-regulation activity and markers of disease progression.

Nef-mediated SERINC5 down-regulation activity is responsible for enhancing virion infectivity (Rosa et al. 2015; Usami, Wu, and Göttlinger 2015), but it is not known to what extent this Nef function influences disease progression. The study's first aim was to determine if the SERINC5 down-regulation ability of Nef in early infection contributed to subsequent HIV-1 subtype C disease progression. Markers of disease progression used in this analysis were viral load set point and the rate of $CD4^+$ T cell decline.

The correlation between other Nef functions (CD4 down-regulation, HLA-I down-regulation and alteration of TCR signalling) and markers of disease progression was investigated in previous studies (Mann et al. 2014; Naidoo et al. 2019). These other Nef functions were described in more detail in the literature review. While the dataset overlapped significantly between the previous study and the current study, there were minor differences: the Nef clones ranged from 101 to 107 in the various studies, the 2014 study used the method of Spearman's correlation only for assessment of the relationship between CD4/HLA-I down-regulation and viral load, and the $CD4^+$ T cell decline values were updated for 5 of the participants following the collection of more follow-up CD4 data after the 2014 study. Furthermore, the restriction of $CD4^+$ T cell decline values to specific ranges in the previous studies (Mann et al. 2014; Naidoo et al. 2019) to approximate a normal distribution resulted in the omission of some data points in those studies. To allow for a fair comparison between the different Nef functions, we reanalysed the relationship between the different Nef functions and the markers of disease progression using the same dataset (Table 3.1).

Table 3.1: The effect of SERINC5 down-regulation on viral load set point.

Variables	Coefficient	Standard Error	P>t
Univariate analysis			
SERINC5 down-regulation	0.002	0.004	0.578
Multivariate analysis			
SERINC5 down-regulation	0.005	0.004	0.281
Cohort [a]			
TRAPS	-0.499	0.211	0.020
Tshedimoso	-0.233	0.244	0.343

[a] The reference group was the HPP cohort.

There was no significant effect of SERINC5 down-regulation on viral load set point in both the univariable and multivariable linear regression (p=0.578 and p=0.281, respectively). Multivariable linear regression included cohort as a variable since there was a significant difference in SERINC5 down-regulation between cohorts. Further, the cohort-adjusted effect of each Nef function on viral load set point from separate multivariate regression models is presented in (Table 3.2).

Table 3.2: The effect of all Nef functions on viral load set point[a].

Nef function	Coefficient	Standard Error	p-value
SERINC5 down-regulation	0.005	0.004	0.281
CD4 down-regulation	0.004	0.004	0.388
HLA-I down-regulation	0.005	0.004	0.203
Alteration of TCR signalling	0.002	0.004	0.615

[a] adjusted for cohort.

We also found no statistically significant relationship between viral load set point and CD4 down-regulation, HLA-1 down-regulation or alteration of TCR signalling (p=0.388, p=0.203, and p=0.615, respectively).

There was no significant effect of SERINC5 down-regulation on the rate of $CD4^+$ T cell decline in univariate or multivariate analysis, which included cohort, follow-up time and baseline CD4 count (p-values 0.695 and 0.484, respectively) (Table 3.3).

Table 3.3: The effect of SERINC5 down-regulation on rate of CD4$^+$ T cell decline[a].

Variable	Coefficient	Standard Error	p-value
Univariate analysis			
SERINC5 down-regulation	0.043	0.110	0.695
Multivariate analysis			
SERINC5 down-regulation	0.080	0.113	0.484
Cohort [a]			
TRAPS	-2.092	6.101	0.732
Tshedimoso	2.096	6.738	0.756
Follow-up time	0.013	0.005	0.016
Baseline CD4 count	-0.038	0.012	0.003

[a] The reference group was HPP cohort.

The cohort-adjusted effect of each Nef function on the rate of CD4$^+$ T cell decline from separate multivariate regression models is presented in (Table 3.4).

Table 3.4: The effect of all Nef functions on rate of CD4$^+$ T cell decline[a].

Nef function	Coefficient	Standard Error	p-value
SERINC5 down-regulation	0.080	0.113	0.484
CD4 down-regulation	0.032	0.118	0.790
HLA-I down-regulation	-0.105	0.101	0.297
Alteration of TCR signalling	0.023	0.099	0.814

[a] adjusted for cohort, follow-up time and baseline CD4.

There was also no significant relationship between the rate of CD4$^+$ T cell decline and CD4 down-regulation, HLA-I down-regulation or alteration of TCR signalling (p =0.790, p=0.297, and p=0.814, respectively).

Together these results show that none of the individual Nef functions studied; namely, SERINC5 down-regulation, CD4 down-regulation, HLA-I down-regulation and alteration of TCR signalling, are significantly associated with viral load set point or the rate of CD4$^+$ T cell decline.

3.5 The contribution of SERINC5 down-regulation to overall Nef function.

The study's second aim was to assess the relative contribution of SERINC5 down-regulation ability to overall Nef function. A dE0 value and a dE90 value (which serve as proxies for *in vivo* Nef fitness) were assigned to each of the 106 clones used in this study and were derived from the Ising and Potts models respectively (Barton et al. 2019). The Potts model accounts for the diversity of amino acids present at each residue. In contrast, in the Ising model, only the consensus amino acid present at each reside is modelled explicitly, and all other amino acids are treated as the same mutant type (Barton et al. 2019). A high dE0 or dE90 value is interpreted as corresponding to low *in vivo* Nef fitness (or a high fitness cost), while a low dE0 or dE90 value is interpreted as corresponding to high *in vivo* Nef fitness.

The relationship between each standardized Nef function and dE0 is presented in (Table 3.5). In univariate analysis, both CD4 down-regulation and SERINC5 down-regulation were significantly associated with dE0 with similar coefficients (-2.288 and -2.227) that were larger than those of the other Nef functions. In multivariate analysis, however, these effects were not significant. A negative coefficient means that a high Nef function is associated with high *in vivo* Nef fitness (a low dE0 value).

Table 3.5: The effect of Nef functions on dE0 (Ising model).

Nef function	Univariate analysis			Multivariate analysis		
	Coef.	Std. Err.	p value	Coef.	Std. Err.	p value
CD4 down-regulation	-2.288	1.106	0.041	-0.370	1.754	0.834
HLA-I down-regulation	-1.657	1.150	0.153	-1.849	1.500	0.221
Alteration of TCR signalling	0.844	1.208	0.486	1.878	1.142	0.103
SERINC5 down-regulation	-2.227	1.059	0.038	-1.925	1.350	0.157

The relationship between each standardized Nef function and dE90 is presented in (Table 3.6). In univariate analysis, CD4 down-regulation and SERINC5 down-regulation were significantly associated with dE90 (p=0.021 and p=0.003, respectively). The greatest relative contribution to the overall Nef function was observed for SERINC5 down-regulation with a model coefficient of -4.882 followed by CD4 down-regulation with a coefficient of -3.654. However, in the multivariate analysis, only SERINC5 down-regulation remained statistically significant and the greatest driver of dE90, with a coefficient of -5.303 (p=0.003).

Table 3.6: The effect of Nef functions on dE90 (Potts model).

Nef function	Univariate analysis			Multivariate analysis		
	Coef.	Std. Err.	p value	Coef.	Std. Err.	p value
CD4 down-regulation	-3.653	1.557	0.021	-0.417	2.241	0.853
HLA-I down-regulation	-2.129	1.610	0.189	0.196	1.917	0.919
Alteration of TCR signalling	0.991	1.695	0.560	1.279	1.459	0.383
SERINC5 down-regulation	-4.882	1.604	0.003	-5.303	1.725	0.003

In summary, the results suggest that CD4 down-regulation and SERINC5 down-regulation are the largest contributors of the Nef functions considered here to overall Nef function and that the contribution of SERINC5 down-regulation is the most significant.

3.6 Potential sequence determinants of Nef- mediated SERINC5 down-regulation activity.

The third aim of this study was to identify Nef amino acids that either increase or decrease Nef's ability to down-regulate SERINC5. To address this aim, a function-sequence analysis was performed using an online codon-by-codon tool (http://brockman-srv.mbb.sfu.ca/~B_Team_iMac/ Codon_by_codon/) which uses Mann-Whitney U tests with q values, on 106 patient-derived Nef clones.

We identified 15 amino acid variants at 11 different codons associated with altered SERINC5 down-regulation activity (Table 3.7). More specifically, eight amino acid variants were associated with increased SERINC5 down-regulation activity, and seven amino acid variants were associated with decreased SERINC5 down-regulation activity.

Table 3.7: Nef amino acids significantly associated with altered SERINC5 down-regulation activity [a].

Codon [b]	AA[c]	Cons [d]	Relative Nef function (%) [e]		No. of Samples [f]		Impact [g]	p-value	q-value
			+AA	-AA	+AA	-AA			
10	K	I	57.6	88.3	6	86	-31	0.008	0.3
10	I	I	88.6	60.9	78	14	28	0.01	0.3
11	V	V	89.5	58.0	79	13	32	0.003	0.3
38	D	D	89.5	65.9	77	29	24	0.005	0.3
38	E	D	55.1	86.7	13	93	-32	0.01	0.3
51	T	N	92.7	79.9	25	81	13	0.01	0.3
65	E	E	81.3	95.1	94	12	-14	0.0005	0.1
65	D	E	95.2	83.2	9	97	12	0.003	0.2
101	V	I	97.7	83.2	5	101	15	0.01	0.3
135	F	Y	59.8	85.9	13	93	-26	0.03	0.4
173	T	M	54.6	85.9	5	101	-31	0.02	0.3
176	T	E	58.8	86.7	9	97	-28	0.02	0.4
188	H	S	94.4	83.2	6	100	12	0.01	0.3
191	H	R	91.0	81.3	16	90	10	0.03	0.4
191	R	R	81.3	91.0	88	18	-10	0.03	0.4

[a] Amino acids (AA) at $n \geq 5$ in the dataset, $p < 0.05$, $q \leq 0.4$.
[b] Numbered according to HXB2.
[c] The amino acids associated with increased or decreased SERINC5 down-regulation activity.
[d] The consensus amino acid at a particular codon from the reference 2004 consensus C Nef sequence from the Los Alamos HIV sequence database.
[e] The median percentage SERINC5 down-regulation activity which was expressed relative to SF2 control of the Nef clones with (+) and without (-) the amino acid.
[f] The number of sequences with (+) and without (-) the amino acid.
[g] The median Nef activity with amino acid – median Nef activity without amino acid.

The most significant association (i.e., lowest p-value) was observed at codon number 65, where Nef clones that encoded the consensus amino glutamic acid (n=94) displayed lower SERINC5 down-regulation activity (median 81.3%) compared with clones that did not (n=12) (95.1%) (p=0.0005). Correspondingly, it was shown that clones that encoded for aspartic acid (n=9) at this codon instead of the consensus amino acid glutamic acid displayed a higher SERINC5 down-regulation activity (median=95.2%) compared with clones that did not (n=97) (83.2%) (p=0.003). A study by (Jin et al. 2019) similarly showed an association between glutamic acid at codon 65 and decreased SERINC5 down-regulation, albeit in subtype B clones.

We also observed that the greatest impact on SERINC5 down-regulation activity was at codons 10, 38 and 173, where non-consensus amino acids decreased activity by more than 30% and at codon 11, where the consensus amino acid increased activity by more than 30%. Together these results show that natural polymorphisms in Nef can lead to both increased and decreased ability to down-regulate SERINC5.

To explore sequence determinants underlying the significant difference in Nef-mediated SERINC5 down-regulation ability observed between the HPP and Tshedimoso cohorts (and therefore also potential determinants of SERINC5 down-regulation ability), we compared the Nef sequences between these cohorts. The VESPA test, available on the Los Alamos HIV sequence database (https://www.lanl.gov/), was used to compare the frequency of each amino acid for each codon between the cohorts. Where the difference in frequency exceeded 15%, we performed a Fisher's exact test to test for significance and used q values to account for multiple comparisons. We identified 11 amino acids at 9 codons that differ significantly between the Tshedimoso and HPP cohorts (Table 3.8).

Table 3.8: Nef amino acid variants differing significantly in frequency between HPP and Tshedimoso cohorts[a].

	Amino acid frequency (%)					
Codon	**Amino acid**	**HPP %**	**Tshedimoso %**	**Difference** [b]	**p-value**	**q-values**
5	W	82	100	-18	0.03	0.1
50	H	53	11	43	0.03	0.1
51	N	88	63	25	0.03	0.1
59	E	25	70	-45	0.0007	0.02
59	Q	66	26	40	0.004	0.05
64	E	91	52	39	0.05	0.1
158	K	59	30	29	0.02	0.1
184	Q	38	67	-29	0.03	0.1
188	H	0	15	-15	0.04	0.1
191	H	6	30	-24	0.03	0.1
191	R	91	67	24	0.05	0.1

[a] $p < 0.05$, $q < 0.2$. [b] The % amino acid frequency in HPP cohort – the % amino acid frequency in Tshedimoso cohort.

The most significant amino acid identified was glutamic acid in codon 59, with a p-value of 0.0007. This amino acid is found in 19 (70.37%) of the clones in the Tshedimoso cohort and 8 (25%) of the clones in the HPP cohort.

Three amino acids identified as significantly different in frequency between the cohorts were also identified as associated with altered SERINC5 down-regulation activity by our codon-by-codon analysis, namely 188H, 191R and 191H. Residues 188H and 191H were associated with moderately increased SERINC5 down-regulation activity, and residue 191R was associated with moderately decreased SERINC5 down-regulation activity (Table 3.7). Both 188H and 191H were more frequent in the Tshedimoso cohort than in the HPP cohort, while residue 191R was more frequent in the HPP cohort than the Tshedimoso cohort (Table 3.8). In addition, the non-consensus amino acid 51T was associated with increased SERINC5 down-regulation in the codon-by-codon analysis (Table 3.7), and here the consensus 51N was more frequent in the HPP cohort (Table 3.8). These amino acid frequency differences between the cohorts may partially explain the overall difference in SERINC5 down-regulation function between the Nef clones derived from the HPP and Tshedimoso cohorts.

CHAPTER

FOUR:

DISCUSSION

4.1 Introduction.

The Nef protein has shown to be an important contributor to HIV-1 pathogenesis (Pereira and daSilva 2016). Previous studies have indicated that Nef-mediated CD4 down-regulation and enhancement of infectivity are likely the major contributors to Nef's effect of enhancing pathogenicity (Iafrate et al. 2000; Watkins et al. 2013), although the relative significance of each Nef function for HIV-1 disease progression remains incompletely understood. As a result of the crucial role Nef-mediated down-regulation of SERINC5 has on enhancing virion infectivity (Rosa et al. 2015; Toyoda et al. 2020; Usami, Wu, and Göttlinger 2015), the main aim of this study was to investigate whether this activity of Nef is a significant contributor to HIV-1 subtype C disease progression. To assess this, we measured the SERINC5 down-regulation ability of 106 patient-derived Nef clones from early/acute infection, spread between three cohorts and two countries, and related this Nef activity to markers of disease progression.

4.2 SERINC5 down-regulation, as well as other Nef functions, and disease progression.

We found that SERINC5 down-regulation ability of Nef clones derived from early infection was not significantly associated with subsequent viral load set point or the rate of CD4$^+$ T cell decline. A previous study on the same clones used in this study revealed that CD4 down-regulation and HLA-I down-regulation correlated with viral load set point and the rate of CD4$^+$ T cell decline, respectively (Mann et al. 2014).

Since the dataset used for analysis differed in some respects from that used in the present study (less CD4$^+$ T cell count follow-up data for some participants, restriction of CD4$^+$ T cell decline data to -50 to +50 cells/mm^3 per month, and use of Spearman's correlation method only for viral load set point analysis in the previous study), the disease progression marker analysis was repeated for all Nef functions using the current dataset and methods to standardise and allow for comparison of results between the different Nef functions. Following re-analysis, we found that no statistical association was observed between the Nef function studied here (SERINC5 down-regulation CD4 down-regulation, HLA-I down-regulation and, alteration of TCR signalling) and viral load set point and the rate of CD4$^+$ T cell decline.

The lack of correlation between HLA-I down-regulation and viral load set point is consistent with our previous study of the same Nef clones (Mann et al. 2014) and with another small study of HIV-1 infected individuals (Lewis et al. 2012) where the researchers observed no correlation between HLA-I down-regulation activity and viral load. This was also shown in an animal model in a study by (Swigut, Shohdy, and Skowronski 2001), where the researchers observed no effect on SIV viral load after disrupting the HLA-I down-regulation function in the macaque model.

The lack of correlation between CD4 down-regulation and SERINC5 down-regulation and disease progression (specifically viral load setpoint) was, however, unexpected due to previous studies showing that Nef-mediated CD4 down-regulation (Iafrate et al. 2000; Watkins et al. 2013), as well as enhancement of infectivity (Iafrate et al. 2000), are likely major contributors to Nef's effect of enhancing pathogenicity.

Furthermore, in chronic HIV-1 infected progressors of the Nef functions studied, only Nef-driven virion infectivity correlated with markers of disease progression (Mwimanzi et al. 2013). The lack of significant correlation between SERINC5 down-regulation and any other individual Nef functions studied here, and markers of disease progression could possibly be explained in several ways. It could result from a requirement for multiple Nef functions to act together to facilitate the enhancement of viral spread and immune evasion *in vivo* (Fackler, Alcover, and Schwartz 2007; Mwimanzi et al. 2013). It could be that there is an unidentified Nef function that is a stronger contributor to HIV-1 pathogenesis than the Nef functions studied here – in fact, (Mwimanzi et al. 2013) show that Nef-mediated CD4 down-regulation, enhancement of infectivity, and one or more unknown Nef functions represent major pathogenic contributors. Another possible explanation for the lack of correlation between SERINC5 down-regulation activity and markers of disease progression despite studies that show that Nef-enhancement of virion infectivity influences HIV-1 pathogenesis is that SERINC5 down-regulation activity, albeit reported to enhance virion infectivity, may not be the only contributor to virion infectivity. Evidence of this possibility was seen in a recent study by (Ramirez et al. 2020), where the researchers corroborate another study that used T-lymphoid cell line (MOLT-3) (Wu et al. 2019) and concluded that Nef appears to modulate a still unidentified host protein(s) to enhance the viral growth rate and infectivity in CD4$^+$ T cells independently of SERINC3 and SERINC5 in CEM cell lines. Perhaps the SERINC proteins work in conjunction with these protein(s) to enhance virion infectivity and replication (in CEM and MOLT-3 cells at least).

4.3 SERINC5 down-regulation and overall Nef function.

The second aim of the study was to determine which Nef function (SERINC5 down-regulation CD4 down-regulation, HLA-I down-regulation and, alteration of TCR signalling) has the most substantial influence on overall Nef function using E values derived from the Ising (dE0) and Potts (dE90) models to represent overall Nef function. Initially, we performed a univariate analysis, and results showed that both CD4 down-regulation and SERINC5 down-regulation were significantly associated with dE0 and dE90 values. However, the difference was that CD4 down-regulation and SERINC5 down-regulation showed similar coefficient sizes in the dE0 model, whereas SERINC5 down-regulation showed a higher model coefficient (and smaller p-value) than CD4 down-regulation in the dE90 model. The association between SERINC5 down-regulation and E values was negative as expected - lower SERINC down-regulation function would correspond to a higher E value (which in the Nef fitness landscape model corresponds to low Nef fitness).

According to (Barton et al. 2019), individual comparisons can be somewhat misleading because if we assume that E is a good proxy for Nef fitness *in vivo*, then a defect in one of the crucial functions of Nef would lead to us inferring a higher E value regardless of the other Nef functions remaining intact. Considering this effect, we then performed a multivariate analysis considering all Nef functions. The multivariate analysis differed significantly from the univariate analysis. None of the Nef functions measured was significantly associated with dE0, and only SERINC5 down-regulation remained statistically significant and the greatest driver of dE90.

We first speculated that the difference between the univariate and multivariate analysis was due to the possible impact of collinearity. This was considered due to the many correlations observed between Nef functions studied here, particularly the correlation between SERINC5 and CD4 down-regulation observed here, together with the correlation between HLA-1 down-regulation and alteration of TCR signalling described previously (Naidoo et al. 2019). This was, however, ruled out by assessing the variance inflation factors. A value greater than the threshold value of 5 would have suggested the influence of collinearity; however, all were below the threshold value of 5.

In terms of predicting the fitness cost of mutations in Nef, which is such a highly mutatable protein (Barton et al. 2019), the Potts model is expected to provide more reliability than the Ising model because it has a residue-specific resolution. In contrast, the Ising model does not consider each amino acid's identity and instead uses a binary approximation, which is all mutant amino acids are denoted as one and the wild-type amino acid is denoted as zero. This principle may not be suitable for Nef, which is a highly variable protein. Nevertheless, in the study by (Barton et al. 2019), both the Ising and Potts models performed similarly in predicting fitness costs in Nef. The possible explanation for this is that the Potts model advantage of residue-specific resolution may be offset by the disadvantage of introducing more noise into the model, and model complexity may need fine-tuning.

Overall, the analysis of the E values indicates that SERINC5 down-regulation activity is one of the strongest contributing factors to overall Nef function. The importance of this function is further highlighted by the fact that this function is highly conserved among the primate lentiviruses for the prevalence of these species (Gonzalez-Enriquez et al. 2017).

It is essential to note that we cannot rule out other Nef functions that were not measured in this study, both known and unknown, which could be just as important if not more important than the functions studied here.

4.4 Natural sequence variation linked to altered SERINC5 down-regulation ability.

The third aim of the study was to identify amino acid variants associated with increased or decreased SERINC5 down-regulation activity. We have identified 15 amino acid variants at 11 different codons that have a significant association with SERINC5 down-regulation activity. Specifically, amino acids 10I, 11V, 38D, 51T, 65D, 101V, 188H and, 191H were associated with increased activity and amino acids 10K, 38E, 65E, 135F, 173T, 176T and, 191R were associated with decreased activity. The codons at which non-consensus amino acids were associated with decreased SERINC5 down-regulation activity (10, 11, 38, 135, 173 and 176) are discussed first, followed by the codons at which non-consensus amino acids increased SERINC5 down-regulation activity (51, 65, 101, 188 and 191). Interestingly, codons 10, 38 and 173, where mutation decreases activity, also had the largest impact on this Nef activity.

4.4.1 Codons at which mutation is associated with decreased SERINC5 down-regulation.

Codons 10 and 11

At codon 10, the non-consensus amino acid lysine (K) was associated with decreased SERINC5 down-regulation activity, and the consensus amino acids for both codons 10 and 11 were associated with increased activity.

This is consistent with a study by (Mwimanzi et al. 2013) on subtype B clones from chronic infection, where the researchers showed that consensus valine (V) (subtype B consensus amino acid) and non-consensus leucine (L) at codon 10 substantially increase and decrease viral infectivity, respectively. However, the opposite was true for codon 11 in a study of subtype B Nef clones where a non-consensus amino acid, in this case, alanine (A), displayed higher SERINC5 down-regulation activity (Jin et al. 2019). As reported in (Mann et al. 2015), the conservation at codons 8-12 in subtype C is associated with lower HLA-I down-regulation function. It was interesting to see the opposite trend occurring here where at codons 10 and 11, the consensus amino acid is associated with higher SERINC5 down-regulation. This could perhaps suggest a trade-off in Nef functions.

Codon 38

At codon 38, consensus amino acid aspartic acid (D) and non-consensus amino acid glutamic acid (E) were shown to increase and decrease SERINC5 down-regulation activity, respectively. It has been reported that codons 12-39, located near the N-terminus, is an interactive platform responsible for the Nef-associated kinase complex (NAKC), which has been identified as an important determinant for numerous Nef activities (Ananth et al. 2019). Codon 38 falls within this range, and this could partly explain the strong association of this codon with altered SERINC5 down-regulation. It is known that E38D is a CTL escape mutation in subtype B (Draenert et al. 2006), which differs in the consensus amino acid compared to subtype C at this codon. It would be interesting to investigate whether the non-consensus 38E in subtype C (which is associated with diminished SERINC5 down-regulation activity) is a CTL escape mutation, as this may have relevance for attenuation-based vaccine design strategies (Chopera et al. 2011).

Codon 135

At codon 135, the non-consensus amino acid phenylalanine (F) was observed to significantly decrease SERINC5 down-regulation activity. According to (Du et al. 2016), Y135F is a known escape mutation in subtype B and is observed frequently in patients with HLA-A*24:02, an HLA Class 1 allele (Han et al. 2014). In a study by (Adland et al. 2013), the findings show that CTL responses to 134-148 are associated with lower viral loads, while a study by (Lewis et al. 2012) found that Y135F ablated HLA-1 down-regulation. Taken together, this could be an important part of Nef as a target for attenuation-based vaccine design (Chopera et al. 2011).

Codons 173 and 176

At codon 173 and 176, clones that encoded amino acid threonine (T) were shown to decrease SERINC5 down-regulation activity. For Nef to down-regulate SERINC5, it must first bind to AP-2 (Shi et al. 2018) and there are several studies that reveal the importance of Nef codon 174 and 175 for Nef's interaction with AP-2 (Jin et al. 2013; Lindwasser et al. 2008; Ren et al. 2014). More recently, as reviewed by (Ramirez et al. 2019), codon 175 has also been identified to be crucial for SERINC5 down-regulation activity. Overall mutations in codons 173 and 176, respectively, could affect the ability of Nef to down-regulate SERINC5 simply due to them being positioned adjacent to codon 174 and 175, which are crucial for Nef-AP-2 binding.

4.4.2 Codons at which mutation is associated with increased SERINC5 down-regulation.

Codon 51

At codon 51, we see that clones encoding threonine (T) displayed higher SERINC5 down-regulation activity when compared with clones that do not. This result is consistent with a study done by (Carl et al. 2001), where the researchers sought to investigate the relevance of amino acid variations in HIV-1 Nef and their association with different stages of disease. They studied 5 Nef alleles, three of which contained the amino acid threonine at codon 51, while the other two alleles encoded the consensus amino acid asparagine (N) at codon 51. The findings from the study by (Carl et al. 2001) showed that Nef alleles with amino acid threonine at codon 51 exhibited two-fold increased virion infectivity.

The data from our codon-by-codon analysis from our study together with further studies by (Jin et al. 2019; Jin et al. 2020), albeit in subtype B Nef clones, showed that Nef clones encoding threonine (T) at codon 51 displayed higher SERINC5 down-regulation activity. It is worth mentioning that there were other amino acid variations in the generated Nef alleles used in the study by (Carl et al. 2001), but there remains a strong possibility that codon 51 is crucial for viral infectivity.

Codon 65

In our study, at codon 65, clones that encoded for aspartic acid (D) were shown to increase SERINC5 down-regulation activity.

Conversely, clones that encoded for the consensus amino acid glutamic acid (E) exhibit lower SERINC5 down-regulation activity than clones that did not. These findings were consistent with the data reported by (Jin et al. 2019), albeit in subtype B.

Codon 188

At codon 188, the encoding of serine (S) is associated with increased SERINC3 down-regulation activity (Kruize et al. 2021). Serine is the consensus amino acid at codon 188, and interestingly from the data, we collected we observed that when histidine (H) is encoded at codon 188, there is a moderate increase in SERINC5 down-regulation activity. According to (Melhem et al. 2014), S188H is a known escape mutation, and considering the associated increase in SERINC5 down-regulation activity observed in our study, this codon may not be a suitable target for attenuation based vaccine design. Overall, codon 188 could possibly be important to both SERINC3 and SERINC5 down-regulation activity, which merits further investigation.

Codon 191

At codon 191, clones encoding for the non-consensus histidine (H) showed an increase in SERINC5 down-regulation activity. Conversely, clones encoding for the consensus amino acid arginine (R) showed a decrease in SERINC5 down-regulation activity. It has been reported that codon 191 is responsible for PAK2 activation (O'Neill et al. 2006).

Further evidence of the possible importance of this codon to viral infectivity was revealed by (Schindler et al. 2007), where the researchers found an F191H and an F191R mutation disrupted the association of PAK2 with Nef. These mutations were shown to impair Nef-mediated virion infectivity in P4-CCR5 cells.

4.5 Nef-mediated SERINC5 down-regulation difference between Tshedimoso and HPP cohorts.

We observed a functional difference between the clones from the HPP (Durban) and Tshedimoso (Botswana) cohorts, where the Tshedimoso clones had a higher function overall. There are two ways to possibly explain the functional difference between the two cohorts from the two countries. One possibility is that population differences or the nature of the epidemic in the two countries may be responsible.

It has been reported previously that the timing and size of the epidemic in the respective countries likely contributed to a difference in HIV-1 Gag function (Payne et al. 2014). The epidemic in Botswana rose to a higher prevalence than South Africa earlier on, and more virus adaptation in the Gag protein to the population immune responses has been reported in Botswana when compared to South Africa. This resulted in attenuation in the Gag protein, which is consistent with a study by (Nomura et al. 2013), where the researchers showed that Gag-driven viral replication capacity attenuated over time.

Our group previously observed a similar trend for Nef-mediated alteration of TCR signalling (Naidoo et al. 2019) where clones from Botswana had lower function than those from Durban, although no such trends were observed for Nef-mediated CD4 and HLA-I down-regulation (Mann et al. 2014), however, we observed the opposite trend for Nef-mediated SERINC5 down-regulation here, which suggests that a different factor is at play for SERINC5 down-regulation. Differences in SERINC expression level in the two populations could be explored as a possible factor contributing to the cohort difference observed in this study.

The other possible way to explain the functional difference between the Tshedimoso and HPP is from a genetic perspective. Using the data acquired in the codon-by-codon analysis in conjunction with the VESPA test, we compared Nef clones' sequences from the Tshedimoso and HPP cohorts. We identified three different amino acids (188H, 191H and 191R) that differed in frequency between the cohorts and were either directly or indirectly associated with altered SERINC5 down-regulation activity in the codon-by-codon analysis performed in this study. To what extent these amino acids affect SERINC5 down-regulation activity remains unknown and merits further study; however, they could partially explain the functional difference between the cohorts.

4.6 Correlation between SERINC5 down-regulation and other Nef functions.

Our results show there is a significant statistical correlation between Nef-mediated SERINC5 down-regulation and CD4 down-regulation. This was also confirmed in a study by (Jin et al. 2019) using subtype B Nef clones. This correlation can be explained by the similar mechanism used for the Nef-mediated down-regulation of both SERINC5 and CD4. Evidence of this was shown in a study by (Shi et al. 2018).

The researchers concluded that to down-regulate CD4 and SERINC5 from the plasma membrane Nef uses the same functional motifs, consistent with previous studies (Geyer and Peterlin 2001; Rosa et al. 2015). They pointed out that Nef internalizes both SERNC5 and CD4 from the plasma membrane to intracellular compartments. Further, their results implicate the AP-2 adaptor complex in Nef-dependent CD4 and SERINC5 down-regulation and highlight the re-localization of SERINC5 by Nef to the early, late, and recycling endosomes in a similar manner to that occurring for CD4 down-regulation.

We found no statistical correlation between SERINC5 down-regulation activity and HLA-1 down-regulation activity in our study; however, in a study by (Jin et al. 2019), a statistical correlation was found between the two, albeit in subtype B clones.

HLA-1 and SERINC5 down-regulation differ in the adapter proteins used by Nef: HLA-1 down-regulation requires interaction with AP-1 (Dirk et al. 2016; Ramirez et al. 2019; Shi et al. 2018), and SERINC5 down-regulation requires interaction with AP-2 (Ramirez et al. 2019; Shi et al. 2018). However, there does appear to be at least one Nef residue required for both functions, namely D123. Nef's ability to down-regulate SERINC5 was diminished by the Nef D123 (Buffalo et al. 2019) mutation, and interestingly this residue is implicated in AP-1 μ1-associated downregulation of HLA-1 (Jia et al. 2012).

4.7 Study limitations

One of the study's limitations was the fact that we only studied one Nef clone per patient. Nevertheless, sequencing was used to confirm that the Nef clone studied was representative of the bulk Nef amplicon, and furthermore, these clones were derived from early infection when virus diversity is limited.

Another possible limitation is the use of cell lines as opposed to primary cells. The advantages of using cell lines are that they are cost-effective, easier to use than primary cells, and, perhaps most importantly, offer reproducible and consistent results (Kaur and Dufour 2012).

It was reported that the structural protein Env could determine SERINC5 resistance independently of Nef, and testing a panel of Env proteins revealed a high frequency of SERINC5-resistant Env (Beitari et al. 2017). Therefore, it is possible that if Nef fails to downregulate SERINC5 well, the Env protein from the same virus could be highly resistant to SERINC5; however, this study only considered Nef-mediated counteraction of SERINC5.

Differing Nef expression levels could influence the results however, our analysis on a subset of Nef clones with Western blot data indicated that there was no significant influence of variability in Nef expression on SERINC5 down-regulation activity.

4.8 Conclusion

In conclusion, none of the Nef functions in our study, including SERINC5 down-regulation, were observed to influence disease progression significantly. However, interestingly we found that CD4 down-regulation and SERINC5 down-regulation were the largest contributors, of the Nef functions considered here, to overall Nef function and that the contribution of SERINC5 down-regulation was the most significant. Taken together, this could be explained by multiple Nef functions acting together to facilitate the enhancement of viral spread and immune evasion *in vivo*, which ultimately enhance disease progression.

We also identified several Nef amino acid variants associated with either increased or decreased ability to down-regulate SERINC5, which could be informative for the development of vaccines or therapies; however, further studies in the form of site-directed mutagenesis experiments are warranted to further understand their effect on SERINC5 down-regulation activity.

CHAPTER

FIVE:

REFERENCE

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