Regulation of TRIM E3 Ligases and Cyclophilin A and the Impact on HIV-1 Replication and Pathogenesis

By

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Preface

The experimental work described in this thesis was carried out in the following labs: HIV Pathogenesis Programme (HPP), Hasso Plattner Research Lab (HPRL), and Centre for the AIDS Programme of Research in South Africa (CAPRISA), located in the Doris Duke Medical Research Institute, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban, 4013, South Africa. This research was carried out from January 2009 to November 2011, under the supervision of Professor Thumbi Ndung'u. The study represents original work by the author and has not been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

The University of KwaZulu-Natal Biomedical Research Ethics Committee has approved the study, refer to Appendix I.

A part of the results depicted in this thesis has been presented at international conferences.

Ravesh Singh, Lise Werner, Koleka Mlisana, Jeremy Luban, SS Abdool Karim, Thumbi Ndung'u, and the CAPRISA Acute Infection Study Team. Association between TRIM22, Type 1 Interferons, Viral Load and CD4 T cells Counts during Primary HIV-1 Infection. *Abstracts* of the 17th Conference on Retroviruses and Opportunistic Infections (CROI 2010), San Francisco, 16-19 February 2010, Abstract no. D-131.

Ravesh Singh, Vinod Patel, Lise Werner, Marianne Mureithi, Koleka Mlisana, Jeremy Luban, Marcus Altfeld, Keertan Dheda, Slim Salim Abdool Karim, and Thumbi Ndung'u. Regulation of huTRIM5α, TRIM22 and IFN-1 in Chronic HIV-1 infection, *Abstracts* of the 6th IAS Conference on HIV Pathogenesis, Treatment and Prevention Rome, Italy, 17-20 July 2011, Abstract no. CDA061.

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SINGH, R., WERNER, L., MLISANA, K., LUBAN, J., KARIM, S. S., & NDUNG'U, T. TRIM11, TRIM19 and TRIM36 induced by type 1 interferons but expression levels do not associate with viral control during primary HIV-1 infection (Manuscript in preparation).

SINGH, R., PATEL, V., MUREITHI, M., NARANBHAI, V., WERNER, L., MLISANA, K., ALTFELD, M., LUBAN, J., KARIM, S. S., DHEDA, K. & NDUNG'U, T. Regulation of huTRIM5α, TRIM22 and IFN-1 in chronic HIV-1 infection (Manuscript in preparation).

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SINGH, R., MADLALA, P., WERNER, L., SIBEKO, S., KARIM, S. S., LUBAN, J., AN, P., WINKLER, C, A. & NDUNG'U, T. Association between Cyclophilin A Gene (PPIA) expression, Viral Load and CD4 T cells Counts during Primary HIV-1 Infection (Manuscript in preparation).

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Ravesh Singh (candidate)

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Professor Thumbi Ndung'u (Supervisor)

Plagiarism Declaration:

I, **Ravesh Singh** declare that:

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Abstract

Type 1 interferons (IFN-1) induce the expression of the Tri-partite interaction motif (TRIM) family of E3 ligases but the contribution of these antiviral factors to HIV pathogenesis is not completely understood. TRIM5α restricts HIV-1 by promoting premature disassembly of HIV-1 capsid while cyclophilin A (CypA), a human protein that is incorporated into the HIV-1 virion increases viral infectivity by facilitating proper uncoating of HIV-1 capsid.

TRIM E3 ligases play an important role in HIV-1 replication and pathogenesis. Ilustration of the mechanism of their regulation can identify novel anti-HIV targets. I hypothesized that increased expression of specific IFN-1 and TRIM isoforms is associated with significantly lower likelihood of HIV-1 acquisition and viral control during primary HIV-1 infection.

I measured IFN-α2b, IFN-β, the universal IFN-1 response marker myxovirus resistance protein A (MxA), huTRIM5α, TRIM11, TRIM19, TRIM36 and TRIM22 messenger RNA (mRNA) levels in peripheral blood mononuclear cells (PBMCs) of high risk, HIV-1 uninfected participants, and HIV-1 positive study participants in primary HIV-1 infection. HIV-1 positive participants had higher levels of IFN-β ($p=0.0005$), MxA ($p=0.007$) and TRIM22 ($p=0.01$) and lower levels of huTRIM5α ($p<0.001$) compared to HIV-1 negative participants. TRIM22 but not huTRIM5α, IFN-α, IFN-β or MxA showed a negative correlation with plasma viral load ($p=0.0307$) and positive correlation with $CD4^+$ T cell counts ($p=0.0281$). Stable TRIM22 knockdown resulted in increased HIV-1 particle release and replication in Jurkat reporter cells. TRIM22 likely acts as an antiviral effector *in vivo*.

The role of these factors in mediating effective anti-HIV immunity during the chronic infection phase or in remote immune privileged site such as the central nervous system is unknown. I measured expression levels of INF-1, MxA, huTRIM5α and TRIM22 in peripheral blood mononuclear cells during primary and chronic HIV-1 infection using messenger RNA real-time PCR. Expression levels of these factors were also analysed in blood- versus the central nervous system-derived cells during chronic infection and association with biomarkers of disease progression investigated.

Primary and chronic HIV-1 infection groups had significantly higher levels of MxA and TRIM22 compared to the HIV-1 negative group, ($p<0.001$, $p<0.05$, $p<0.0171$ and $p<0.0052$) respectively. Chronically HIV-1 infected group had lower levels of huTRIM5α compared to the HIV-1 primary infection group ($p<0.0001$) or HIV-1 negative group ($p<0.0001$). In matched CNS-derived and peripheral blood mononuclear cell samples, higher levels of MxA ($p<0.001$) was noted in the CNS when compared to the periphery, however, huTRIM5α levels were lower in the

periphery when compared to the CNS compartment, ($p<0.0001$). Similar levels of TRIM22 were noted in the CNS compartment when compared to the periphery. There was weak but significant negative association between TRIM22 levels and viral load in the periphery during chronic HIV-1 infection, ($r= -0.397$, $p=0.044$).

Both huTRIM5α and TRIM22 were expressed in PBMC and CNS-derived cells. TRIM22 but not huTRIM5α was associated with low viremia in PBMCs. However, TRIM22 was not associated with viral control in the CNS. Data suggest an intricate balance between the roles of TRIM22 as a marker of viral control versus immune activation, which warrants further investigation.

To further explore what role these antiviral factors may play in HIV pathogenesis in the CNS, I investigated *in vitro* the expression of TRIM5 isoforms and TRIM22 in some well characterized T-cell lines, B-cell lines and neuroblastic cell lines. The levels of huTRIM5α, TRIM5δ and TRIM5γ in the neuronal cells where comparable to the immune cells, however lower levels of TRIM22 was noted in neuronal cells. The modulation of these TRIM E3 ligases was dependant on the cytokine and was also cell line dependant. Neuroblasts stimulated with TNF-α showed the highest level of huTRIM5α induction when compared to neuroblasts stimulated with IFN-α, IL-2 and IL-10 and other immune cell lines

Human cyclophilin A, or CypA, encoded by the gene peptidyl prolyl isomerase A (*PPIA*), is incorporated into the HIV type 1 (HIV-1) virion and promotes HIV-1 infectivity by facilitating virus uncoating as opposed to TRIM5α that has been shown to promote premature disassembly of HIV-1 capsid. In light of this I hypothesized that increased levels of CypA may raise HIV-1 susceptibility risk. The HIV negative individuals showed differential expression of CypA mRNA levels. CypA levels did not significantly change during HIV-1 primary infection. There was no association between CypA and viral load or CD4 T cell counts. I dissected the result of differential expression of CypA in my cohort by looking at the expression of CypA in validated immune cell lines. There were two distinct patterns of CypA expression with CEM, HOS and 293T cells expressing higher levels of CypA compared to HeLa, H9, SupT and Jurkat cells. LPS stimulation of the cell lines did not induce CypA expression. I hypothesized that regulatory polymorphisms in the Cyclophilin A gene (*PPIA*) control the mRNA levels. The A1650G single nucleotide polymorphism in the *PPIA* promoter region was significantly associated with higher CypA expression in PBMCs from HIV-1 infected individuals but not HIV-1-uninfected PBMCs and PBMCs from individuals with this polymorphism supported higher HIV-1 replication *in vitro*.

I postulate that that the modulation of TRIM22 is dependent on the activation state of the cell. This would explain the lack of association of TRIM22 with viral load in the

CNS compartment where there is high levels of immune activation, while in the periphery we still see an association between TRIM22 and viral load. huTRIM5α and TRIM22 expressed in neuronal cells has a biological function, as the suppression of huTRIM5α or TRIM22 by specific siRNA led to increased pseudotyped HIV-1 replication in these cells. My data suggests that TRIM E3 ligases may play a part in innate defense against HIV-1 in the CNS. I have demonstrated in this study that TRIM22 could be a candidate for novel therapeutics against HIV-1 in numerous stages of disease progression. I speculate that specific enhancement of TRIM22 without concomitant immune activation may block viral replication. Further studies to pursue this potential antiviral strategy are warranted. We have also demonstrated that CypA, a HIV-1 cellular cofactor plays a part in HIV-1 replication and we speculate that blocking CypA may be advantageous as an antiviral strategy.

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There is one person that I have gained an invaluable amount of knowledge from during the course of this PhD that is Prof. Thumbi Ndung'u. I would like to deeply and sincerely thank Prof Ndung'u for the excellent mentorship as well as bringing out my passion for science and helping to become a better scientist.

To all the members of the HPRL/HPP lab, thank you guys for all the support and help throughout my degree. I would not be at this stage of my life without the help of you. I am deeply grateful to each of you with a special thank you to Mrs Keshni Hiramen and members of Room 213.

To my parents, I am extremely grateful for everything that you have done for me. I know it's because of you I reached this stage of my life. I cannot thank you enough.

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Dedication

To my parents and God

Abbreviations

µg	microgram
AIDS	Acquire Immunodeficiency Syndrome
APOBEC	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like
APOBEC3G	APOlipoprotein B mRNA Editing enzyme, Catalytic polypeptide-like 3G
CA	Capsid
CCR5	C-C chemokine Receptor type 5
CD28	Cluster of Differentiation 28
CD3	Cluster of Differentiation 3
CD4	Cluster of Differentiation 4
cDNA	complementary DNA
CXCR4	C-X-C chemokine Receptor type 4
CypA	Cyclophilin A
DC	Dendritic Cell
DNA	DeoxyriboNucleic Acid
dsRNA	double-stranded RNA
Env	Envelop polyprotein
ER	Endoplasmic Reticulum
Fv1	Friend Virus susceptibility-1 protein
Gag	Group specific antigen polyprotein
GAPDH	GlycerAldehyde 3-Phosphate DeHydrogenase
h	hour
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus type 1

HIV-2	Human Immunodeficiency Virus type 2
HLA	Human leukocyte Antigen
HLE	Human Leukocyte Elastase
huTRIM5α	human TRIM5α
IL-1	InterLeukine 1
IL-7	InterLeukine 7
IN	Integrase
JAK	Janus Kinase
kD	kiloDalton
LTR	Long Terminal Repeat
MA	MAtrix
MHC I	Major Histocompatibility Complex class I
MHC II	Major Histocompatibility Complex class II
min	minute
miRNA	microRNA
ml	milliliter
MLV	Murine Leukemia Virus
mM	miliMolar
mRNA	messenger RNA
NC	NucleoCapsid
NCBI	National Center for Biotechnology Information
Nef	NEgative Regulatory Factor
NES	Nuclear Export Signal
NFκB	Nuclear Factor-Kappa B
NFAT	Nuclear Factor of Activated T-cells
ng	nanogram

NIAID	National Institute of Allergy and Infectious Diseases
NLS	Nuclear Localization Signal
nm	nanometer
NPC	Nuclear Pore Channel
PPFIBP1	PTPRF Interacting protein, Binding Protein 1 (liprin beta 1)
PR	PRotease
Rev	Regulator of virus protein expression
rhTRIM5α	rhesus macaque TRIM5α
RISC	RNA-Induced Silencing Complex
RNA	RiboNucleic Acid
RNAi	RNA interference
RNAPol II	RNA Polymerase II
RNase H	RiboNuclease enzyme H
RRE	Rev Responsive Element
RT	Reverse Transcriptase
RTC	Reverse Transcription Complex
shRNA	short hairpin RNA
siRNA	small interfering RNA
SIV	Simian Immunodeficiency Virus
SIVmac	SIV from macaques
STAT	Signal Transducers and Activator of Transcription
Tat	Transcriptional transactivator protein
TNF-α	Tumor Necrosis Factor-alpha
TRIM5α	Tripartite Motif Protein 5 alpha
TRIM11	Tripartite Motif Protein 11
TRIM19	Tripartite Motif Protein 19

TRIM22	Tripartite Motif Protein 22
TRIM36	Tripartite Motif Protein 36
tRNA	transference RNA
UNAIDS	United Nations Joint Programme on HIV/AIDS
Vif	Viral infectivity factor
Vpr	Viral protein R
Vpu	Viral protein U
VSV-G	Glycoprotein G from Vesicular Stomatitis Virus

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1 Literature Review

1.1 Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)

Human immunodeficiency virus (HIV) is the causative agent of the progressive disease acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983, Gallo et al., 1983). It is one of the leading causes of death worldwide (UNAIDS, 2012).

Approximately 34 million people live with HIV globally, with 23.5 million people living in sub-Saharan Africa (UNAIDS, 2012). A HIV infection is characterized by a progressive depletion of cluster differentiation 4 (CD4) + T cells which in turn causes immune dysfunction. This eventually leads to the final stage of the disease where infected individuals are more susceptible to opportunistic infections and they are now clinically defined as having AIDS (Fauci et al., 1985, Phillips et al., 1991).

There are two types of human immunodeficiency virus described, type 1 (HIV-1) (Barre-Sinoussi et al., 1983, Gallo et al., 1983) and type 2 (HIV-2) (Clavel et al., 1986). Both types cause AIDS, however HIV-2 represents a significant minority of all HIV infections. HIV-2 infection differs from HIV-1 infections as patients have been described as having a slower disease progression and transmission rate, a lower plasma viral load and a low rate of CD4 T cell decline (Marlink et al., 1994, Whittle et al., 1994, Jaffar et al., 1997).

Numerous efforts have been made to develop ways to prevent and treat this disease since the discovery of HIV. Several drugs have been developed and combinations of these drugs have been successfully used to treat HIV infections. The current antiviral therapy accepted by the scientific and medical community is known as the highly active anti-retroviral therapy (HAART) or combination antiretroviral therapy (cART). HAART consists of antiviral drugs that act against HIV proteins (De Clercq, 2009, Morse et al., 2012).

HAART targets reverse transcriptase (RT); (nucleoside and RT inhibitors such as zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, emtricitabine and tenofovir and non nucleoside RT inhibitors; nevirapine, delavirdine, efavirenz and etravirine), and the viral protease (saquinavir, ritonavir, indinavir, nellfinavir, amprenavir, lopinavir, atazanivir, fosamprenavir, tipranavir and darunavir) (Arhel and Kirchhoff, 2010). In recent years antiretroviral drugs that inhibit viral intergrase (IN), (raltegravir), and the six helix bundle core formation of gp41 transmembrane protein required for virus cell fusion (enfuvirtide) have been approved for clinical trials (Arhel and Kirchhoff, 2010, Chow et al., 2012). HAART reduces viral loads to almost undetectable levels but does not cure HIV; however there are several drawbacks such as toxicity and the formation of viral drug resistant mutants (Stebbing et al., 2006, Schiffer et al., 2012). Current HAART regimens are also very expensive and require an infrastructure with a functional health care system that also allows for medical monitoring of infected individuals. The vast majority of people currently infected with HIV live in developing countries and may not have access to antiretroviral therapy (Arhel and Kirchhoff, 2010).

Although there have been major breakthroughs in the development of these antiviral drugs, the eradication of HIV and the cure of AIDS has still not been achieved (Mamo et al., 2010). Due to the various limitations of current combination antiretroviral therapies, the field of antiretroviral drug research remains very active, where the HIV biology and the interaction between the virus and its host cells can provide new insights for the development of new antiviral therapeutic strategies.

1.2 HIV-1 taxonomy

HIV-1 belongs to the genus Lentivirus of the *Retroviridae* family. Retroviruses are characterized by their single strand positive RNA diploid genome and by the virion polymerase that is capable of RNA-directed DNA synthesis (reverse transcription) generating a DNA intermediate that is integrated in the host genome (Bishop, 1978, Coffin et al., 1997, Jayappa et al., 2012).

Lentiviruses are different from other retroviruses because of their long incubation period before manifestations of clinical illness. Lentiviruses share a common morphogenesis and morphology, tropism for macrophages, genetic and antigenic variability, and additional regulatory genes not found in the other groups of retroviruses (Narayan and Clements, 1989, Coffin et al., 1997).

Phylogenetic analysis of HIV-1 sequences have distinguished 4 major groups among HIV-1 isolates such as group M (Major), group O (Outlier), group N (non-M, non-O) and group P. The M group is subdivided into eleven clades (A to K) and accounts for more than 90% HIV/AIDS cases (Geretti, 2006). There is a dominance of HIV-1 (B) and HIV-1 (C) subtypes (Novitsky et al., 2010). Recently Vallari and colleagues reported a new group, P group, isolated from a Cameroonian woman. The

viral sequence, RBF168, for the P group forms a distinct HIV-1 lineage that includes SIV sequences from western gorillas (SIVgor; *Gorilla gorilla gorilla*) (Vallari et al., 2010).

1.3 HIV-1 structure

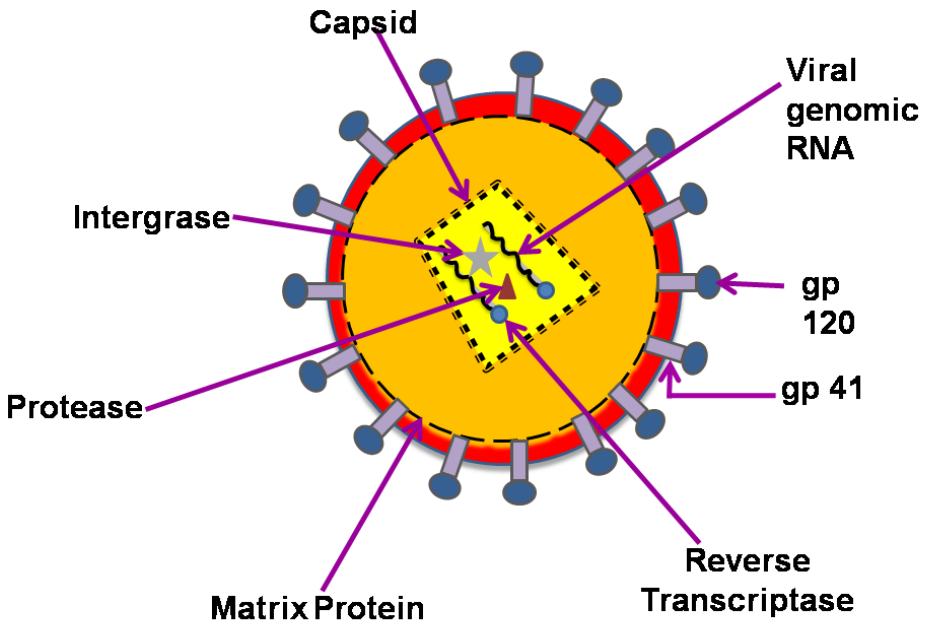


Figure 1.1 Morphology of the mature HIV virion. adapted from (Freed, 1998)

Mature HIV-1 virions are spherical shaped that have a diameter of approximately 100nm. HIV-1 is similar to other lentiviruses as it has both an outer and an inner membrane. The outer viral membrane is derived from the host cell membrane, which consists of a lipid bilayer where the viral surface (gp120 or SU)

glycoprotein is anchored via interaction with the viral transmembrane (gp41 or TM) glycoprotein (Turner and Summers, 1999, Wang et al., 2000, Taylor et al., 2008).

The outer viral membrane is also made of proteins derived from the host cell, such as the major histocompatibility antigens, actin and ubiquitin (Arthur et al., 1992). The viral inner membrane is composed by several matrix proteins (p17 or MA) that involve the conical capsid core, formed by the capsid protein (p24 or CA). Subsequently, the capsid core encompasses two RNA double strand molecules of approximately 9 Kb that are stabilized as a ribonucleoprotein complex. The ribonucleoprotein complex is formed by several copies of the nucleocapsid protein (p7 or NC) and by the viral enzymes integrase (p31 or IN), reverse transcriptase (p66/p51 or RT) and protease (p11 or PR). The viral proteins, negative regulatory factor (Nef), virion infectivity factor (Vif) and viral protein R (Vpr) is also incorporated in the viral particle (Turner and Summers, 1999, Hoffmann, 2007).

1.4 HIV-1 genome

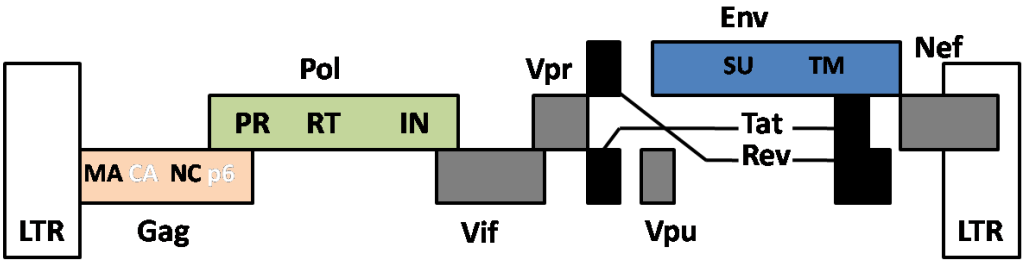


Figure 1.2 The HIV-1 genome adapted from (Freed, 2001)

HIV-1 carries its genetic information in double strand RNA molecules that are synthesized by the host DNA-dependent RNA polymerase II (RNAPol II). Similar to eukaryotic cellular mRNAs, HIV-1 RNA has post-translational modifications such as 5´ cap and 3´poly A tract (Whitcomb and Hughes, 1992). During the HIV-1 life cycle, RNA molecules are reverse transcribed by RT into viral complementary DNA (cDNA). This cDNA is then integrated into the host chromosome and remains part of the host genome as long as the cell survives. At this stage the viral genome is referred to as the provirus and relies on the host replication machinery for its replication (Coffin et al., 1997, Onafuwa-Nuga and Telesnitsky, 2009).

The HIV-1 genome, similar to all other replication competent retroviruses, has three major genes. The three major genes are *gag* (*group specific antigen polyprotein*, coding for structural proteins), *pol* (*polymerase,* coding for viral enzymes) and *env* (*envelope*, coding for envelope glycoproteins). They are flanked by long term repeats (LTR) sequences (Frankel and Young, 1998, Wang et al., 2000). Long term repeats (LTR) regions are composed by short directed repeat sequence (R) flanked by unique 5´ (U5) and 3´ (U3) sequences (U5-R-U3) (Whitcomb and Hughes, 1992). The LTR contains functional regions that are essential for HIV-1 transcription. These include the transactivation response element (TAR), the basal promoter and the core enhancer (Pereira et al., 2000). HIV-1 gene expression is mediated *via* numerous viral proteins through regulatory mechanisms that have overlapping reading frames and also have alternative messenger RNA (mRNA) splicing (Wang et al., 2000).

The *gag* gene encodes for a 55kDA (p55Gag) precursor protein (Porter et al., 1996). During virus maturation p55Gag is cleaved by the viral protease into the

structural proteins matrix, capsid and nucleocapsid, and into peptides p1, p2 and p6 (Freed, 1998). The *pol* gene codes for three enzymes namely protease, reverse transcriptase and intergrase (Scarlata and Carter, 2003). These enzymes are essential during the HIV-1 life cycle (Frankel and Young, 1998). These proteins originated from a fusion polyprotein precursor p160GagPol which is synthesized through a frameshift event that occurred during p55Gag translation (Doyon et al., 1998). The *env* gene encodes for the polyprotein Env precursor gp160 (Freed, 2001). This is cleaved by a cellular protease resulting in the surface (gp120) and the transmembrane (gp41) glycoproteins (Frankel and Young, 1998).

HIV-1 is different from other retroviruses as HIV-1 codes for six additional proteins (Scarlata and Carter, 2003, Stevenson, 2012). These are two regulatory proteins, transcriptional transactivator (Tat) and regulator of virus protein expression (Rev), and four accessory proteins Nef, Vif, Vpr and viral protein U (Vpu) (Frankel and Young, 1998, Stevenson, 2012). The regulatory proteins (Tat and Rev) are essential for virus replication (Dayton et al., 1986, Li et al., 2012). They control HIV-1 expression in host cells (Lopez-Huertas et al., 2010). The accessory proteins play major roles in virus persistence, spread pathogenesis *in vivo* and they carry out many important functions during the HIV-1 life cycle by interacting with cellular proteins (Li et al., 2005a).

Tat is a multifunctional protein and acts mainly as a transactivating protein (Frankel and Young, 1998, Li et al., 2012). It induces the expression of functional genes and acts together with Rev to stimulate the expression of structural genes (Ruben et al., 1989). Tat functions also include chromatin remodeling, induction of

phosphorylation of RNAPol II, transactivation of viral genes and binding to specific structures of HIV-1 mRNA (Romani et al., 2010). Rev is a regulator of viral mRNA production that binds to the rev responsive element (RRE) in the viral RNA (Malim et al., 1989, Suhasini and Reddy, 2009). It facilitates the nuclear export of the single spliced viral RNAs (Hope, 1999).

Vif plays an essential role in *in vivo* infectivity and pathogenesis and its main function is to counteract the host innate antiretroviral defence mediated by cytidine deaminases such as apoliprotein B mRNA editing enzyme, catalytic polypeptide like 3G (APOBEC3G) (Sheehy et al., 2002, Lackey et al., 2012) and the apoliprotein B-editing catalytic polypeptide 3F (APOBEC3F) (Liddament et al., 2004, Wiegand et al., 2004, Zheng et al., 2004, Albin et al., 2010) by different mechanisms (Henriet et al., 2009).

Nef, Vpr and Vpu act as multiple-functional proteins in regulating HIV-1 infectivity and pathogenesis (Subbramanian and Cohen, 1994). Nef induces downregulation of CD4, major histocompatibility complex class I (MHC I) and II (MHC II) and cluster differentiation 28 (CD28) from the surface of HIV-1-infected cells; enhances virion infectivity; stimulates viral replication; and modulates T cell activation state of its cell host (Jere et al., 2010, Landi et al., 2011). Vpr modulates transcription of the virus genome, promotes nuclear transport of the HIV-1 pre-integration complex (PIC), facilitates reverse transcription, causes G2 cell cycle arrest, induces apoptosis, induces defects in mitosis, suppresses immune activation and balances HIV-1 mutation rate (Romani and Engelbrecht, 2009, Romani et al., 2010). Vpu is responsible for CD4 degradation, induction of apoptosis, enhancement of viral

particle release and downregulation of MHC I and MHC II (Nomaguchi et al., 2008). Recently, it was reported that Vpu counteracts the cellular protein Tetherin, that specifically inhibits virion release from the host cells (Neil et al., 2008). .

1.5 HIV-1 life cycle

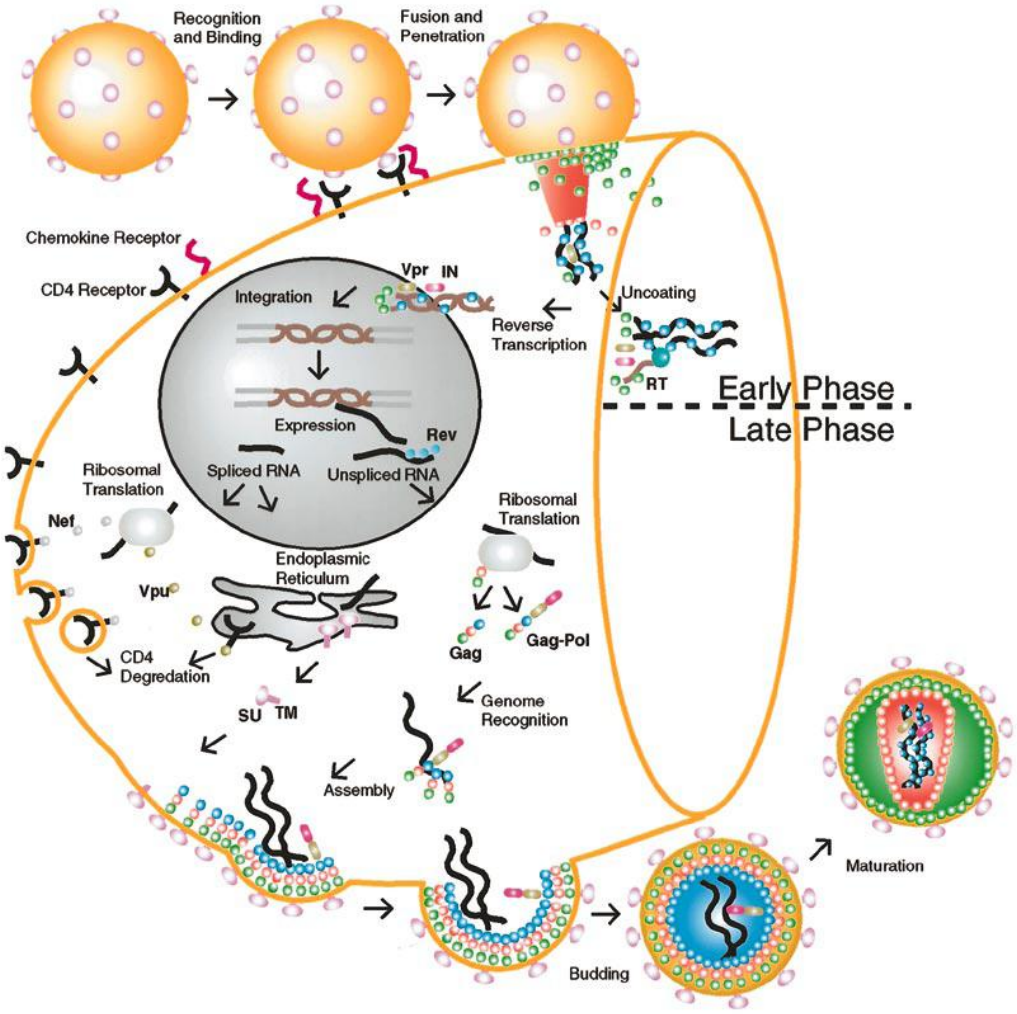


Figure 1.3 General features of the HIV-1 replication cycle. Adapted from (Turner and Summers, 1999).

The HIV-1 life cycle is similar to all other retroviruses and is divided into two distinct phases, an early and a late phase (Morrow et al., 1994, Murakami et al., 2004). The early phase begins with the recognition of the target cell by the infectious virion (Canki et al., 2001). It involves all the following steps until integration of the genomic DNA into the chromosome of the host cell (Li et al., 2001, Coffin et al., 1997). The late phase begins with the expression of the intergrated proviral genome (Greene and Peterlin, 2002). It involves all the processes up to and including virus budding and maturation of the progeny virions (Turner and Summers, 1999). All of these processes depend both on viral proteins and the host cell machinery which is exploited by the virus during its replication (Turner and Summers, 1999).

1.5.1 Early phase

1.5.1.1 Virus entry

The HIV-1 life cycle begins when an infectious particle comes into contact with the host cell (Shah et al., 2010, Hartman and Buckheit, 2012). The majority of the host cells that HIV infects are helper T cells, monocytes and macrophages (Vaishnav et al., 1991, Weiss, 2002, Shah et al., 2010). Other cells, such a Langerhans, follicular dendritic, glial and certain colon tumor cell lines are also susceptible to HIV-1 infection (Vaishnav et al., 1991, Weiss, 2002, Hartman and Buckheit, 2012).

When a helper T cell is infected by HIV, the virion attaches to cell surface through a high affinity interaction between the viral glycoprotein gp120 and the primary cell receptor CD4 (Dalgleish et al., 1984, McDougal et al., 1985). This

binding causes structural alterations in gp120 and enhances its affinity for a co-receptor which triggers the fusion of the viral envelope to the cellular membrane (Melikyan, 2008).

HIV-1 co-receptors belong to a G protein coupled receptor super family of seven transmembrane domain proteins (Berger et al., 1999). The two major co-receptors for HIV-1 infection *in vivo* are C-X-C chemokine receptor type 4 (CXCR4) (Feng et al., 1996) and C-C chemokine receptor type 5 (CCR5) (Alkhatib et al., 1996, Deng et al., 1996, Dragic et al., 1996).

CCR5 is the major receptor for primary HIV-1 infection, and CXCR4 emergers' later (Schramm et al., 2000). HIV-1 strains are classified based on the chemokine receptor being used (Hoffman et al., 2002). HIV isolates that use the CCR5 coreceptor are classified as R5 viruses and isolates that use the CXCR4 co-receptor are classified as X4 viruses and isolates that use both co-receptors are classified as R5X4 viruses (Berger et al., 1998). The co-receptor binding triggers conformational changes in gp120 and gp41 viral glycoproteins, namely the exposure of the gp41 fusion peptide and consequently insertion into the target cell membrane (Doms and Moore, 2000). This process leads to the fusion of the viral envelope with the target cell membrane and to the delivery of viral core into the cytoplasm of the target cell (Doms and Moore, 2000, McKnight et al., 2001, Murooka et al., 2012).

1.5.1.2 Post-entry: Uncoating and Reverse Transcription

Immediately after release of the viral core into the host cytoplasm, HIV-1 undergoes a process called uncoating, this is where the viral core is rearranged into a new structure known as the reverse transcription complex (RTC) (Lehmann-Che and

Saib, 2004). The RTCs are large dense complexes that contain viral RNA genome, proteins and the host cellular proteins. Viral proteins originate from viral core, like MA and RT; and several cellular host proteins that are necessary for reverse transcription, translocation and intergration processes, such as actin, barrier-to-autointegration factor (BAF) and lens epithelium-derived growth factor (LEDGF/p75) (McDonald et al., 2002, Warrilow and Harrich, 2007). Exposure of RTC to a significant concentration of deoxyribonucleotides in the cytoplasm is thought to trigger the initiation of reverse transcription (Lehmann-Che and Saib, 2004). Reverse transcription, although a very complex process, is very well regulated (Gotte et al., 1999).

During reverse transcription, the viral RNA genome is converted into a double-stranded DNA molecule, by the action of the viral heterodimer RT protein (p51/p66) (Held et al., 2006). The p66 subunit contains both Polymerase and RiboNuclease enzyme H (RNase H) enzyme activities (Andreola et al., 1992). The p66 subunit is essential for the reverse transcription process however the role of p51 is mainly structural in nature (Kohlstaedt et al., 1992). Reverse transcription begins with the binding of the cellular tRNA(Lys3) that is present in the RTC to the primer binding site (PBS) sequence of the viral RNA (Warrilow et al., 2009). This continues with a series of steps that involve several *cis* acting elements of the viral genome, deoxyribonucleotides addition and RT enzymatic activity (Miller et al., 1997). The newly synthesized viral DNA remains associated with viral and cellular proteins, leading to the maturation of RTC and generation of post-integration complex (PIC) (Miller et al., 1997, Gotte et al., 1999). PIC differs from RTC mainly by enclosing

viral cDNA instead of viral RNA, nevertheless loss of viral proteins, and reduction of capsid molecules are also observed (Warrilow et al., 2009).

1.5.1.3 Nucleus import and Integration

When the viral DNA is synthesized, HIV-1 has to deliver the recently retrotranscribed viral DNA that is enclosed in the PIC into the nuclease, crossing the nucleus membrane, in order to integrate the viral DNA into the host chromosome (Suzuki and Craigie, 2007). In the cytoplasm, the PIC was shown to connect with the cytoskeleton components that favour its proximity to the nuclear membrane (Bukrinskaya et al., 1998), however, the PIC nuclear import is an active process (Suzuki and Craigie, 2007). PIC has a Stoke diameter of 56nm which exceeds the central channel of the nuclear pore channel (NPC) of 25nm excluding passive diffusion as a viable mechanism (Miller et al., 1997, McDonald et al., 2002).

Some HIV-1 PIC proteins such as MA, IN, CA and Vpr have been identified as having karyophilic signals and are known to interact with cellular proteins such as Importin-α, Transportin 3, (TNPO3), Lens epithelium derived growth factor, (LEDGF/p75) (Maertens et al., 2004). These karyophilic signals and cellular proteins allow the direct transport across the intact nuclear envelope through the NPCs (Lehmann-Che and Saib, 2004, Suzuki and Craigie, 2007). Small viral DNA derived from reverse transcription, the DNA flaps, has also been implicated in PIC nuclear import acting as *cis* acting determinant of HIV-1 genome nuclear import (Zennou et al., 2000).

Following nuclear import of the PIC the viral DNA is covalently integrated into the host chromosome through the viral IN catylytic activity (Maertens et al., 2004,

Mousnier et al., 2004). This process occurs preferentially in transcription units that are transcriptionally active (Ciuffi and Bushman, 2006). IN catalyzes endonucleolytic cleavage of the viral genome extremities (Delelis et al., 2008). It cleaves the cellular target DNA and promotes a strand transfer reaction leading to the insertion of the viral DNA into the cellular target DNA (Van Maele and Debyser, 2005, Delelis et al., 2008). Integration is completed when the opposite short gaps in the complementary strands, resulting from the integration process, are repaired (Sakurai et al., 2009). This process is designated the postintegration repair and seems to be accomplished by host cellular proteins (Yoder and Bushman, 2000, Sakurai et al., 2009). Although IN is necessary and sufficient for all basic catalytic activities during the integration process, several cellular proteins have been implicated as important partners for IN and/or integration process (Van Maele and Debyser, 2005). After integration, the viral DNA, referred to as provirus, persists in the host cell and serves as template for the transcription of viral genes and replication of the viral genome (Freed, 2001).

1.5.2 Late phase

1.5.2.1 HIV-1 LTR Transcription

HIV-1 provirus may either remain latent (post-integration latency) or become transcriptionally active (Colin and Van Lint, 2009). Provirus acts as a template for the synthesis of viral RNA (Nazari and Joshi, 2009). It can be used as genomic RNA for virions progeny or can be translated into viral proteins (Nazari and Joshi, 2009). HIV-1 transcription is controlled by the inducible viral promoter located at U3 of 5´LTR (Sodroski et al., 1985).

HIV-1 LTR is a prototypic promoter of the cellular RNAPol II (Harrich et al., 1990, Marcello et al., 2004). The LTR enclosing a consensus TATA motif, where the host cell transcription factor II D (TFIID) binds; Sp1 and NFκB binding sites, that are recognized by endogenous host cell transcription factors and control LTR expression in the absence and in the presence of the transcription transactivator, Tat; and an additional DNA sequence-binding sites for several further cellular proteins (Garcia et al., 1989, Harrich et al., 1990, Steffy and Wong-Staal, 1991).

Tat protein acts as a transactivator of LTR by triggering viral transcription more than 100 fold greater than its basal expression (Bieniasz et al., 1999, Bres et al., 2002). Tat function is dependent of its binding to a stem-loop element of the nascent RNA transcript, TAR (for trans-activating response element) (Centlivre et al., 2008). TAR tethers Tat and brings it upstream of the promoter, endorsing TAT interaction to transcription factors and other cellular proteins (Steffy and Wong-Staal, 1991). Tat recruits the heterodimer positive transcription elongation factor b (P-TEFb) formed by cyclin T and cyclin-dependent kinase 9 (CDK9) proteins (Wei et al., 1998) leading to the phosphorylation of RNAPol II and consequently, to an increase in viral RNA transcription (Romano et al., 1999). The first rounds of proviral transcription and translation occur independently of Tat, resulting in basal amounts of Tat, Rev and Nef. Once sufficient amounts of Tat have been produced, Tat further controls transcription of HIV-1 genes (Sierra et al., 2005).

1.5.2.2 Gene expression, Assembly and Budding

Transcribed viral RNAs are divided into three groups according to their splicing processes (Houzet et al., 2007):

1) The unspliced RNAs that are the precursors for Gag and Gag-Pol polyproteins which can be used as genomic RNA that is subsequently packaged in progeny virions (Shehu-Xhilaga et al., 2001).
2) The partially spliced RNAs that encode Env, Vif, Vpu and Vpr (Asai et al., 2003).
3) The multiple spliced RNAs that are translated into Tat, Rev and Nef are expressed (Van Ryk and Venkatesan, 1999).

Initially, only multiple spliced RNAs are produced and the regulatory proteins Tat, Rev and Nef are expressed (Van Ryk and Venkatesan, 1999). When sufficient amounts of the Rev protein are produced, Rev binds to the *cis* acting RNA element, the Rev responsive element (RRE) of the unspliced or the single spliced RNAs, leading to the formation of a protein complex that interacts with the cellular nuclear export machinery, which consequently promotes RNA transportation out of the nucleus, where translation occurs (Wang et al., 2000, Freed, 2001).

The Env precursor polyprotein (gp160) is synthesized in the endoplasmic reticulum (ER) using the single spliced *env* mRNA (Bultmann et al., 2001). It then undergoes posttranslational modification in the ER and in the Golgi apparatus (Turner and Summers, 1999). During trafficking through the Golgi apparatus, gp160 is cleaved by the host cell protease generating the mature gp120 and gp41 proteins that subsequently form trimmer complexes (gp120-gp41) (Frankel and Young, 1998). These complexes are transported to the cell surface *via* the secretory pathway and gp41 protein anchors the complexes in the cell membrane for virus assembly (Turner and Summers, 1999).

Gag polyprotein is synthesized in the ribosomes from unspliced RNAs and a translation frameshift leads to the formation of smaller amounts of Gag-Pol precursor proteins (Turner and Summers, 1999). Gag polyprotein is responsible for the assembly of new immature viral particles (Wills and Craven, 1991). During assembly, Gag is moves to the plasma membrane (Ono and Freed, 2001). The anchored Gag leads to the induction of Gag multimerization and subsequently incorporation of the viral genomic RNA, Env glycoproteins and Gag-pol precursor into the viral particle (Wills and Craven, 1991). MA is responsible for the targeting of Gag to the plasma membrane and for the incorporation of gp120-gp41 complexes and Gag-Pol precursor into the viral particle, whereas C-terminal domain of CA (CA-CTD) and NC are responsible for Gag multimerization (Frankel and Young, 1998). In addition, NC due to its zinc-finger motifs, it also mediates the specific recognition of viral RNA genome for packaging into the virions (Ono, 2010) . Viral enzymes, accessory viral proteins, the cellular tRNAlys3 primer and cellular proteins also associate to the immature core for incorporation into the viral particle (Jewell and Mansky, 2000, Adamson and Freed, 2007, Bieniasz, 2009). Subsequently, the immature core associated to the plasma membrane buds through the plasma membrane. The viral P6 protein helps in this process through the recruitment of the endosomal sorting complex required for transport (ESCRT), facilitating fission of virions from the plasma membrane to the extracellular medium (Demirov and Freed, 2004, Morita and Sundquist, 2004).

1.5.2.3 Maturation

After the release of the immature budded virus from the host cell, Gag and Gag-Pol polyproteins are cleaved by the viral PR to produce independent enzymes, as

well as the MA, CA and NC structural proteins (Hill et al., 2005). The structural proteins rearrange *via* a process called maturation to form the infectious virus particle (Turner and Summers, 1999). Cleavage of Gag appears to occur *via* an ordered, sequential cleavage process that is controlled by different intrinsic proteolysis rates at the different cleavage sites (Wiegers et al., 1998). After maturation, HIV-1 life cycle is completed with the formation of newly infectious viral particles ready to infect a new host cell (Freed, 2001).

1.6 Cellular Co factors and HIV-1 replication

HIV-1 uses both its own cell machinery and the host cell machinery to replicate and also evade the host cellular antiviral responses to assure progeny spread (Sorin and Kalpana, 2006, Goff, 2007). Much interest in studying host cellular factors that could regulate HIV-1 replication started soon after the characterization of the virus (Goff, 2007). Many studies have shown that the interaction between the virus and host proteins is very tight, and it has been seen at every step of the HIV-1 life cycle (Sorin and Kalpana, 2006, Goff, 2007, Komano et al., 2005).

The HIV-1 and host interactions are complex, with an extensive list of interactions represented in the National Institute of Allergy and Infectious Diseases (NIAID) HIV Protein Interaction Database (Fu et al., 2009). Host proteins interact directly or indirectly with HIV-1 (Fu et al., 2009). Despite the many interactions so far demonstrated, it is believed that many cellular factors' interactions with HIV remain to be described or discovered (Chu et al., 2009, Goff, 2007). Cellular proteins can act as cellular co-factors to HIV-1 where these factors are needed for HIV-1 replication and for HIV-1 to complete its life cycle, or as HIV-1 restriction factors

where they have antiviral properties and inhibit HIV-1 replication at different steps of its life cycle (Sorin and Kalpana, 2006, Goff, 2007).

Understanding the complex interaction between HIV-1 and its host will provide new insights or better understanding of the mechanisms underlying the various steps of retroviral replication and this may lead to the development of novel antiviral therapeutic strategies (Arhel and Kirchhoff, 2010).

1.6.1 The role of cyclophilin A in HIV-1 replication

Among the most abundant cellular proteins related to HIV-1, and the first cellular protein ever found in HIV-1 virions, is the *cis*/*trans* peptidyl-prolyl isomerase (PPIase) cyclophilin A (CypA). CypA was found to be specifically incorporated into HIV-1 virions but not into virions of other retroviruses (Franke et al., 1994, Luban et al., 1993, Thali et al., 1994). Originally CypA was described as the binding partner of cyclosporine A (CsA), an immunosuppressive cyclic undecapeptide used clinically to suppress organ rejection after allograft transplantation. CypA was the first representative of the cyclophilins to be discovered, a family of enzymes that were later identified as PPIases and found in organisms ranging from prokaryotes to humans that catalyze the otherwise relatively slow *cis*/*trans*-interconversion of imidic bonds of proline residues *in vitro* (Handschumacher et al., 1984). Although CypA was shown to be required for numerous cellular functions (Ivery, 2000), it is dispensable for cell growth *in vitro*, as demonstrated by gene targeting of both *ppia*-alleles in Jurkat T-cells (Braaten and Luban, 2001) and is also not essential *in vivo*, as shown by targeted disruption of the CypA encoding *ppia*-gene in mice (Colgan et al., 2000).

CypA specifically binds to a proline rich sequence in CA proteins from HIV-1 and the closely related SIVcpz from chimpanzees (Franke et al., 1994, Luban et al., 1993, Thali et al., 1994, Braaten et al., 1996, Colgan et al., 1996, Yoo et al., 1997). Numerous possible functions of CypA in the HIV-1 replication cycle have been intensively investigated, but the biological function of this interaction still remains unclear (Luban, 2007, Sokolskaja and Luban, 2006). Blocking this interaction by CsA, RNAi (RNA interference), or mutation of the binding site in CA, reduced the virus titer produced from human cells, however, it releases HIV-1 from an antiviral restriction activity in cells from non human primates. Efforts to understand these effects of CypA finally led to the hypothesis that CypA modulates HIV-1 susceptibility to restriction factors expressed from the tripartite interaction motif (TRIM) 5α (Stremlau et al., 2004, Towers et al., 2003). The discovery of TRIM-CypA fusion proteins in the New World monkey species of owl monkeys and later in the two Old World monkeys, the long tailed macaques and pigtail macaques pointed towards a connection between both proteins (Sayah et al., 2004, Newman et al., 2008, Brennan et al., 2008). Indeed, TRIM5α mediated restriction in non human primates clearly is CypA dependent. However, in human cells TRIM5α and CypA independently regulate HIV-1 infection (Keckesova et al., 2006, Sokolskaja et al., 2006).

TRIM5α restricts HIV-1 by promoting premature disassembly of HIV-1 capsid while Cyclophilin A (CypA), a human protein that is incorporated into the HIV-1 virion increases viral infectivity by facilitating proper uncoating of HIV-1 capsid (Stremlau et al., 2006). CypA is a member of the cyclophilin family, members of which all possess peptidyl-prolyl cis/trans isomerase activity (Sherry et al., 1998,

Sokolskaja et al., 2004). Peptidyl prolyl cis/trans isomerases (PPIAses) catalyze the cis/trans isomerization of prolyl peptide bonds and are believed to be involved in protein folding (Schmid, 1995). The incorporation of CypA into the HIV-1 virion capsid is mediated through the direct binding between prolyl peptide bond located in a proline-rich loop of the fourth and fifth helices of the HIV-1 capsid and the active sites of CypA (Braaten et al., 1997).

CypA interacts with Gag in infected cells and this leads to its recruitment into HIV-1 virions (Thali et al., 1994, Franke et al., 1994). CypA also interacts with the core of HIV-1 in newly infected cells and this interaction is important for infectivity (Towers et al., 2003, Kootstra et al., 2003, Hatziioannou et al., 2005). CypA performs cis/trans isomerisation at CA G89-P90 on the outer surface of the capsid (Bosco et al., 2002, Bosco and Kern, 2004) and this leads to infectivity (Keckesova et al., 2006, Berthoux et al., 2005). Reducing CypA expression with small interfering RNA reduces susceptibility to HIV-1 (Keckesova et al., 2006, Sokolskaja et al., 2006). Infectivity to HIV-1 is also reduced by blocking CypA activity with cyclosporine A (CSA) (Keckesova et al., 2006, Sokolskaja et al., 2006). TRIM5α and CypA seem to have opposing effects on HIV-1 replication (Berthoux et al., 2005). Genetic variations in CypA, a host cellular factor, also affect the clinical outcome of HIV-1 infection (Yang and Aiken, 2007, An et al., 2007b, Bleiber et al., 2005).

1.6.2 Genome-wide screenings for identification of Cellular Co factors and Restriction factor for HIV-1

RNAi is a powerful technique for studying loss of function phenotypes by specific down-regulation of gene expression, allowing the evaluation of its function during HIV-1 infection. To date there have been four genome-wide RNAi screens performed to discover genes essential for HIV-1 replication (Brass et al., 2008, Konig et al., 2008, Zhou et al., 2008, Yeung et al., 2009). It was discovered from these screens that hundreds of human proteins were playing an important role for HIV-1 replication acting as cellular co-factors during HIV infection (Brass et al., 2008, Konig et al., 2008, Zhou et al., 2008, Yeung et al., 2009). However, when these studies were compared to each other, there was very little overlap in the results (Goff, 2008). Some of the reasons for the low overlap in the results could be as follows: different experimental design, the cell lines used and the criteria for analyzing data (Goff, 2008). Despite the low overlap between genes, their functional analysis indicated they belong to common pathways, like ubiquitination, SUMOylation, DNA repair, vesicular transport, and energy production (Goff, 2008, Kok et al., 2009).

Among the identified pathways from the screens, some pathways were already identified as being important for HIV replication, however there were also new unknown pathways that have not been described before (Kok et al., 2009). There have been novel pathways that are important in HIV replication that have been discovered using RNAi screens such as : MEDs, mediator complex that regulates transcription have been shown to be implicated in activation of HIV-1 LTR transcription, Golgi proteins were hypothesized as critically involved in the intracellular transport and

processing of viral nucleic acids and proteins; and many genes encoding proteins that are involved in mitochondrial function and energy production were also identified (Goff, 2008, Kok et al., 2009).

1.6.3 RNA Interference (RNAi)

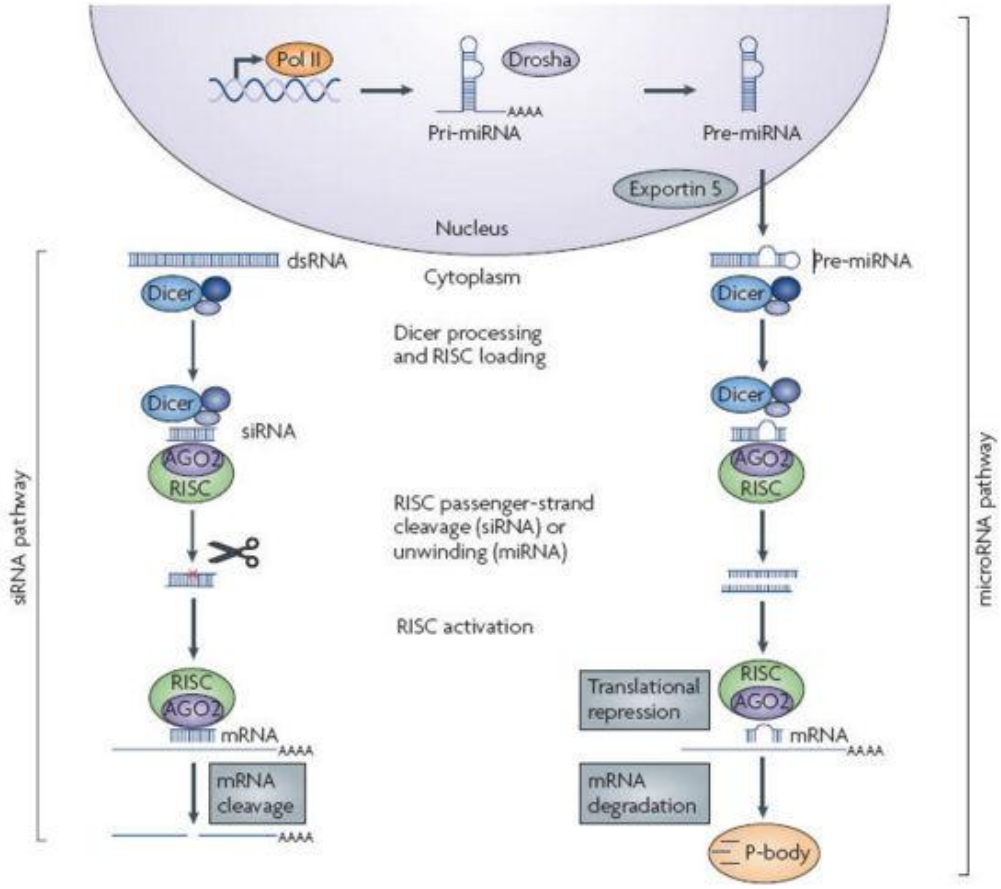


Figure 1.4 Mechanism of RNA interference in mammalian cells. Schematic representation of the RNAi pathway in mammalian cells, guided by small RNAs that include siRNA and miRNAs that lead ultimately to knockdown of the target gene, adapted from (de Fougerolles et al., 2007).

RNA interference (RNAi) was first observed in the late 1980s as an unknown phenomenon (Ecker and Davis, 1986). Fire and colleagues showed that exogenous double-stranded RNA (dsRNA) could induce potent and sequence-specific silencing of endogenous gene expression in *Caenorhabditis elegans* (Fire et al., 1998).

The same mechanism was shown to exist in mammalian cells by using a synthetic small interfering RNA (siRNA) that was able to knock down genes (Elbashir et al., 2001). RNAi was proven to be an evolutionarily conserved mechanism that leads to a sequence-specific gene silencing and is involved in processes as diverse as defence against viral infection, mobilization of transposable genetic elements, cell fate specification, and regulation of developmental timing (Rao and Sockanathan, 2005).

Initially, two types of naturally occurring small dsRNAs acting as gene silencers were described: the short interfering siRNAs (siRNAs) and the microRNAs (miRNAs) (Rao and Sockanathan, 2005). siRNAs derive from RNA long duplexes that are often produced during the course of viral infection within cells (foreign dsRNA) or by hybridization of overlapping transcripts from repetitive sequences in the genome, such as transposons or latent viruses (Rao and Sockanathan, 2005).

Each long dsRNA is cleaved by a ribonuclease III family (RNase III) enzyme known as a Dicer into 21-25 base pairs (bp) called siRNAs (Zamore et al., 2000). The siRNA has two nucleotides (nt) 3′ overhangs that allow them to be recognized by the enzymatic machinery of RNAi. The resulting small RNAs are incorporated into the RNAi-induced silencing complex (RISC), where Argonaute 2 (AGO2), a multifunctional protein contained within RISC, cleaves the passenger (sense) strand.

The activated RISC, which contains the antisense strand of the siRNA which is directed to the mRNA target, recognizes it and degrades the mRNA complementary to the antisense strand, cleavage carried out by the catalytic domain of AGO2 (Whitehead et al., 2009).

siRNAs are normally transfected in cells, activate RNAi machinery and effectively promote gene silencing (Leung and Whittaker, 2005). Although siRNAs were proven to be effective, their expression is transient and their effect is dependent on the number of siRNA molecules that are transfected and their persistence in the cell (Leung and Whittaker, 2005). One way to overcome this problem was the construction of short hairpin RNAs (shRNAs) expressed vectors. shRNAs construction was based on cellular miRNA and are constituted by perfectly double-stranded stems of 19–29 bp that have an identical sequence to the target mRNA. The two strands of the stem are connected by a loop of 6–9 bases, which is removed *in vivo* by Dicer to generate effective siRNAs (Paddison et al., 2002a, Paddison et al., 2002b). These shRNA vectors are plasmid-based shRNAs that have a constitutive efficient promoter, like U6 and a RNAPol III promoter that is active in all cell types and efficiently directs the synthesis of small, non-coding transcripts (Mittal, 2004).

Transfection can be easily performed with these plasmids and stable cell lines can be generated through a drug resistance marker selection. These vectors lead to an efficient and stable knockdown of target genes. Nevertheless, plasmid vectors also have limitations in terms of transfection efficiency. Consequently, retroviral, lentiviral and adenoviral vector systems for shRNA delivery were constructed, permitting the

efficient introduction and stable integration of these shRNA-expression cassettes into the host genome (Bos et al., 2009). Lentivirus-mediated delivery has advantages over the others systems once the virus can efficiently integrate into the genome of dividing and non-dividing cells, and transgenes carried by lentiviral vectors are resistant to silencing (Mittal, 2004).

The discovery of RNAi functionality in mammalian cells, all developments in "RNAi technology", together with the complete genome sequencing of a large number of organisms led to the construction of siRNA and shRNA libraries. These libraries allow the targeting of any gene of the studied genome and permit the development of genome-wide screens to evaluate individual gene function through loss-of-function analyses and interaction between genes and biological pathways (Cullen and Arndt, 2005, Root et al., 2006).

1.6.4 RNAi and HIV-1

The constant search for a cure to HIV-1 infection have intensified the hunt for new strategies to develop anti-HIV therapies (Qin et al., 2003). RNAi, is a very powerful candidate to overcome some viral infections including HIV-1 (Corbeau, 2008). RNAi can be used against HIV-1 using as target the HIV-1 provirus integrated in the host genome, the viral RNA transcripts, or cellular genes essential for HIV-1 replication (Singh and Gaur, 2009, Soejitno et al., 2009).

Several studies were performed by targeting HIV-1 genes, involved in early and late infection. Effective siRNAs were developed against *gag* (Novina et al., 2002, Chang et al., 2005, Lee et al., 2005, Morris et al., 2005, Song et al., 2005d, Cave et al., 2006), *pol* (Chang et al., 2005, Morris et al., 2005, Lau et al., 2007), *vif* (Barnor et al.,

2005, Lee et al., 2005), *tat* (Coburn and Cullen, 2002, Lee et al., 2003, Dave and Pomerantz, 2004, Li et al., 2005b), *rev* (Coburn and Cullen, 2002, Lee et al., 2002, Dave and Pomerantz, 2004, Lee et al., 2005, Li et al., 2005b), *env* (Park et al., 2003, Hayafune et al., 2006a, Hayafune et al., 2006b) and *nef* (Dave and Pomerantz, 2004, Chang et al., 2005, Yamamoto et al., 2006). Viral RNA was also targeted with RNAi by targeting LTR promoter or by inducing transcriptional gene silencing, through TAR inhibition (Barnor et al., 2005, Puerta-Fernandez et al., 2005, Barichievy et al., 2007, Christensen et al., 2007).

Alternatively, anti-HIV strategies with RNAi were constructed to inhibit host genes that have an essential role during HIV-1 replication. siRNAs targeting the receptor CD4 (Novina et al., 2002) and the co-receptors CXCR4 and CCR5 (Martinez et al., 2002, Lee et al., 2003, Qin et al., 2003, An et al., 2007a) have been developed. Host factors that are important for HIV-1 LTR driven transcription were also targeted and it was demonstrated that its knockdown led to an efficient inhibition of HIV-1 replication (Brass et al., 2008). Other proteins that influence HIV-1 replication, like actin related protein (ARP) 2/3 complex (Komano et al., 2004), PARP1 (Kameoka et al., 2004), P-TEFb (Chiu et al., 2004) and TSG101 (Garrus et al., 2001) were also used for RNAi targeting, having an effect during HIV-1 infection.

The RNAi engineering seems to be effective against HIV-1, however it has some drawbacks. Indeed, although siRNAs have a smaller size then dsRNA, several studies have indicated that siRNA and shRNA expressed by vectors can trigger the interferon pathway, triggering host innate immunity (Robbins et al., 2009). Another concern is the propensity of HIV-1 to emerge with rapid mutation, leading to RNAi

resistance (Westerhout et al., 2005). The use of cellular factors overcomes this problem (Goff, 2008). Nevertheless, cellular target selection has to be made carefully, taking into account that RNAi effect can affect both virus and cell host (Yeung et al., 2009). To counteract these weaknesses co-expression of multiple siRNAs targetting different HIV-1 RNA sequences or HIV-1 and cellular RNA sequences was used and combinatory shRNA strategies were developed with the construction of long hairpin RNAs (lhRNA). These RNAi molecules are transcribed by RNAPol III and are processed by Dicer into multiple siRNAs (Saayman et al., Konstantinova et al., 2006).

Other RNAi molecules that can be efficient against HIV-1 are cellular and native miRNAs. Cellular miRNAs have already been described to target HIV-1, namely against Nef (Ahluwalia et al., 2008). Cellular miRNA against viral proteins together with miRNAs that target cellular genes essential for HIV-1 replication could also be used for an antiviral strategy (Corbeau, 2008). Despite the drawbacks, RNAi against HIV-1 can be used in therapeutics against HIV-1 and it seems a very promising strategy (Sibley et al., 2010). The continuous development of this technology could lead to future treatments for HIV-1 infection. Beyond the potential of the RNAi as a therapeutic approach to combat HIV-1, this mechanism is also very powerful to study HIV-1 replication, viral proteins function and HIV-host interaction (Sibley et al., 2010, Lenz, 2005). The development of artificial siRNA/shRNA against viral and cellular proteins as described above has helped to identify the importance of those proteins in HIV-1 life cycle. Nevertheless, maybe the most striking utility of RNAi as a tool for loss-of-function studies in HIV-1 was the construction of the siRNA and shRNA libraries that permitted the emergence of RNAi screens to identify host proteins

required for HIV-1 replication. siRNA and shRNAs genome wide screens were performed (Brass et al., 2008, Konig et al., 2008, Zhou et al., 2008, Yeung et al., 2009) and hundreds of new human genes were identified as important for HIV-1 replication (Goff, 2008). This screening approach expanded enormously the field of HIV-host interaction and provided new hints for cellular pathways involved in HIV-1 replication (Brass et al., 2008, Konig et al., 2008, Zhou et al., 2008, Yeung et al., 2009).

1.7 Interferons

Interferons (IFNs) represent a large family of cytokines that contribute to antiviral, antitumor and immunomodulatory activities (Stark et al., 1998, MacMicking, 2012). IFNs, classified as α, β, γ and ώ on the basis of their structure and antigenic properties, are grouped into two families; type I and type II IFNs (Samuel, 2001). IFN α, β, and ώ, which belong to type I family, are produced by most cell types in response to stimuli like viral infection or double-stranded RNA (Stark et al., 1998).

IFN-γ is the only member of the type II family and is secreted by T lymphocytes and NK cells in response to specific antigens or mitogens (Pfeffer et al., 1998). Type I and II IFNs interact with cell surface receptors, resulting in the activation of specific Janus protein kinase (JAK)/signal transducers and activators of transcription (STAT) pathways, leading to the transcription of a distinct set of genes that mediate the biological responses of these cytokines (Hervas-Stubbs et al.). IFN-stimulated response elements (ISREs) drive the expression of most IFN α/ β regulated genes and a few IFNγ regulated genes (Stark et al., 1998).

IFNs are essential for the survival of higher vertebrates because they provide an early line of defense against viral infections, hours to days before immune

responses. The best-characterized IFN-induced antiviral pathways are the dsRNA-dependent protein kinase (PKR), the 2-5A system, and the Mx proteins (Barber, 2001). Any stage in viral replication appears to be a point of attack for inhibition by IFN-regulated pathways; entry, uncoating, RNA stability, initiation of translation, maturation or assembly and release (Barber, 2001).

IFNs inhibit cell growth and control apoptosis, activities that are important for the suppression of cancer and infection (Stark et al., 1998). Cells in culture exhibit varying degrees of sensitivity to the antiproliferative activity of IFNs. Growth arrest may be due to differentiation, particularly when IFNs are used in combination with other agents, i.e. retinoids (Higuchi et al., 1991, Nason-Burchenal et al., 1996). IFN-α has been shown to target specific components of the cell-cycle control, including c-myc, pRb, cyclin D3 and cdc25A (Stark et al., 1998).

1.8 HIV-exposed seronegative (HESN): CSM (Commercial Sex Workers)

Highly exposed persistently seronegative cohorts, which include individuals exposed to HIV-1 to such an extent that they can be classified as resistant according to epidemiologic models, consist almost exclusively of CSW cohorts in Africa and Asia (Horton et al., 2010). The Pumwani CSW cohort in Nairobi, Kenya was the first described and most comprehensively followed cohort (Fowke et al., 1996). Although participants had counseling and condoms where made available, these women have a high frequency of unprotected sex, with as many as 15 clients per day (Lau et al., 2008, Luchters et al., 2008). Such cohorts can be a key resource for research into preventing infection in women (Horton et al., 2010).

HIV-exposed seronegative (HESN) individuals have been shown to immunological advantages against HIV and provide clues for protective mechanisms (Burgener et al., 2012). Numerous studies have shown that they have unique adaptive and innate mucosal immune responses, including HIV-specific cellular $CD4^+$, $CD8^+$ immune responses and HIV-neutralizing IgA, as well as elevated RANTES and other CC-chemokines (Kaul et al., 1999, Kaul et al., 2000, Levinson et al., 2009, Hirbod et al., 2008).

There is still no clear understanding why some individuals seem to have reduced susceptibility to HIV infection (Burgener et al., 2012). The only validated exception is the finding that homozygosity for a CCR5 Delta-32 mutation which confers protection against sexual transmission of HIV, although new genetic determinants are being discovered (Liu et al., 1996, Limou et al., 2012).

1.9 Genetic polymorphisms that restrict HIV

In addition to the conventional innate and acquire immune responses, mammalians have evolved mechanisms to efficiently counteract retroviral infections (Bieniasz, 2004). This phenomenon is mainly due to genetic factors, mainly polymorphisms in host responsive genes or in the receptors of these genes, or to inhibitory gene products that when expressed in the host cell prevent the progression of particular steps of the retrovirus life cycle (Rowland-Jones et al., 2001, Goff, 2004b).

Genetic outcomes of HIV-1 infection are well recognized and can indicate potential mechanisms in the pathogenesis of infection. One of the best studied

polymorphism or missence varriant is in the CCR5 chemokine-receptor gene (Dean et al., 1996, Liu et al., 1996, Samson et al., 1996). The CCR5Δ32 allele expresses a truncated protein that is no longer transported to the cell surface. Therefore, homozygotes for this polymorphism are resistant to HIV-1 infection (Benkirane et al., 1997).

Moreover, many other genetic polymorphisms have been described to affect susceptibility to HIV infection (Lama and Planelles, 2007). The cellular proteins named host restriction factors function as inhibitors of viral replication and are constitutively expressed or enhanced by interferons (Neil and Bieniasz, 2009). Although restriction factors can be enhanced by viral infection, they do not require virus-triggered signaling (Bieniasz, 2004, Neil and Bieniasz, 2009). These proteins are active in the host cell and are an important part of the innate immunity against viral infection (Bieniasz, 2004). Nevertheless, as host cells have evolved to block viral infection, retroviruses have co-evolved to counteract these restrictions, with several viral proteins now described that can efficiently neutralize the inhibitory effect of some restriction factors (Strebel et al., 2009).

Evolution is an ongoing process and it is also driven by gene duplication. Many TRIM genes also have shown to undergo extensive gene duplication in both primates and teleost fish (Han et al., 2011). The *TRIM5/6/22/34* gene cluster likely arose through tandem gene duplication, as these four *TRIM* genes are close human paralogs and because major gene rearrangements have been documented in this chromosomal region (Han et al., 2011, Sawyer et al., 2007). Apobec genes have arisen

through gene duplication and their number varies from one gene in mice to seven genes in humans (LaRue et al., 2009).

1.10 Host restriction factors that are part of the innate/intrinsic immunity

Host Restriction factors

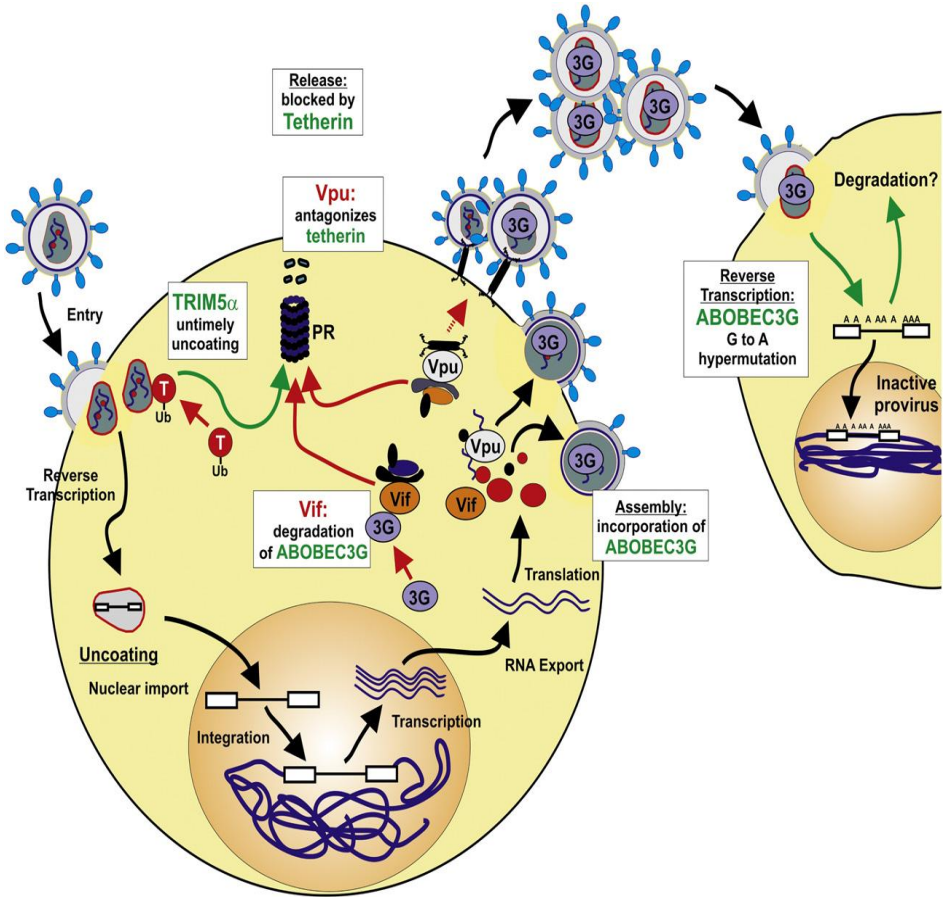


Figure 1.5. Intrinsic host restriction factors and their viral antagonists. Adapted from (Arhel and Kirchhoff, 2010).

1.10.1 Friend Virus susceptibility-1 protein (Fv1)

The friend virus susceptibility 1 protein (Fv1) was one of the first studied retrovirus restriction factors (Hartley et al., 1970). The *Fv1* gene is naturally encoded in the mouse and its expression promotes mice resistance to murine leukemia virus (MLV) infection (Lilly, 1967). The *Fv1* gene has two different alleles, *Fv1n* and *Fv1b* and they confer different MLV tropism (Hatziioannou et al., 2004a). *Fv1n* allows replication of N-tropic strains of MuLV and blocks B-tropic MuLV viruses (Jolicoeur and Baltimore, 1976). The *Fvb* allele permits replication of B-tropic viruses and blocks N-tropic viruses (Goff, 2004a). *Fv1n/b* heterozygotes animals are resistant to both N- and B-tropic virus (Hatziioannou et al., 2004a). The amino acid 110 of the CA domain in *gag* gene has been shown to be responsible for MLV virus tropism, with an arginine specifying the N-tropism and a glutamate the B-tropism (Kozak and Chakraborti, 1996). The *Fv1* restriction mechanism is thought to occur after reverse transcription, where *Fv1* detects the CA protein of the incoming PIC and blocks virus replication (Goff, 2004a).

1.10.2 APOBEC family

A very important group of restriction factors are included in Apoliprotein B-editing catalytic polypeptide-like (APOBEC) family of cytidine deaminases, APOBEC3G being the most studied in the HIV-1 context (Conticello et al., 2005, Albin and Harris, 2010). APOBEC3G is a strong inhibitor of HIV-1 and other retroviruses infections, acting after uncoating of the viral capsid, during reverse transcription, and is counteracted by the viral Vif protein (Sheehy et al., 2002). In the

absence of Vif, APOBEC3G can be incorporated into HIV-1 particles in the producer cell (Bishop et al., 2008). Upon infecting a new cell, during reverse transcription, APOBEC3G deaminates the cytosine bases of the viral minus-strand DNA to uracil resulting in G to A hypermutations in the complementary positive sense DNA strand (Mangeat et al., 2003, Mariani et al., 2003, Zhang et al., 2003, Harris, 2008).

These U-rich transcripts are either degraded by activation of the cellular uracil-DNA-glycosylase causing the failure of reverse transcription or yields proviruses that are largely non-functional due to G-to-A hypermutation (Goff, 2003, Harris, 2008). HIV-1 Vif protein counteracts APOBEC3G by blocking its incorporation into the viral particles (Mariani et al., 2003, Stopak et al., 2003). Vif interacts directly with APOBEC3G and *via* a conserved SOCS box-like sequence, recruits a RING-finger E3 ubiquitin complex containing elongin B and C, cullin 5, and RING-box protein 1 (Rbx1), inducing the poly-ubiquitination of APOBEC3G and subsequently, its degradation by the proteasome machinery (Conticello et al., 2003, Marin et al., 2003, Sheehy et al., 2003, Stopak et al., 2003, Mehle et al., 2004, Yu et al., 2004b). Vif also impairs APOBEC3G mRNA translation by a not well-understood mechanism (Stopak et al., 2003, Mercenne et al., 2010). APOBEC3F, other protein from APOBEC family has been also demonstrated to be an efficient inhibitor of HIV-1 in human cells (Liddament et al., 2004, Wiegand et al., 2004, Zheng et al., 2004, Albin et al., 2010). Reddy and colleagues showed in a South African cohort infected with subtype C HIV-1 those genetic polymorphisms of the APOBEC3G gene affect HIV-1 disease progression. They showed that the H186R missence variant, a codon-changing variant in exon 4, and a 30 extragenic mutation (rs35228531) were associated with

high viral loads (P=0.0097 and P<0.0001) and decreased $CD4^+$ T-cell levels (P=0.0081 and P<0.0001), respectively(Reddy et al., 2010). An and colleagues reported that a genetic variant of APOBEC3G, the H186R mutation, is associated with an AIDS-accelerating effect in African–Americans infected with HIV-1B (An et al., 2004)

APOBEC3F is also efficiently inhibited by HIV-1 Vif in a similar mode as APOBEC3G, excluding it from the viral particles (Liddament et al., 2004, Wiegand et al., 2004, Zheng et al., 2004, Liu et al., 2005, Albin et al., 2010). Other members of the APOBEC family, such as Apoliprotein B mRNA editing enzyme, catalytic polypeptide-like 3B (APOBEC3B) and 3C (APOBEC3C) have also been shown to have a potent antiviral activity against simian immunodeficiency virus (SIV), although not efficient against HIV-1 (Yu et al., 2004a, Lackey et al., 2012).

1.10.3 Tetherin

Tetherin also known as BST-2, CD317 or HM1.24 is a potent antiviral factor that inhibits HIV-1 replication (Neil et al., 2008, Van Damme et al., 2008). Interferon-α induces Tetherin expression in T cells (Neil et al., 2007, Le Tortorec et al., 2011). Tetherin inhibits the release of newly formed virions from the host cell surface by binding to the cell surface at the site near to were the virions bud (Neil et al., 2008, Van Damme et al., 2008). The mechanism of this restriction is still unclear (Neil et al., 2007). It was recently discovered that tetherin associates with a RING-type E3 ubiquitin ligase breast cancer associated gene 2 (BCA2) that can accelerate internalization and degradation of tethered HIV-1 virions (Miyakawa et al., 2009). However, it is not yet understood if the virions hijacked by tetherin are generally

degraded or remain intact within the cell (Mangeat et al., 2009). Retroviruses have also evolved to counteract the action of tetherin (Sauter et al., 2009).

Specifically, HIV-1 counteracts tetherin action through the viral Vpu protein. Vpu interacts directly with the transmembrane domain of tetherin, promoting the reduction of tetherin levels at the cell surface and a decrease of the total amount of this protein in the cell (Van Damme et al., 2008, Neil et al., 2008, Le Tortorec et al., 2011). In the absence of Vpu expression, cell-free HIV-1 particles are poorly released from CD4+ T cells and macrophages, and mature virions accumulate on the cell surface and in vacuolar structures (Klimkait et al., 1990, Gottlinger et al., 1993). Although the exact mechanism of tetherin down-modulation is not unclear, it has been hypothesized that Vpu targets tetherin to the trans-Golgi network or to the early endosomes for proteosomal or lysosomal degradation (Douglas et al., 2009, Goffinet et al., 2009, Mangeat et al., 2009, Mitchell et al., 2009).

Similarly to other restriction factors, tetherin antiviral activity is also effective against other retroviruses (Neil et al., 2007, Le Tortorec et al., 2011). The antiviral mechanism seems to be common but the viral counteracting protein differs. Most primate lentiviruses do not contain the *vpu* gene and use Nef protein to antagonize tetherin, in a similar way as Vpu (Jia et al., 2009, Sauter et al., 2009, Zhang et al., 2009). HIV-2 uses Env protein to counteract tetherin by sequestering it away from virus assembly sites (Le Tortorec and Neil, 2009).

1.10.4 Tripartite Motif Family

Many mammalian genes were demonstrated to have restriction activities against retrovirus capsids (Pertel et al., 2011). Members of the tripartite motif (TRIM) family of proteins have been described to have a restriction activity against several retroviruses (Nisole et al., 2005). There are 70 members in this family (Uchil et al., 2008). TRIM proteins are composed of a RING finger, B-box, and coiled-coil domains (Nisole et al., 2005). The most studied member of this family is the tripartite motif 5-alpha (TRIM5α) which was demonstrated to be an important restriction factor in mammals blocking infection by retroviruses in a species-specific manner (Luban, 2007, Towers, 2007, Stremlau et al., 2004).

1.10.4.1 Tripartite Motif Family: Structure

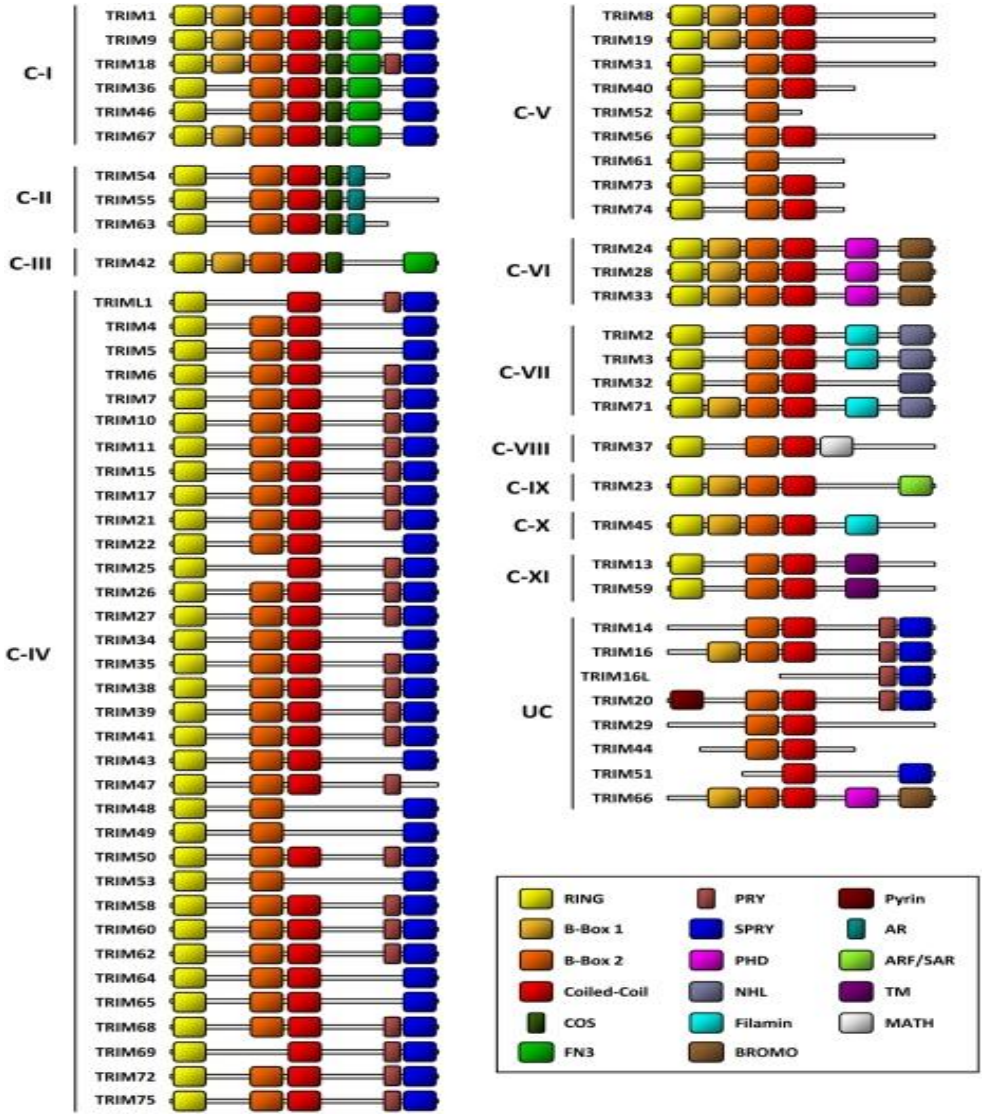


Figure 1.6 Human TRIM proteins: The TRIM protein family is composed of 11 sub-families, from C-I to C-XI, whereas some TRIM proteins remain unclassified (UC), since they do not have a RING finger domain as ‘‘true’’ TRIM proteins. Adapted from (Carthagena et al., 2009)

The Tripartite motif (TRIM) family is made up of a wide variety of proteins that have diverse functions that are only now starting to be elucidated (Meroni and Diez-Roux, 2005, Nisole et al., 2005). The TRIM family of genes is characterized by the presence of a RING domain, followed by one or two B-Box domains, and a stretch of amino acids that form the coiled coil structures, and a sequence called the RBCC or tripartite motif (Ohkura et al., 2006). The C2 RING domain that is found on TRIM proteins employs a "cross brace" system of cysteins and histidines to coordinate two Zinc ions (Freemont, 1993). The C2 RING domain has been described to be associated with ubiquitination (Meroni and Diez-Roux, 2005), TRIM proteins have E3 ubiquitin ligase activity (Uchil et al., 2008). The RING domain is defined by a specialized zinc finger of 40-60 amino acid residues that binds two zinc atoms and seems to be involved in protein-protein interactions (Nisole et al., 2005, Borden, 2000). RING finger domains have been found to play an essential role in mediating the transfer of ubiquitin to substrates. This domain is therefore a characteristic feature of many E3 ubiquitin ligases (Joazeiro and Weissman, 2000). In the TRIM family ubiquitin ligase activity has been shown for the TRIM5 delta isoform, TRIM18/MID1, TRIM25/EPF, TRIM32/HT2A and TRIM35/ARD1 (Meroni and Diez-Roux, 2005).

Characteristic B-boxes (B1 and B2) with two zinc-finger motifs each follow the RING domain (Short and Cox, 2006). B boxes are found exclusively in TRIM proteins and are perhaps a critical determinant of the tripartite motif (Reymond et al., 2001). If both B-boxes are present, B1 always precedes B2, but if only one is present it is always of the B2 variety (Sardiello et al., 2008). So far no function has been clearly assigned to these domains (Reymond et al., 2001). The B-Box domains have

been suggested to function as Zinc ion coordinators, which have been confirmed by structural NMR studies (Borden et al., 1993, Borden et al., 1995, Massiah et al., 2006). There are two classes of B-box domains, B-box1 and B-box2 (Massiah et al., 2006). B-box1 adopts the "cross brace" system which is employed by the RING domain and is able to chelate two Zinc ions (Nisole et al., 2005). The B-box2 can only chelate one Zinc ion (Torok and Etkin, 2001). TRIM protein B-Box domains show very strong adherence to their consensus sequence and TRIM proteins (Borden et al., 1993, Borden et al., 1995, Massiah et al., 2006).

The greatest variability from the conserved tripartite motif is seen in the coiled-coil structure (Li et al., 2006a). The coiled-coil structure is often composed of two or three smaller coiled-coil regions (Meroni and Diez-Roux, 2005). The region does not show any strong consensus except for hydrophobic residues such as leucine in areas to facilitate the "knobs in holes" coiled-coil stacking structures, suggesting that the coiled-coil structures are used for homo-oligomerization (Meroni and Diez-Roux, 2005, Reymond et al., 2001). It has also been suggested that cellular localization is disrupted by the disruption of the coiled coil region (Reymond et al., 2001). The coiled-coil domain has the capacity to merge with other coiled-coils and by these means mediate homo- and hetero- interactions (Peng et al., 2002). The homo interactions are much more common than the hetero interactions among the TRIM family members (Meroni and Diez-Roux, 2005). This tendency for homo interactions stimulates the formation of TRIM high-molecular-weight complexes (Massiah et al., 2006). These are compartmentalized either in the nucleus or the cytoplasm, suggesting that TRIM proteins might define subcellular compartments (Reymond et al., 2001).

Song and collogues showed that many primate species shows diffuse cytoplasmic staining of TRIM5α, however TRIM5α from spider monkeys did not form cytoplasmic bodies and all TRIM5α screened restricted retroviruses such as SIV_{mac}, SIV_{agm} and HIV-1 (Song et al., 2005a)

The amino acids in the C-terminal RBCC motif show great diversity among the family members and even between splice variants of the same gene (Nisole et al., 2005). This suggests that the conserved RBCC motif provides a specific functional role which is targeted by the C-terminus (Meroni and Diez-Roux, 2005, Nisole et al., 2005). Despite the great variation in C-termini composition, the C-termini of the various TRIM proteins do show certain frequent motifs (Ohmine et al., 2011). One of the most common is the B30.2/SPRY domain which are found in almost two-thirds of the known TRIM species (Song et al., 2005b).

The B30.2 domain has been identified in 11 protein families and has been shown to be involved in for example developmental processes and the immune defense (Rhodes et al., 2005, Bradley and Pober, 2001). Short and Cox subclassified the TRIM/RBCC family on the basis of their varied C-terminal domain compositions and described a novel motif, COS box, which was shown to be necessary for microtubule binding, suggesting the involvement of the TRIM/RBCC family in subcellular compartments (Short and Cox, 2006).

1.10.4.2 TRIM/RBCC proteins as E3 ubiquitin ligases

TRIM proteins are involved in many diverse cellular processes such as antiviral response and cell differentiation, their conserved structure may suggest a common biochemical function (Meroni and Diez-Roux, 2005). There is a strong relationship

between the presence of a RING domain and ubiquitin ligase activity (Pickart, 2001). The covalent attachment of ubiquitin to selected cellular proteins is used by eukaryotic cells to control different cellular functions, the most well known being proteasome mediated degradation (Yang and Yu, 2003).

The ubiquitination process involves at least three classes of enzymes, and begins with the formation of a thiol-ester linkage between the C-terminus of Ubq and the active site cysteine (Cys) of the Ubq activating enzyme (E1) (Deshaies and Joazeiro, 2009). Ubq is then transferred to an Ubq conjugating enzyme (Ubc or E2), after which the complex is transferred to the E3 ligase (Glickman and Ciechanover, 2002, Deshaies and Joazeiro, 2009). The E3 ligase interacts with E2 and substrate and mediates formation of isopeptide bonds between the C terminus of Ubq and lysines on the target protein (Glickman and Ciechanover, 2002). E3 ligases are considered to be the primary specificity determining factors in ubiquitination, since they can specifically recognize different substrates (Deshaies and Joazeiro, 2009). Many proteins are regulated by ubiquitination which demands high specificity and accounts for the growing number of discovered E3 ligases (Yang and Yu, 2003, Schwartz and Hochstrasser, 2003). However, there are only a few E1s but at least 20 E2s known to date (Yang and Yu, 2003, Schwartz and Hochstrasser, 2003). Ubiquitination affects the function and location of targeted proteins either through monoubiquitination (regulating for example of endocytic processes, chromatin remodelling and budding of retroviruses from plasma membrane) or polyubiquitination (regulating proteasomal degradation) (Hicke, 2001).

The RING domain can be one of the responsible domains for E3 ligase activity (Orimo et al., 2000). The strong relationship between the RING domain and ubiquitination has led to the suggestion that the TRIM/RBCC family represents a subclass of RING finger E3 ligases (Meroni and Diez-Roux, 2005). Some of the family members possess E3 ubiquitin ligase activity (Meroni and Diez-Roux, 2005). TRIM25/EFP has been shown to regulate the protein levels of the cell cycle regulator 14-3-3-δ (Urano et al., 2002). TRIM33/Ecto modulates the TGFβ signaling pathway by ubiquitination of Smad4 (Dupont et al., 2005).

In addition, the nature of the modifier does not have to be the classical ubiquitin peptide (Deshaies and Joazeiro, 2009). A growing number of ubiquitin-like (UBL) peptides have been discovered and found to determine different cellular events, i.e. SUMO, Nedd8 and ISG15 (Schwartz and Hochstrasser, 2003, McNab et al., 2011).

Bell and colleagues demonstrated that TRIM16 which does not have a classic ring domain exhibits E3 ligase activity via the B-boxes (Bell et al., 2012). They also found that TRIM16 can homo and hetero-dimerize, a common feature found in other TRIMs (Bell et al., 2012). Similar to other TRIM family members, TRIM38 is localized in the cytoplasm TRIM38 increases ubiquitination of other cellular proteins and catalyzes self-ubiquitination(Liu et al., 2011). TRIM38 has E3 ubiquitin ligase activity and can be degraded during virus infection (Liu et al., 2011). Duan and colleagues demonstrated that TRIM22 underwent self-ubiq-uitination in a RING finger domain-dependent manner in vitro and in vivo, indicating that TRIM22 may function as a RING finger E3 ligase (Duan et al., 2008).

1.10.4.3 Immunological Functions of TRIMs

A large number of TRIM proteins have diverse immunological functions attributed to them (Nisole et al., 2005). Ubiquitin-mediated degradation and ubiquitinmediated activation play key steps in many immune pathways (Chen, 2005).

TRIM19 or Promyelocytic Leukemia protein (PML) an interferon stimulatory gene (ISG) is one of the most studied TRIMs for its immunological characteristics (Stadler et al., 1995). Many studies have shown that TRIM19 expression modulates the susceptibility of a cell to various retroviruses (Nisole et al., 2005). The exact role of TRIM19 functioning as an antiviral agent has not been completely elucidated however TRIM19's SUMOyalation targets the protein to nuclear bodies and induces its degradation (Zhu et al., 2002). Its disruption appears to abrogate TRIM19's antiviral effects (Zhu et al., 2002, Van Damme and Van Ostade, 2011).

TRIM5α has the ability to mediate "Lv1 restriction" of a number of viral strains including HIV-1 in certain primate species (Stremlau et al., 2005). This viral restriction is believed to be mediated through the viral capsid protein and the C-terminal SPRY domain in TRIM5α (Yap et al., 2005). Many other TRIMs exhibit viral restriction (Nisole et al., 2005). TRIM1 restricts murine leukemia virus (MLV), TRIM32 and TRIM22 can mediate transcriptional repression of HIV-1 genes by interacting with the Tat proteins and HIV-1's long terminal repeats (Bouazzaoui et al., 2006, Tissot and Mechti, 1995, Yap et al., 2004).

TRIM proteins have been shown to play many other immunological roles apart from direct viral restriction (Uchil et al., 2008). Some TRIM proteins have been shown to reduce immune signaling (Liu et al.). TRIM45 has been shown to repress the

transcriptional activity of AP-1 (Wang et al., 2004). TRIM27 was shown to interact with the repressing activities of IKKα, IKKβ, IKKε, and TBK1 (Zha et al., 2006).

Many TRIMs enhance the immune response. TRIM8 has been shown to ubiquitinate and degrade the protein Suppressor of Cytokine Signaling-1 (SOCS-1), which is responsible for shutting down IFN signaling (Toniato et al., 2002). TRIM25 has been shown *in-vitro* to mediate the covalent attachment of ISG15, an IFN stimulated ubiquitin like molecule, to various substrates (Zou and Zhang, 2006, Zou et al., 2007). Gack and colleagues linked TRIM25 to the viral detection pathway mediated by the RNA helicase RIG-I (Gack et al., 2007). The study demonstrated that the C-terminal SPRY domain of TRIM25 can directly interact with the CARD domains of RIG-I which mediate RIGI's interaction with mitochondrial antiviral signaling protein (MAVS). Following this interaction, TRIM25 ubiquitinates RIG-I which then increases its downstream signaling activity vis-a-vis MAVS as shown by the greatly compromised ability of cells to fight off viral infection and produce IFNβ in TRIM25-deficient cells (Gack et al., 2007).

1.10.4.4 TRIM proteins and viral defense

Although the TRIM E3 ligases are a large family, less than 20 members have been individually characterized (Nisole et al., 2005). They have been shown to be involved in cell proliferation, development, differentiation, oncogenesis or aptosis (James et al., 2007). Only 3 of the 70 TRIM proteins have been identified as being IFN inducible they include TRIM19, TRIM22 and TRIM5 suggesting a involvement in viral defense by inducing the innate system (Lavau et al., 1995, Gongora et al., 2000, Asaoka et al., 2005, Jefferies et al., 2011, Hattlmann et al., 2012).

Other TRIM proteins such as TRIM1 and TRIM32 have been found to interfere with viral replications that are not interferon inducible like TRIM19, TRIM22 and TRIM5 (Yap et al., 2004, Fridell et al., 1995). It has been shown that in the absence of interferon, constitutive overexpression of TRIM19 mediates resistance to infection by vesicular stomatitis virus (VSV) and influenza A virus (Chelbi-Alix et al., 1998, Van Damme and Van Ostade, 2011). TRIM19 inhibits both VSV mRNA and protein synthesis and has also been shown to bind human foamy virus transactivator Tas, inhibiting retroviral transcriptional (Chelbi-Alix et al., 1998, Regad et al., 2001). Using a yeast two-hybrid system, TRIM32 was demonstrated to bind to the activation domain of Tat proteins of HIV-1, giving further evidence for the role of TRIM proteins in inhibition of viral replication (Fridell et al., 1995)

TRIM5α is the largest isoform found in the TRIM5 gene and has been shown to block the replication of many retroviruses, HIV-1 and MLV (Song et al., 2005b, McNab et al., 2011). TRIM5α blocks the replication at a stage in the viral cycle following reverse transcription but before integration of viral DNA into the host chromosome (Stoye, 1998). During the characterization of TRIM5α, other TRIM members were tested for antiviral activity, and TRIM1 was identified to restrict infection of MLV in a similar fashion to TRIM5α (Bouazzaoui et al., 2006).

Bouzzaoui and colleagues showed that TRIM22 inhibits HIV-1 replication in human monocyte derived macrophages (Bouazzaoui et al., 2006). TRIM22 acts as a potent inhibitor of transcription from the LTR promoter of HIV-1 (Bouazzaoui et al., 2006, Tissot and Mechti, 1995, McNab et al., 2011). This suggests that TRIM22 acts as a transcriptional regulator and plays a negative role in the HIV-1 replication cycle

(Bouazzaoui et al., 2006, Tissot and Mechti, 1995). TRIM proteins play a role in innate immunity and viral defense (Nisole et al., 2005).

1.10.4.5 TRIM5

TRIM5α was first identified in rhesus monkey (rh) as factor that blocks HIV-1 replication (Stremlau et al., 2004). Shortly after, several groups demonstrated that the restriction factors previously described in human, redox factor 1 (Ref1), and in primates, Lv1, restricting MuLV and HIV-1 and/or SIV from macaques (SIVmac) respectively, were species-specific variants of TRIM5α protein (Hatziioannou et al., 2004b, Keckesova et al., 2004).

Although rhTRIM5α has potent inhibitory effect against HIV-1, the human TRIM5α (hTRIM5α) appears to only modestly inhibit HIV-1 replication (Stremlau et al., 2004). The exact antiviral mechanism of TRIM5α is so far not clear. It is known that TRIM5α trimers interact with hexameric capsids and block replication (Ohkura et al., 2011). TRIM5α is ubiquitinylated within cells and is rapidly turned over by the proteasome in a RING-domain-dependent way, suggesting that auto-ubiquitination might promote CA degradation (Diaz-Griffero et al., 2006). Other studies have indicated that TRIM5α can disrupt the uncoating of the capsid before reverse transcription; or alternatively can somehow act after reverse transcription blocking PIC access to the nucleus (Towers, 2007).

1.10.4.6 TRIM22 or Staf50 (Stimulated Trans acting Factor of 50kDa

TRIM22 was identified in a screen for interferon inducible genes, where it was observed that TRIM22 mRNA and protein levels could be induced with both type I and type II IFN (Tissot and Mechti, 1995, Barr et al., 2008). When determining

endogenous expression of TRIM22 in different tissues, it was shown that in the absence of exogenous IFN treatment, TRIM22 was mainly expressed in peripheral blood leukocytes, in lymphoid tissue such as spleen and thymus, and in the ovary (Tissot and Mechti, 1995). TRIM22 expression levels decreased during T lymphocyte activation with CD3/CD2/CD28, which was indicative of the antiproliferative effects for TRIM22 (Gongora et al., 2000).

The transcription of TRIM22 was found to be upregulated in response to p53, and subsequently direct binding of p53 to the p53 response element in intron 1 of the TRIM22 sequence was identified (Obad et al., 2004). When Wei *et al* performed a global screening of p53 binding sites in the human genome; TRIM22 was confirmed as a p53 target gene (Wei et al., 2006).

TRIM22 mediates inhibition of HIV- 1 replication in monocyte-derived macrophages, which was consistent with Tissot and Mechtis' discovery that TRIM22 down-regulates the transcription regulated by the LTR promotor region of human HIV-1 (Bouazzaoui et al., 2006, Tissot and Mechti, 1995). This notion is further supported by the fact that the TRIM22 mouse ortholog Rpt-1 gene is selectively expressed in resting helper/inducer T cells and has been shown to down-regulate gene expression directed by the HIV-1 promoter (Patarca et al., 1988, Hattlmann et al., 2012).

1.11 Purpose of Study

Several studies have identified individuals who remain uninfected despite repeated exposure to human immunodeficiency virus type 1 (HIV-1) (Yang et al., 2005, Devito et al., 2000). On the other hand, the disease course in HIV-1 infected

people is characterized by heterogeneity; with a significant proportion of people displaying clinical long-term non-progression, characterized by low viral loads and high CD4 counts (O'Connor et al., 2007, Crotti et al., 2006). Several possible mechanisms of protection against infection and disease progression have been suggested.

In the past decade, a new system of antiviral resistance has been identified in the mammalian cells and termed intrinsic immunity (Goila-Gaur and Strebel, 2008). Intrinsic immunity is thought to be distinct from traditional innate and adaptive immune systems and is mediated by host restriction factors that are thought to play an important role usually as species-specific barriers to viral infections (Bieniasz, 2004, Zhang et al., 2006). Relatively little is known about the contribution of intrinsic immunity to protection against retroviral infections *in vivo*, although in some cases, host restriction factors can completely block infection (Chiu and Greene, 2008).

TRIM E3 ligases represent a recently described family of proteins with potent antiviral activity (Uchil et al., 2008). Rhesus TRIM5α (TRIM5αrh) was shown to be responsible for the complete block of HIV-1 replication in Old World monkey cells (Sayah et al., 2004, Stremlau et al., 2004). This effect was shown to be mediated through the interaction of TRIM5αrh with the HIV-1 capsid (Stremlau et al., 2004). Further studies have suggested that in addition to the effects of TRIM5αrh on HIV via binding to capsid, other mechanisms of viral inhibition are possible (Perron et al., 2004, Sakuma et al., 2007). TRIM5α is responsible for species-specific post entry restriction of retroviruses, such as murine leukemia virus, (N-MLV) and HIV-1 in

primate cells (Yap et al., 2004, Stremlau et al., 2004). Other TRIM E3 ligases with antiviral activity have also been described (Yap et al., 2004).

TRIM family proteins affect specific steps in the HIV life cycle (Hatziioannou et al., 2004b). Interference with uncoating of the viral pre-integration complex was noted for huTRIM5α and rhTRIM5α (Nisole et al., 2005), repression of viral gene expression by TRIM 11 and inhibition of viral assembly by TRIM 22 (Ozato et al., 2008). TRIM 19 has been suggested to affect trafficking of viral proteins (Uchil et al., 2008, Yap et al., 2004). Thus TRIM E3 ligases may mediate antiviral responses at multiple steps in the HIV-1 life cycle.

Although the antiretroviral activity of TRIM E3 ligases is now well established, the contribution of this family of proteins to protection against HIV-1 infection or to control of disease progression is largely unknown. Many *in vitro* studies have suggested that human TRIM5α has little or no effect on HIV replication. However, genetic association studies have demonstrated that certain polymorphic variants of the human TRIM5α gene are associated with reduced susceptibility to HIV infection or are overrepresented among HIV negative individuals compared to HIV positive ones (Javanbakht et al., 2006, Speelmon et al., 2006, Price et al., 2010), suggesting that huTRIM5α may have some protective role against HIV-1 infection. It has also been reported that huTRIM5α genetic variants can influence the rate of disease progression, although the effects appear to be either phase of infection dependent or rather modest (Goldschmidt et al., 2006, van Manen et al., 2008). One study has also suggested that human TRIM5α may select for escape mutants after a prolonged duration of HIV-1 infection (Kootstra et al., 2007).

In a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, we have recently shown that elevated expression of human TRIM5α (huTRIM5α) is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). Furthermore, we found that huTRIM5α messenger RNA levels were neither actively down-regulated nor up-regulated among individuals in this cohort who eventually became HIV-1 infected. The latter finding was surprising because previous studies have demonstrated that type 1 interferons are dysregulated during HIV-1 infection (Hardy et al., 2009, Kamga et al., 2005) and TRIM5α is a well known type 1 interferon inducible gene (Sakuma et al., 2007, Asaoka et al., 2005) and therefore we would be expected to show similar trends for human TRIM5α as has been reported for type 1 interferons. In addition to TRIM5α, several TRIM proteins with antiviral activity have recently been described and shown to be interferon-inducible (Uchil et al., 2008, Barr et al., 2008) although there are also notable exceptions to this rule (Carthagena et al., 2009, Rajsbaum et al., 2008). Interferons are themselves the main mediators of innate immunity against viral infection, and they play a significant role by up-regulating the expression of many antiviral effectors within the cell (Fensterl and Sen, 2009, Sadler and Williams, 2008).

In this thesis study I characterized the expression of TRIM E3 ligases, cyclophilin A and type I interferon *in vivo* in order to answer the following questions:

1) Do the expression levels of TRIM E3 ligases, cyclophilin A and type I interferons in peripheral blood mononuclear cells affect relative susceptibility to HIV-1 infection in a well characterized cohort of high risk South African individuals? Is there correlation of TRIM E3 ligases and IFN-1 expression *in vivo*?

2) Do the expression levels of these factors correlate negatively with viral loads and positively with CD4 T cell counts?

3) How do cells from the central nervous system compare to peripheral blood mononuclear cells in expression of TRIM E3 ligases and IFN-1? Do expression levels correlate with markers of disease progression/pathogenesis?

4) Which HIV-1 target cells express TRIM E3 ligases and cyclophilin A? Can we demonstrate *in vitro* that knockdown of particular TRIM E3 ligases enhances HIV-1 replication?

All five TRIM E3 ligases screened, TRIM5α, TRIM11, TRIM19, TRIM22 and TRIM36, have a coil-coil, RING and B-Box2 domains (Short and Cox, 2006, Carthagena et al., 2009, Ozato et al., 2008) and this could be attributing to their anti-viral activity.

1.12 Hypothesis and Aims of study

1.12.1 Hypotheses being tested

- Novel HIV-1 restriction factors such as TRIM E3 ligase, (huTRIM5α, TRIM11, TRIM19, TRIM22 and TRIM36) have anti-viral effects in human cells *in-vivo* while cellular co-factors to HIV-1, (CypA) play an important role in viral replication *in-vivo*.
- Furthermore, I hypothesized that increased expression of particular TRIM E3 ligases is associated with reduced susceptibility to HIV-1 infection or low viral load and high $CD4^+$ T cell counts during HIV-1 infection.

- I also tested the hypothesis that TRIM E3 ligases play a significant role in viral control in compartments without significant adaptive immune responses, such as the central nervous system.
- In contrast, I hypothesized that high expression of cyclophilin A (CypA), a HIV-1 replication cofactor with opposite effects to TRIM5α is associated with high likelihood of HIV-1 acquisition and/or high viral load and low $CD4^+$ T cell counts.

1.12.2. Aims of study

The specific aims of this study were to:

1) Investigate the expression of TRIM E3 ligases (TRIM5α, 11, 19, 22, 36), CypA and type 1 interferon (IFN-1) from HIV positive versus HIV negative peripheral blood mononuclear cells. I tested the hypothesis that HIV-1 infection is associated with dysregulation of these factors. Furthermore, using matched samples (PBMC from persons who were recruited while HIV negative and later became HIV positive versus those from non-seroconverters). I tested the hypothesis that increased expression of particular innate and intrinsic immunity factors (TRIM E3 ligases and IFN-1) is associated with reduced likelihood of HIV-1 acquisition. Conversely, I hypothesized that high baseline expression of CypA, an HIV-1 replication cofactor is associated with increased likelihood of HIV-1 infection. I also correlated the expression of these factors with markers of disease progression (viral load and CD4+ T cell counts).

2) Characterize the expression of TRIM E3 ligases and IFN-1 in primary cells derived from the central nervous system (CNS) cells versus peripheral blood

mononuclear cells and correlate their expression with viral control. I tested the hypothesis that intrinsic immunity factors are more highly expressed in CNS cells compared to PBMCs, and that they are more effective in viral control in the CNS compartment where adaptive immunity has a more limited role.

3) Characterize the expression of TRIM E3 ligases and CypA in different immune cells, particularly those known to be targeted by HIV-1 *in vivo*. In addition, mechanistic studies were undertaken to verify the effects of IFN-1 on the expression of selected TRIM E3 ligases and to assess effects on HIV-1 replication *in vitro*.

2. Methods

2.1 Study Participants-

2.1.1 Study Participants-Cohort A

Study participants for specific aim 1 were from the CAPRISA 002 Acute Infection (AI) study cohort which was an observational natural history study of HIV-1 subtype C infection established in Durban, South Africa in 2004 (van Loggerenberg et al., 2008). A urine dipstick is done on clinical suspicion of pregnancy, or if requested by the participant. In addition, a routine laboratory assessment, urinalysis, and STI screening are performed at 6-monthly intervals (van Loggerenberg et al., 2008).

245 females at high risk for HIV infection were enrolled into Phase 1 of the study. Participants were screened monthly and seroconverters were identified by two HIV-1 rapid antibody tests, Determine (Abbott Laboratories, Abbott Park, IL) and Capillus (Trinity Biotech PLC, Bray, County Wicklow, Ireland). Antibody negative samples underwent pooled PCR testing for HIV-1 RNA (Ampliscreen v1.5, Roche Diagnostics), (figure 2.1.).

Participants were enrolled into the Acute Infection phase if they became antibody positive within 5 months of a previous antibody negative test or if they had evidence of viral replication without HIV-1 antibodies as assessed by rapid tests and PCR testing. Participants with acute HIV infection (HIV antibody negative, RNA positive) and those from other seroincidence cohorts who fulfilled the acute infection or recent infection criteria as defined above were also recruited into Phase 2. Currently, there are 62 acute infections identified in this cohort. Acutely infected

participants are followed weekly for 3 weeks, fortnightly until 3 months post-infection, monthly until 12 months post-infection and thereafter quarterly for a maximum of 5.5 years.

In this study, we used samples from individuals within 1 year of infection because we hypothesized that as part of the innate immune response, intrinsic immune factors are more likely to have an impact during the primary phase of infection.

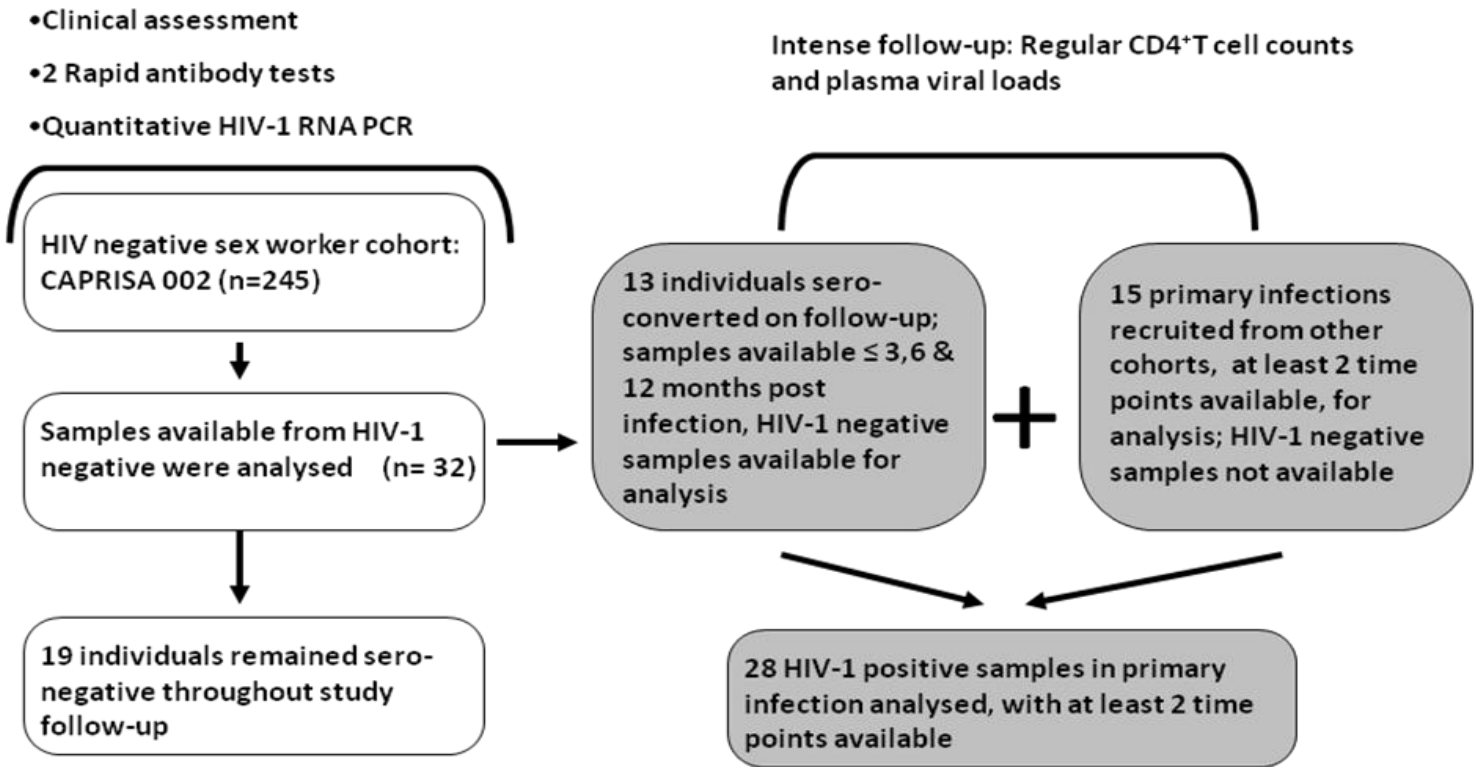


Figure 2.1. Cohort A

2.1.2. Study Participants-Cohort B

Study participants for specific aim 2 were part of a prospective case control study developed by Dr Patel who recruited one hundred and fifty consecutive patients with suspected Tuberculous meningitis (TBM) (Patel et al., 2010). We performed gene expression studies on selected TRIM E3 ligases where samples were available. We performed studies on 25 matched CSF and PBMC samples (figure 2). Fluorecence activated cell sorting (FACS) analyses were performed on 9 matched CSF and PBMC samples. Briefly one hundred and fifty consecutive patients with suspected TBM were prospectively recruited, between January 2008 and April 2009, at the Inkosi Albert Luthuli Central Hospital (IALCH), in Durban, South Africa. The study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and all participants provided written informed consent. All patients were clinically assessed by a neurologist and had a computerised tomography (CT) scan done to exclude contraindications to a lumbar puncture (LP).

All patients had routine blood tests including full blood count, urea and electrolytes, liver function tests, blood glucose at the time of LP, immunoglobulin (Ig) levels, testing for human immunodeficiency virus (HIV), and if appropriate, a CD4 T cell count. Cerebrospinal fluid (CSF) was obtained by LP and the following tests were performed: microscopy (Gram stain and for acid-fast bacilli), bacterial culture, *M.tb* culture, fungal culture, TB PCR (Roche Amplicor), routine chemistry (protein, glucose, chloride), viral PCR (Roche Amplicor) for [cytomegalovirus (CMV), herpes simplex (HSV) and varicella zoster virus (VZV)], fluorescent treponemal antigen

(FTA) test and Wasserman reaction (WR) for neurosyphilis, cysticercal enzyme linked immunosorbent assay (ELISA), and a cryptococcal antigen latex agglutination test (CLAT) which has a high specificity and sensitivity.

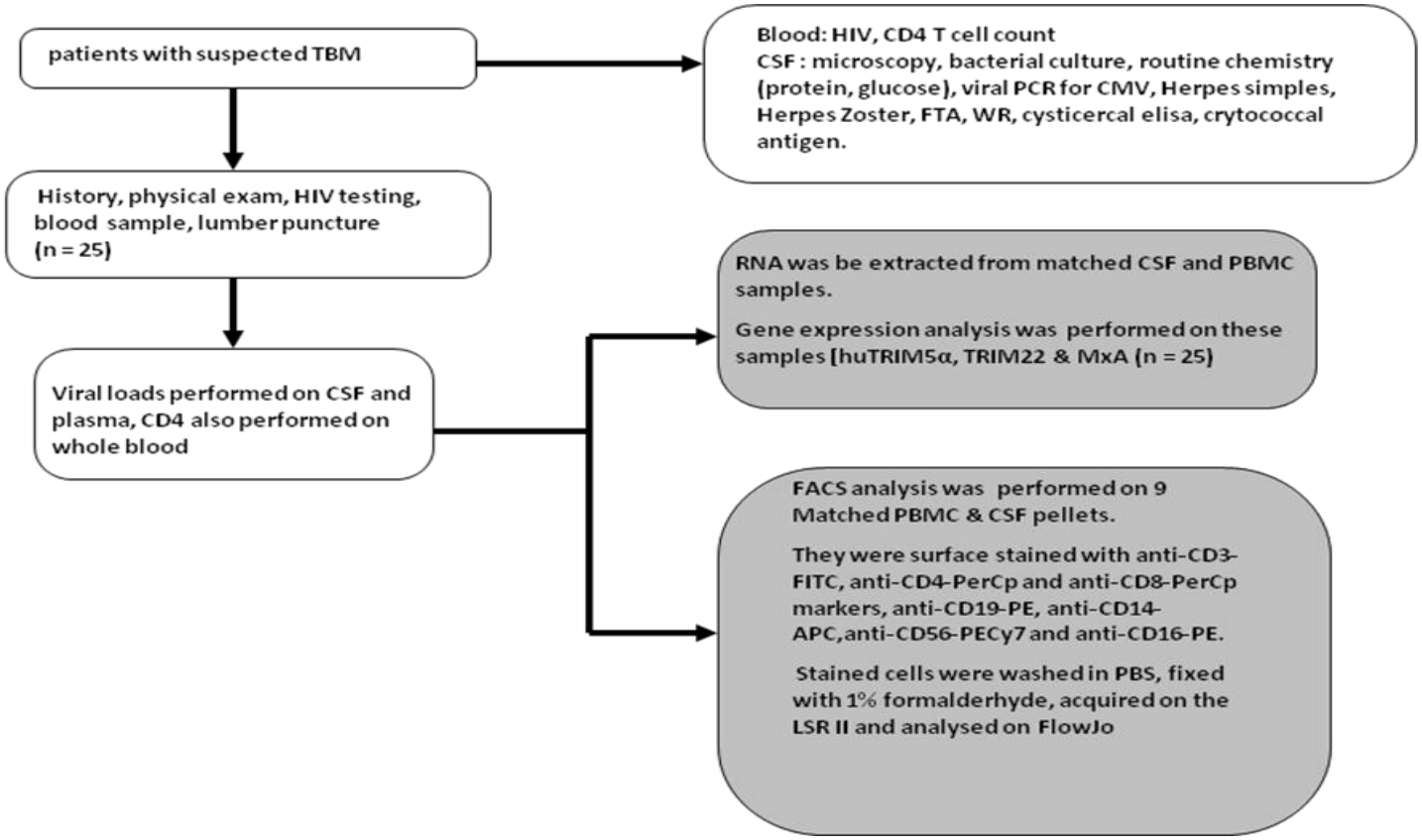


Figure 2.2. Cohort B

2.2 Sample processing, Viral Load Quantification and CD4 cell enumeration

PBMCs were isolated by Ficoll-Histopaque (Sigma) density gradient centrifugation from blood within 6 hours of blood collection and frozen in liquid nitrogen until use. Viral load was determined using the automated COBAS AMPLICOR HIV-1 Monitor Test v1.5 (Roche). $CD4^+$ cells were enumerated by using the Multitest kit (CD4/CD3/CD8/CD45) on a four-parameter FACSCalibur flow cytometer (Becton Dickinson).

2.3 Cell lines

CEM-SS (Human T4-lymphoblastoid cell line, cat. no. 776, NIH AIDS Research and Reference Reagent Program), Jurkat T-Cells CypA -/- (Human T cell lymphoblast-like cell line, cat. no. 10095, NIH AIDS Research and Reference Reagent Program) and B lymphoblasts (Daudi, CCL-213, ATCC, Rockville, MD) were maintained in RPMI with penicillin and streptomycin, 10% fetal calf serum. Human astroglioma cells, (U87.CD4.CCR5, cat. no. 4031, NIH AID Reagent Repository) and neurobalstoma cell lines (cortical neuron, cat. no. CRL-2137, CRL-2142, ATCC, Rockville, MD) where maintained in DMEM with 2mM L-glutamine, 100U/ml penicillin 100µg/ml streptomycin, and 10% FBS.

2.4 Reagents

Recombinant human interferon α-2b, (IFN-α, cat. no. CYT-205), interleukin -2, (IL-2, cat. no. CYT-209), interleukin-10, (IL-10, cat. no. CYT-500) and tumour necrosis factor-α, (TNF-α, cat. no. CYT-223) where purchased from Prospec, (Prospec, East Brunswick, NJ).

2.5 IFN-α stimulation of cell lines

CEM-SS (Human T4-lymphoblastoid cell line, cat. no. 776, NIH AIDS Research and Reference Reagent Program), Jurkat T-Cells CypA -/- (Human T cell lymphoblast-like cell line, cat. no. 10095, NIH AIDS Research and Reference Reagent Program), B lymphoblasts (Daudi, CCL-213, ATCC, Rockville, MD), Human astroglioma cells, (U87.CD4.CCR5, cat. no. 4031, NIH AID Reagent Repository) and neurobalstoma cell lines (cortical neuron, cat. no. CRL-2137, CRL-2142, ATCC, Rockville, MD) in 24 well plates ($1x10^6$ cells/well) were stimulated with 0, 10, 100 and 1000U IFN-α for 6 hours where upon cells where washed and resuspended in cell lysis buffer from RNA extraction kit, [(RNAEasy kit (Qiagen)], gene expression was assessed for huTRIM5α, TRIM5δ, TRIM5γ, TRIM22, IFN-α and IFN-β.

2.6 Cytokine stimulation of cell lines

CEM-SS (Human T4-lymphoblastoid cell line, cat. no. 776, NIH AIDS Research and Reference Reagent Program), Jurkat T-Cells CypA -/- (Human T cell lymphoblast-like cell line, cat. no. 10095, NIH AIDS Research and Reference Reagent Program), B lymphoblasts (Daudi, CCL-213, ATCC, Rockville, MD), Human astroglioma cells, (U87.CD4.CCR5, cat. no. 4031, NIH AID Reagent Repository) and neurobalstoma cell lines (cortical neuron, cat. no. CRL-2137, CRL-2142, ATCC, Rockville, MD) in 24 well plates ($1x10^6$ cells/well) were stimulated with 1000U IFN-α, (Prospec, East Brunswick, NJ), IL-2 (83 ng/µl), (Prospec, East Brunswick, NJ), IL-10 (10ng/µl), (Prospec, East Brunswick, NJ), TNF-α (10ng/µl), (Prospec, East Brunswick, NJ) for 24 hours where upon cells where washed and resuspended in cell

lysis buffer from RNA extraction kit, (RNAEasy kit (Qiagen), gene expression was assessed for huTRIM5α, TRIM5δ, TRIM5γ and TRIM22

2.7 Stable knockdown in JLTRG Reporter cells

A gene knockdown experiment was performed on TRIM22 that has shown to be associated with low viral load and high $CD4^+$ T cell counts or reduced susceptibility to HIV-1 infection. sh RNAs against specific TRIM22 was designed, a total of 5 plasmids was tested. *sh* plasmids for the gene knockdown experiments was purchased from Dana-Farber Cancer Institute, [The RNAi Consortium (TRC)].

2.7.1 Use of Glycerol Stocks and Preparation of Transfection-Quality Plasmid DNA

A. Storage and handling of glycerol stocks

Hairpin-pLKO.1 bacterial glycerol stocks were stored at -80 °C.

B. Inoculation and growth in 96-well plates

96-well sterile growth plate was pre-filled with 1.2 mL TB media containing 100 μg/ml carbenicillin. Hairpin-pLKO.1 bacterial glycerol stock(s) was thawed, and mixed by pipetting, and 5 μL was transfered per well into the deep well growth plate. After inoculation, growth plate were sealed with a gas-permeable seal and shaken (300 rpm) at 37 °C for 17 hours.

C. Duplication of glycerol stocks

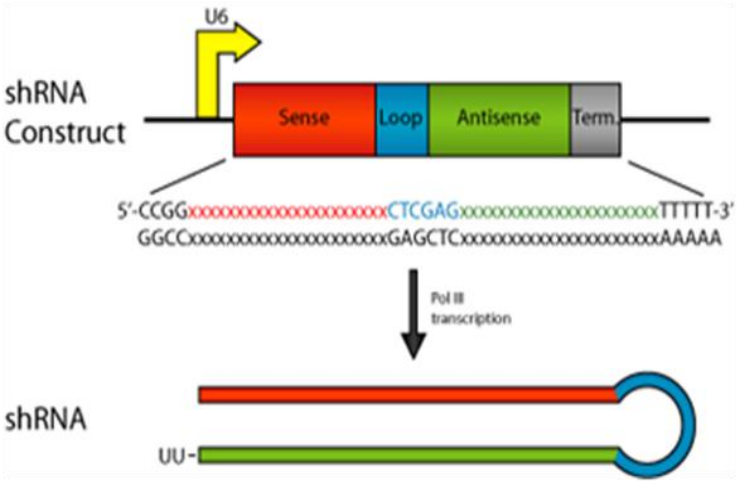
Round-bottom 96-well glycerol storage plates were pre-filled with 40 μL autoclaved 50% glycerol and 80 μL of culture from the 17 hour deep well growth plate was transfered into each destination plate to make replicate copies. Plate was frozen immediately and store at -80 °C.

D. Inoculation and growth in 96-well plates

96-well sterile growth plate was pre-filled with 1.2 mL TB media containing 100 μg/ml carbenicillin. Hairpin-pLKO.1 bacterial glycerol stock(s) was thawed, and mixed by pipetting, and 5 μL was transfered per well into the deep well growth plate. After inoculation, growth plate were sealed with a gas-permeable seal and shaken (300 rpm) at 37 °C for 17 hours.

E. Measurement of cell density, harvesting cells and Plasmid DNA preparation

40 μL of each bacterial culture was removed and diluted with 80 μL fresh media. Optical density of the diluted samples was measured at 600nm (OD_{600}). If the OD_{600} of the diluted samples are between 0.4 - 0.6, we proceeded with harvesting cells. Growth plate was centrifuged at 1,500 x g for 8 minutes at 4 °C. TB media was removed by inverting the growth plate. The inverted plate was gently tapped against a paper towel to remove as much liquid as possible without disturbing the bacterial pellets. Plasmid DNA was isolated from each well using the Qiagen mini prep kit as per manufacturer's instructions.



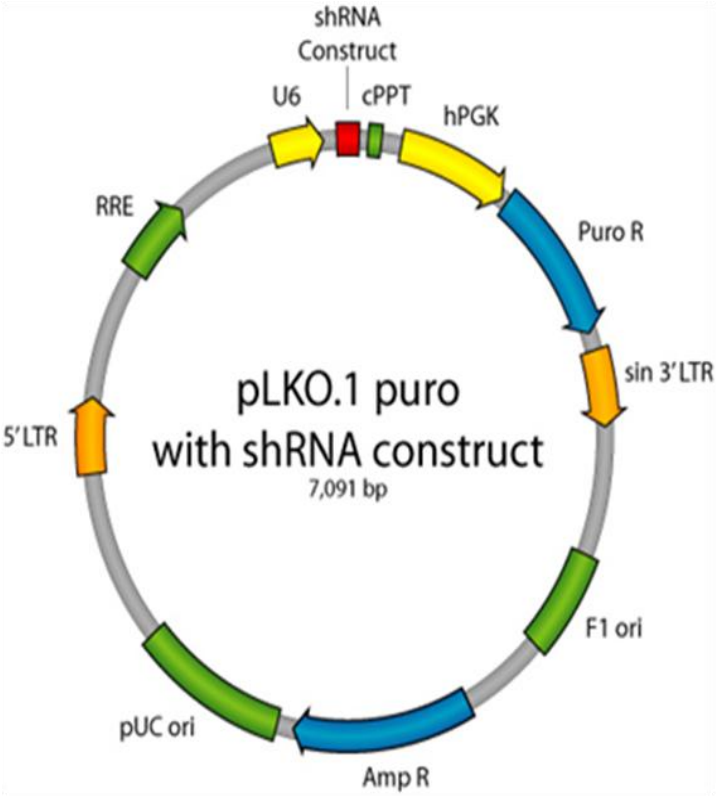


Figure 2.3 sh Constructs used

2.7.2 Lentiviral Production

293T packaging cells were seeded at 1.3-1.5x10^5 cells/mL (6 mL per plate) in low-antibiotic growth media (DMEM + 10% heat inactivated FBS + 0.1x penicillin/streptomycin) in 6cm tissue culture plates. Cells were incubated for 24 hours (37°C, 5% CO_2). Packaging cells were transfected with a mixture consisting of 3 tranfection plasmids: packaging plasmid (pCMVdR8.2 dvpr, Addgene), pRSV-Rev (Addgene) 900 ng, envelope plasmid (VSV-G/pMD2.G, Addgene) 100 ng, hairpin-pLKO.1 vector 1 μg, OPTI-MEM to total volume 10 to 30 μL. TransIT-LT1 transfection reagent (Mirus Bio, MIR 2300/5/6) was diluted in OPTI-MEM (Invitrogen, #31985-070). TransIT-LT1 reagent was added dropwise and mixed by swirling the tip or gently flicking the tube. This mixture was incubated for 5 minutes at room temperature. For a 6cm plate 6 μL TransIT-LT1 and OPTI-MEM were added to total volume 90 μL. The 3 plasmids mix was added dropwise to the diluted TransIT-LT1 reagent and mixed by swirling the tip. The transfection mixture was incubated for 30 minutes at room temperature. The transfection mixture was transferred onto the packaging cells. The cell where incubated for 18 hours (37 °C, 5% CO_2). The media was changed to remove the transfection reagent and replaced with 6ml high serum growth media for viral harvests. Cells where incubated for a further 24 hours (37 °C, 5% CO_2). Media containing lentivirus at ~40 hours post-transfection and stored in a polypropylene storage tube. 6 mL high serum growth media for viral harvests was added. 24 hours later lentivirus was harvested.

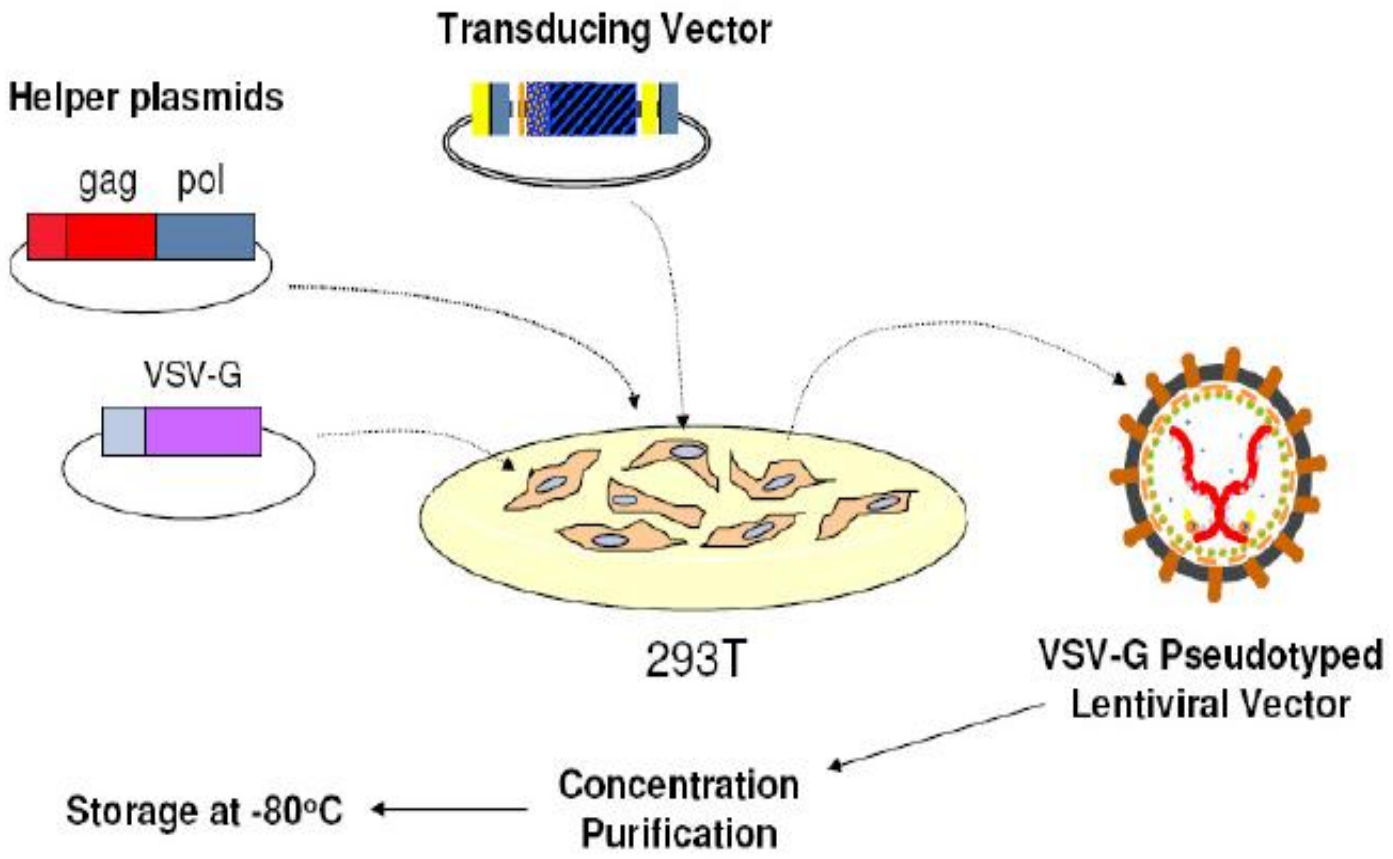


Figure 2.4 Overview sh RNA experiment

2.8. Infection Experiment in JLTRG Reporter cells:

JLTRG cells are of Jurkat T cell lineage in which HIV-1 infection is directly correlated to enhanced green fluorescence protein (EGFP) expression (JLTRG-R5 cells). These cells hold a stably integrated copy of an HIV-1 long terminal repeat (LTR)-EGFP reporter construct, which is induced through the presence of HIV-1 Tat protein following infection. Infection levels can thus be directly quantified using flow cytometric analysis (Ochsenbauer-Jambor et al., 2006).

JLTRG reporter cells was transduced by lentivirus containing the pLKO Empty Vector (Vector), control shRNA (Control), or shRNA targeting the 3'UTR of TRIM22 (3'UTR) was incubated in the presence or absence of IFN-α (1000U/mL) before spinnoculation with HIV IIIB (2h, 2,500 RPMs, 37^{o}C) at 2x10^{5} cells/well in a 96-well plate. Virus was subsequently removed, cells was washed once, and then allowed to incubate before analysis of GFP expression by flow cytometry on day 2 and 7 (GFP expression is a Tat-dependent reporter which indicates successful infection by HIV). Cell culture supernatants from day 7 samples was harvested and analyzed by p24 ELISA. JLTRG cells transduced with lentivirus was assessed for TRIM22 expression by Western blotting and real-time PCR.

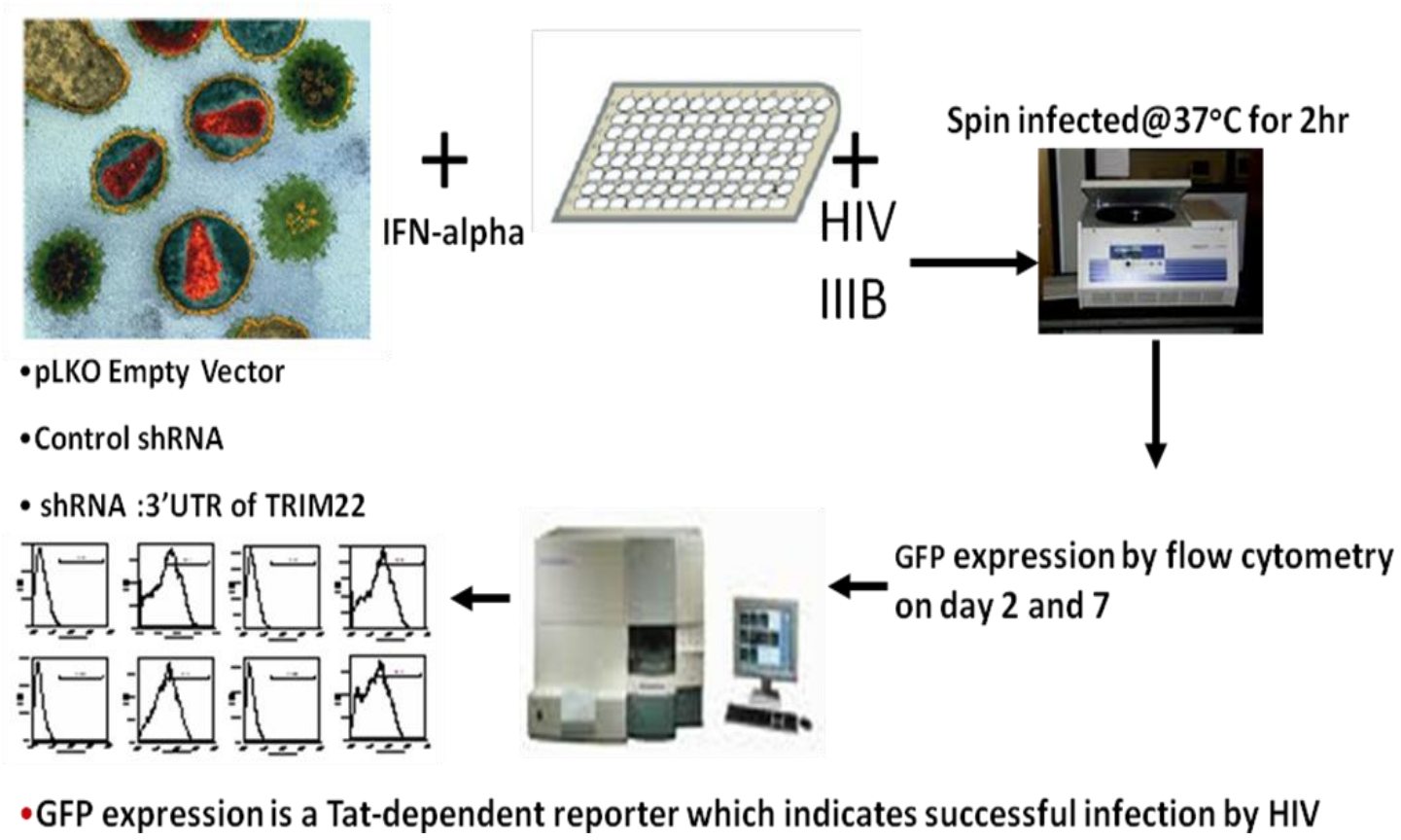


Figure 2.5 Overview sh RNA experiment

2.9 Short interfering RNA transfection and pseudotyped HIV-1 infection

TRIM22 (cat. no. Acell SMARTpool E-006927-00-0005, Human TRIM22, NM-006074, 5nmol), TRIM5α (cat. no. Acell SMARTpool E-007100-00-0005, Human TRIM5, NM-033092, 5nmol) and control, (GAPDH (cat. no. Acell GAPD siRNA-Human D-001930-01-05,5nmol) and non targeting (cat. no. Acell Non-targeting siRNA no. 1D-001910-01-05,5nmol)) siRNA where chemically synthesized by Dharmacon (Dharmacon Lafayett, Co). Human astroglioma cells, (U87.CD4.CCR5, NIH AIDS Research and Reference Reagent Program) and neuroblastoma cell lines (cat. no. CRL-2137, ATCC, Rockville,MD) were transfected with 100nM TRIM5 and TRIM22 specific siRNA or control siRNA using the Acell delivery media (cat. no. B-005000, Dharmacon Lafayett, Co) as per manufacturer's instruction. Forty eighty hours after transfection, the Acell delivery media was removed and replaced with culture media.

Cells where incubated for a further twenty four hours before phenotypic assays where performed. The above mentioned cells where stimulated with or without 1000U IFN-α in a 96 well plate, ($1x10^5$ cells/well) for twenty four hours. All experiments where performed in triplicate. Half of the cells where collected for gene expression analysis, huTRIM5α and TRIM22. Another half of the cells where infected with VSG-env-pseudotyped HIV-1 (20ng of p24/ml) for 48 hours. Cell lysates were harvested and analyzed by p24 Vironostika® HIV-1 antigen Microelisa kit (bioMerieux, Netherlands). Half of the cells where collected for gene expression analysis, huTRIM5α and TRIM22. Uninfected transfected cells with above mentioned siRNA was used as controls, they were also stimulated with or without 1000U IFN-α

in a 96 well plate, ($1x10^5$ cells/well) for twenty four hours washed and incubated for a further forty eight hours and resuspended in cell lysis buffer from RNA extraction kit, (RNAEasy kit (Qiagen), gene expression for TRIM22 and huTRIM5α was performed.

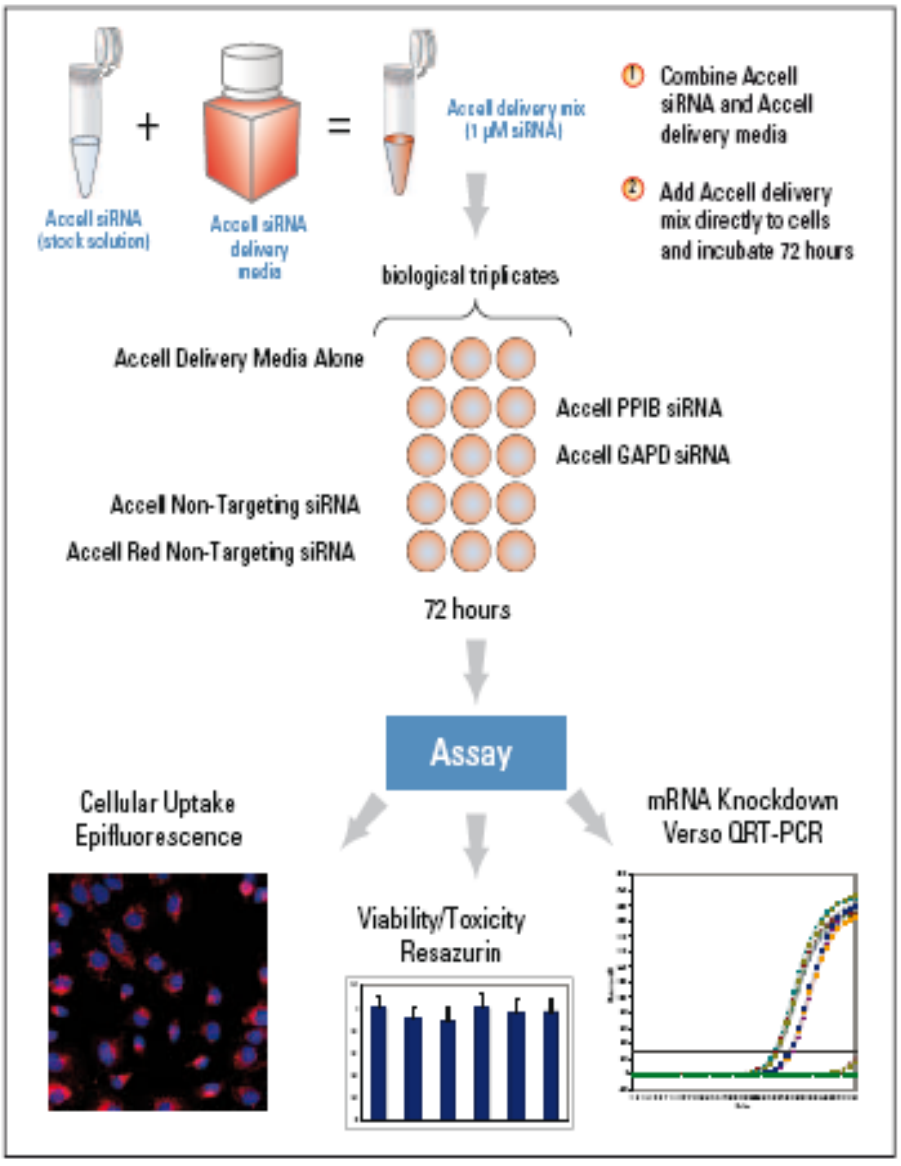


Figure 2.6 Overview si RNA experiment

2.10 RNA isolation, cDNA synthesis and analysis

2.10.1 Trizol Protocol

RNA was extracted from peripheral blood mononuclear cells (PBMCs) using a modified Trizol method (Perou et al., 1999). PBMCs were homogenized in 1ml Trizol reagent, followed by incubation for 10 minutes at room temperature. The homogenate was centrifuged for 10 minutes at 4^{o}C at 12,000 rpm followed by the addition of 200µl of chloroform. The chloroform homogenate was shaken vigorously for 30 seconds and incubated at room temperature for 5 minutes, followed by centrifugation for 15 minutes at 4^{o}C at 12,000 rpm. The supernatant which contained the total RNA was transferred into a new 1.5 ml eppendorf tube. The total RNA was precipitated with the addition of 500µl isopropanol and 1.5µl glycogen and incubated for 10 minutes at room temperature. This was followed by centrifugation for 10 minutes at 4^{o}C at 12000 rpm. The supernatant was discarded and the RNA pellet was washed with 1ml 75% ethanol, followed by centrifugation for 5 minutes at 4^{o}C at 12000 rpm. The supernatant was discarded and the RNA pellet was air dried. The RNA pellet was resuspended in 30µl Diethylpyrocarbonate (DEPC) water.

2.10.2 Qiagen RNA easy method

For all samples, RNA was extracted immediately after thawing cell lysates. RNA was extracted from 1 x 10^6 cells and 1 x 10^5 cells for the gene knockdown experiments using the RNAEasy kit (Qiagen cat. no. 74106) as per manufacturer's instructions.

Briefly PBMCs or cells where washed and counted, followed by lysis of the cell pellet with 500µl RLT buffer. Cells where incubated for 10 minutes. The cell

lysates were diluted with an equal volume of 70% ethanol. This was applied to the RNA column and span at 12000 rpm for 1 minute. The column was washed once with 600µl of RW1 buffer by spinning at 12000 rpm for 1 minute. The column was washed twice with 500µl of RPE buffer by spinning at 12000 rpm for 1 minute. We applied 30µl of nuclease free water onto the membrane and incubated the membrane for 5 minutes at room temperature. RNA was eluted by spinning spin column for 5 minutes at 12000 rpm.

RNA integrity was confirmed by (3-[N-Morpholino] propanesulfonic acid) (Sigma cat. no. M-1254) MOPS/formaldehyde gel electrophoresis. The total RNA concentration was quantified and samples were used only if the OD_{260}/OD_{280} ratio was 1.90 or greater. All RNA samples were DNAse treated using the fermentas DNASE kit (Fermentas cat. no. EN0521).

2.10.3 Reverse Transcription

1 µg of total RNA from each sample was reversed transcribed using the iScript cDNA synthesis kit (Biorad). Briefly cDNA synthesis was performed using the BioRad iScript cDNA synthesis kit according to the manufacturer's protocol. The reaction mixture was constituted as follows: 4µl of 5x iScript reaction mix, 1µl of iScript reverse transcriptase, 1µg RNA and nuclease free water to a volume of 15µl. For the CSF samples all RNA extracted was reversed transcribed.

The above reagents were added to a sterile PCR tube and kept on ice. The reaction protocol entailed incubating the reaction mixture at 25^{o}C for 5minutes, 42^{o}C for 30 minutes, 85^{o}C and holding at 4^{o}C for 45 minutes in the GeneAmp 9700 PCR System (Applied Biosystems, California, USA).

2.11 Real-time PCR RNA quantitation

The PCR primers and cycling conditions used for beta-actin, huTRIM5α, TRIM5δ, TRIM5γ, IFN-α, IFN-β, MxA and TRIM22 real-time quantitative PCR were validated in our laboratory and are shown in table 1. GAPDH was validated from among 5 genes as the most suitable reference gene based on PCR efficiency. Each PCR reaction comprised of 3 mmol/μl $MgCl_2$, (0.5 pmol/μl for huTRIM5α, TRIM5δ, TRIM5γ, IFN-α, IFN-β, TRIM22, β-Actin), (0.25 pmol/μl for GAPDH) of each primer, 5 μl Fast Start SYBR Green I (Roche), 1 μg cDNA and water to 10 μl. Reactions were run in duplicate on a Roche LightCycler 480 version 1.5 with 1 cycle at 95 ˚C (10 min), followed by 45 cycles consisting of denaturation, annealing and extension (table 1). Detection of the fluorescent products was carried out at the end of the 72 ˚C extension period. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and agarose gel electrophoresis. Serial dilutions of cDNA from total RNA were performed for each target gene. These served as standard curves for quantitative analysis.

Table 1 - Primers used in study (Roche LightCycler 480)

Gene	Accession number	Sequence 5'-3'	Cycling conditions: denaturation, annealing and extension
MxA	**NM_002446 2**	F: 5'-AAGCTGATCCGCCTCCACTT-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-TGC AAT GCA CCC CTG TAT ACC-3'	
INF-α	**NM_000069**	F: 5'-GAAACCACTGACTGTATATTGTGTGAAA-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-CAGCGTCACTAAAAACACTGCTTT-3'	
INF-β	**L41942**	F: 5'-AGTCAGAGGGAATTGTTAAGAAGCA-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-TTTGGAATTAACTTGTCAATGATATAGGTG-3'	
huTRIM5δ	**NM_033093**	F**:** 5'-GTGGATGGCGTCATAAAAAGGA-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-AGTAGCGTCGGACATCTGTCA-3'	
huTRIM5γ	**NM_033092**	F: 5'-ACGCTACTGGGGTAAGGAGAA-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-GGAGGGGTAAGTTATGTGTGTCT-3'	
huTRIM5α	**NM_033034**	F: 5'-AGGAGTTAAATGTAGTGCT-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-ATAGATGAGAAATCCATGGT - 3'	
TRIM22	**NM_006074**	F: 5'-GGTTGAGGGGATCGTCAGTA-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'- TTGGAAACAGATTTTGGCTTC-3'	
GAPDH	**NM_002046**	F: 5'-AAGGTCGGAGTCAACGGATT-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-CTCCTGGAAGATGGTGATGG-3'	
β-Actin	**NM_001101**	F: 5'-GTCCACCTTCCAGCAGATGT-3'	(95 ˚C, 15 s), (60 ˚C, 15 s) and (72 ˚C, 15s)
		R: 5'-AAAGCCATGCCAATCTCATC-3'	

The PCR primers and cycling conditions used for huTRIM5α, TRIM22, interferon-α, (IFN-α), interferon-β, (IFN-β), Myxovirus Resistance Protein A, (MxA) TRIM11, TRIM19 and TRIM36 real-time quantitative PCR were validated in our laboratory and are shown in table 2. GAPDH was validated from among 5 genes as the most suitable reference gene based on PCR efficiency (Sewram et al., 2009). Each PCR reaction comprised of 3 mmol/µl $MgCl_2$, (0.5 pmol/µl for MxA, TRIM11, TRIM19, TRIM36, TRIM 22, CypA), (0.25 pmol/µl for IFN-α, IFN-β and GAPDH) of each primer, 1 µl Fast Start SYBR Green I (Roche), 1 µg cDNA and water to 10 µl. Reactions were run in duplicate on a Roche LightCycler version.1.5 with 1 cycle at 95 ˚C (10 min), followed by 45 cycles consisting of denaturation, annealing and extension (table 2.). Detection of the fluorescent products was carried out at the end of the 72 ˚C extension period. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis and agarose gel electrophoresis. Serial dilutions of cDNA from total RNA were performed for each target gene. These served as standard curves for quantitative analysis.

Table 2 - Primers used in study (Roche LightCycler 1.5)

Gene	Accession number	Sequence 5'-3'	Cycling conditions: denaturation, annealing and extension
MxA	**NM_0024462**	F: 5'-AAGCTGATCCGCCTCCACTT-3'	(95 ˚C, 6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-TGC AAT GCA CCC CTG TAT ACC-3'	
TRIM22	**NM_006074**	F: 5'-GGTTGAGGGGATCGTCAGTA-3'	(95 ˚C, 6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'- TTGGAAACAGATTTTGGCTTC-3'	
TRIM11	**BC069227**	F: 5'-GAGTCCTGTTGCCTCTCCTG-3'	(95 ˚C, 6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-GGTAAGGGCTGTTGGCATTA-3'	
huTRIM5α	**NM_033034**	F: 5'-AGGAGTTAAATGTAGTGCT-3'	(95 ˚C, 6 s), (60 ˚C, 15 s) and (72 ˚C, 6s)
		R: 5'-ATAGATGAGAAATCCATGGT - 3'	
GAPDH	**NM_002046**	F: 5'-AAGGTCGGAGTCAACGGATT-3'	(95 ˚C, 6 s), (65 ˚C, 6 s) and (72 ˚C, 6s)
		R: 5'-CTCCTGGAAGATGGTGATGG-3'	
TRIM19	**AF230404**	F: 5'-ACAGAGCACAGAGAGCCATC-3'	(95 ˚C, 6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-GAGACCAAGTCCGAATAGGGG-3'	
TRIM36	**NM_018700**	F: 5'-GAGCTGTTTACCCACCCATTG-3'	(95 ˚C,6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R:5'-CTGATCCCACATCGTTGAATGA-3'	
INF-α	**NM_000069**	F: 5'-GAAACCACTGACTGTATATTGTGTGAAA-3'	(95 ˚C, 6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-CAGCGTCACTAAAAACACTGCTTT-3'	
INF-β	**L41942**	F: 5'-AGTCAGAGGGAATTGTTAAGAAGCA-3'	(95 ˚C,6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-TTTGGAATTAACTTGTCAATGATATAGGTG-3'	
CypA	**NM_021130**	F: 5'-GTCAACCCCACCGTGTTCTTC-3'	(95 ˚C,6 s), (60 ˚C, 6 s) and (72 ˚C, 10s)
		R: 5'-TTTCTGCTGTCTTTGGGACCTTG -3'	

2.12 FACS analysis

Nine matched PBMC or CSF pellets were surface stained on ice with the following murine anti-human monoclonal antibodies for T cells anti-CD3-FITC, anti-CD4-PerCp and anti-CD8-PerCp markers, B cells anti-CD19-PE, monocytes, anti-CD14-APC, NK cells anti-CD56-PECy7 and anti-CD16-PE (Becton Dickson, Franklin Lakes, NJ). Stained cells were washed in PBS and fixed with 1% formaldehyde. Between 50,000-100,000 events were acquired per sample on the LSRII flow cytometer and data analysis was performed using FlowJo version 8.8.2 (TreeStar, Inc.).

2.13 Cell sorting Experiments

It is important to understand the basal levels of huTRIM5α and TRIM22 expression in CD4 lymphocytes and monocytes *ex vivo* as these are the main targets of HIV-1 *in vivo*. Few studies have addressed whether intrinsic antiviral factors and/or HIV replication cofactors are differentially regulated and expressed in different cells in the body. We employed magnetic cell sorting (MACS) to isolate the different cell populations from frozen PBMCs. Purity was assessed by FACS analysis.

We employed magnetic cell sorting (MACS and STEMCELL technologies) to isolate the different cell populations from fresh HIV-1 negative PBMCs from six donors. Purity was assessed by FACS analysis. The $CD4^+$ T Cell Isolation kit II (Miltenyi) is an indirect magnetic labeling system for the isolation of unstimulated $CD4^+$ T helper cells from human PBMCs. Non-$CD4^+$ T cells, ie $CD8^+$ T cells, B cells, NK cells, dendritic cells, monocytes, granulocytes and erythroid cells, are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD8,

CD14, CD16, CD19, CD36, CD56, CD123, TCR and Glycophorin A, and anti-Biotin microbeads, isolation of highly pure $CD4^+$ T cells is achieved by depletion of magnetically labelled cells. The Monocytes Isolation Kit II (Miltenyi) was used to obtain monocytes. Natural killer cells were isolated using the Easy Sep negative selection human NK cell enrichment kit (STEMCELL technologies).

2.14 Western blotting

Antibodies used in this study: Rabbit polyclonal anti-TRIM22 (Sigma Prestige, Cat #, HPA003575), mouse monoclonal anti-Ran (Sigma, Cat #R4777) or rabbit polyclonal anti-IFITM1 (Abcam, Cat # ab70477)). Briefly cell lysates were mixed with lamli buffer (BioRad) and boiled for 10 minutes. Samples were loaded on 4% PAGE gels and electrophoresis for 45 minutes at 150V. The running buffer was SDS. The proteins where transferred onto PVDF overnight using a Dry Transfer Apparatus (BioRad). The PVDF was blocked with 5% milk in TBST (BioRad). The membrane was incubated in the primary antibody (TRIM22) in 5% milk TBST at a 1:500 dilution for two and a half hours on a rocker, followed by three washes in TBST for five minutes. The membrane was incubated in the primary antibody (Ran) at a 1:500 dilution in 5% milk TBST for two and a half hours on a rocker, followed by three washes in TBST for five minutes. The membrane was incubated in the secondary antibody (anti-Rabit) at a 1:500 dilution in 5% milk TBST for one hour on a rocker, followed by three washes in TBST for five minutes. Antibody-antigen complexes were detected using enhanced chemiluminescence reagents (Invitrogen).

2.15 IFN-1, Cytokine stimulation and viral infection of CEM cell line

CEM-SS (CEM-SS, NIH AIDS Research and Reference Reagent Program) cells in 6 well plates ($2x10^6$ cells/well) were stimulated with IFN-α, (1000U/ml), (Prospec, East Brunswick, NJ), lipopolysaccharide (LPS) (100ng/ml), (Sigma, St Louis, MO, USA) or activated with IL-2 (83 ng/µl), (Invitrogen, Nivelles, Belgium), IL-15 (20ng/µl), (Prospec, East Brunswick, NJ), or purified mouse anti-human CD28 together with purified mouse antihuman CD2 and purified mouse anti-human CD3 (5 ng/µl each), (Becton Dickson, Franklin Lakes, NJ) for 20h. Purified mouse anti-human CD28, CD2 and CD3 were added individually into the cell culture wells. Purified mouse anti-human CD3, CD2, and CD28 are referred to as CD3/CD2/CD28. CEM-SS, (CEM-SS, NIH AIDS Research and Reference Reagent Program) that were stimulated with the above mentioned cytokines or antibodies were then infected by spin inoculation, (2h, 2,500 rpms, 37^{o}C) at $2x10^6$ cells/well in a 12-well plate with NL4-3 (NIH AIDS Research and Reference Reagent Program), (5ng/ml). Virus was subsequently removed; cells were washed once, and then allowed to incubate for an additional two day before analysis of huTRIM5α and TRIM22 expression by real-time PCR. Gene expression was also assessed on day four and day seven. Cell culture supernatants from day two, four and seven samples were harvested and analyzed by p24 Vironostika® HIV-1 antigen Microelisa kit (bioMerieux, Netherlands).

2.16 Genotyping for regulatory SNPs (A1650G and C1604G) in the *PPIA* gene.

DNA samples of 52 seropositive (SP) and 195 seronegative (SN) participants from the CAPRISA AI 002 cohort were available for genotyping. For analysis of these polymorphisms in the promoter region of *PPIA*, DNA samples were amplified by PCR

using Taq DNA polymerase (Invitrogen) in the presence of 1.25 mM $MgCl_2$ with primer pair CYPAex-1s (5'-AAGTCGCAGACCCGATTG-3') and CYPAex-1a (5'-ACTTTCTGGGCCCCATTC-3'). The following amplification cycles were used: 10 min 95^{o}C; 35 cycles of 30 s 94^{o}C, 30 s 60^{o}C, 45 s 72^{o}C; 5 min 72^{o}C. Subsequently, PCR products were subjected to a restriction digest with RsaI and HAEIII (4 hours to overnight at 37^{o}C; New England Biolabs) to detect polymorphisms and analyzed on a 4% agarose gel. A PCR product containing a 1650A allele will result in a 250 bp fragment (undigested) product. A PCR product containing 1650G allele will be digested with Rsa1 resulting in a 250 bp; 130 bp and 120 bp fragments. A PCR product containing a 1604C allele will be digested with HAEIII resulting in 138 bp; 40 bp; 36 bp; and 19 bp fragment. A PCR product containing a 1604G allele will be digested with HAEIII resulting in 138 bp; 110 bp, 40 bp, 36 bp, 28, and 19 bp fragments.

2.17 Viral infection of PBMCs

Replication capacity of HIV-1 in PBMCs ($2x10^6$) isolated from 26 healthy HIV-negative donors that were genotyped for A1650G was investigated. Nine 1650A wild type genotypes and 17 AG/GG mutant genotypes were placed in a 12-well plate in R20 and incubated for 3 days at 37^{o}C, 5% CO_2. Following stimulation with IL2, cells were washed with R10 and then infected with HIV IIIB (NIH AIDS Reagent Repository) by spinoculation (2h, 2,500 RPMs, 37^{o}C) at $2x10^5$ cells/well in a 96-well plate. Virus was subsequently removed; cells were washed once, and then allowed to incubate for an additional 2-7 days before analysis of CypA expression real-time PCR.

This was described in section 2.9 to 2.10. Cell culture supernatants from day 2 and 7 samples were harvested and analyzed by p24 ELISA (Becton Dickinson).

2.18 Statistical analysis

Generation of bar graphs and non-parametric statistical analysis were performed using the statistical program Instat Graphpad Prism V.5. Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison test.

Generation of dot plots, non-parametric statistical analysis and correlations [Pearson] were performed using the statistical program Instat Graphpad Prism V.5 and SAS. Values are expressed as medians. Differences between groups were evaluated by using a Student's t-test.

We correlated huTRIM5α and TRIM22 to IFN-1 gene expression values to each other. Pearson correlations where performed on log transformed data. Univariate and multivariate Generalized Estimating Equations (GEE) models were fitted for huTRIM5α, TRIM22, MxA, IFN-α and IFN-β expression, viral load and CD4 cell counts. Viral loads and expression levels were log-transformed, while square-root transformation was applied to CD4 count data, to ensure normality.

The difference in allele frequency distribution between the SN and SP group was determined by Fisher's exact test (FET) for A1650G to test the null hypothesis that allele frequencies were the same in the two groups.

The effect of A1650G on HIV-1 viral load and $CD4^+$ T cell count was determined using a Generalized Estimating Equation (GEE) model (Zeger and Liang,

1986) taking into account longitudinal measures for each participant. Viral loads were log-transformed and the square root of $CD4^+$ T cell count was used to normalize their measurements.

Kaplan-Meier survival statistics and the Cox proportional hazards model (Cox model) were used to assess the effect of each SNP on time to HIV-1 infection after enrollment and on the rate of progression to AIDS defined as $CD4^+$ T cell decline to less than 350 cells/μl (CD4 < 350). Decline in CD4 levels was determined and compared for the group with one or two copies of the minor allele to a reference group with two copies of the major frequency allele (dominant genetic model). The significance of genotypic associations and relative hazard (RH) was determined by unadjusted Cox regression analysis for the dominant genetic model.

CypA mRNA expression levels were compared between SNs and SPs by performing dot plot graphical representation, nonparametric statistical analysis, and correlation (Pearson). Values were expressed as median values. Differences between the 2 groups were evaluated using Dunn's multiple comparison test, whereas the Mann-Whitney *U* test was used for any 2-group comparisons. Primers are available on request. The software used for the analysis was SAS version 9.1.3 (SAS Institute Inc., Cary, NC). A result was considered significant if the P-value was < 0.05.

Generalized Estimating Equations (GEE) models:

The generalized estimating equation (GEE), is a method of analyzing correlated data that otherwise could be modeled as a generalized linear model. GEEs have become an important strategy in the analysis of correlated data (Zeger and Liang, 1986). These data sets can arise from longitudinal studies, in which subjects are

measured at different points in time, or from clustering, in which measurements are taken on subjects who share a common characteristic such as belonging to the same litter (Zeger and Liang, 1986). In epidemiological cohort studies, GEE models could be used to assess the probability of developing a disease for individuals in a treatment/intervention group when compared with that of a control group (Zaihra and Paul, 2010). Genberg and colleagues used GEE models to estimate medication-specific adherence estimates on detectable HIV-RNA (> 400copies/mL) (Genberg et al., 2012). They looked at the patterns of adherence that confer the greatest benefit across different ART regimens, and showed adherence was consistently strongly associated with treatment response across ART regimens (Genberg et al., 2012).

3 Results

3.1 Association of TRIM22 with Type 1 Interferon Response and Viral Control during Primary HIV-1 Infection

3.1.1 Abstract

Type 1 interferons (IFN-1) induce the expression of the Tri-partite interaction motif (TRIM) family of E3 ligases but the contribution of these antiviral factors to HIV pathogenesis is not completely understood. We hypothesized that increased expression of specific IFN-1 and TRIM isoforms is associated with significantly lower likelihood of HIV-1 acquisition and viral control during primary HIV-1 infection. We measured IFN-α, IFN-β, myxovirus resistance protein A (MxA), huTRIM5α and TRIM22 messenger RNA (mRNA) levels in peripheral blood mononuclear cells (PBMCs) of high risk, HIV-1 uninfected participants, and HIV-1 positive study participants. Samples were available from 32 uninfected subjects, and 28 infected persons, all within one year of infection. HIV-1 positive participants had higher levels of IFN-β ($p=0.0005$), MxA ($p=0.007$) and TRIM22 ($p=0.01$) and lower levels of huTRIM5α ($p<0.001$) compared to HIV-1 negative participants. TRIM22 but not huTRIM5α correlated positively with IFN-1 (IFN-α, IFN-β and MxA) (all $p<0.0001$). In a multivariate model, increased MxA expression showed a significant positive association with viral load ($p=0.0418$). Furthermore, TRIM22 but not huTRIM5α, IFN-α, IFN-β or MxA showed a negative correlation with plasma viral load ($p=0.0307$) and positive correlation with $CD4^+$ T cell counts ($p=0.0281$). *In vitro* studies revealed that HIV infection induced TRIM22 expression in PBMCs obtained

from HIV-negative donors. Stable TRIM22 knockdown resulted in increased HIV-1 particle release and replication in Jurkat reporter cells. Collectively, these data suggest concordance between IFN-1 and TRIM22 but not huTRIM5α expression in PBMCs, and that TRIM22 likely acts as an antiviral effector *in vivo*.

3.1.2 Background

TRIM E3 ligases represent a recently described family of proteins with potent antiviral activity (Uchil et al., 2008). There are approximately 70 TRIM family members and they are characterized by the presence of the tri-partite motif, which consists of a RING domain, one or two B-box motifs and a coil-coil region (Uchil et al., 2008, Reymond et al., 2001, Meroni and Diez-Roux, 2005). The presence of the RING domain suggests that these proteins function as E3 ubiquitin ligases and mediate ubiquitylation events (Gack et al., 2007). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (Reymond et al., 2001, Towers, 2005).

The prototype member of this family, TRIM5α is responsible for the complete block of HIV-1 replication in Old World monkey cells (Sayah et al., 2004, Stremlau et al., 2004). This effect is mediated through the interaction of rhesus monkey TRIM5α (TRIM5αrh) with the HIV-1 capsid (Stremlau et al., 2004). Further studies have suggested that in addition to the effects of TRIM5αrh on HIV *via* binding to capsid, other mechanisms of viral inhibition are possible (Perron et al., 2004, Sakuma et al., 2007). TRIM5α is responsible for species-specific post entry restriction of retroviruses, such as murine leukemia virus, (N-MLV) and HIV-1 in primate cells (Yap et al., 2004, Stremlau et al., 2004). Other TRIM E3 ligases with antiviral activity

have been described (Yap et al., 2004). TRIM family proteins affect specific steps in the HIV life cycle (Hatziioannou et al., 2004b). TRIM proteins appear to mediate their antiviral activities via diverse mechanisms; interference with uncoating of the viral pre-integration complex was noted for TRIM5α (Nisole et al., 2005), inhibition of viral budding has been described for TRIM22 (Ozato et al., 2008).

Although the antiretroviral activity of TRIM E3 ligases is established, the contribution of this family of proteins to protection against HIV-1 infection or to control of disease progression is largely unknown. Many *in vitro* studies have suggested that human TRIM5α (huTRIM5α) has little effect on HIV replication.

However, some huTRIM5α genetic variants have been associated with reduced susceptibility to HIV infection (Javanbakht et al., 2006, Speelmon et al., 2006), suggesting that huTRIM5α may have a protective role in infection. Modest effects of huTRIM5α genetic polymorphism on the rate of disease progression have also been reported (Goldschmidt et al., 2006, van Manen et al., 2008) and it has been suggested that human TRIM5α may select for HIV-1 escape mutants after a prolonged duration of infection (Kootstra et al., 2007).

In a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, we have recently shown that elevated expression of huTRIM5α is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). Furthermore, we found that huTRIM5α mRNA levels were neither actively downregulated nor upregulated among individuals in this cohort who eventually became HIV-1 infected. The latter finding was surprising because previous studies have demonstrated that IFN-1 are dysregulated during HIV-1 infection (Hardy et al.,

2009, Kamga et al., 2005) and TRIM5α is a well known IFN-1 inducible gene (Sakuma et al., 2007, Asaoka et al., 2005). Therefore, we would have expected to observe similar trends for huTRIM5α as has been reported for IFN-1. In addition to TRIM5α, several TRIM proteins with antiviral activity have recently been described and shown to be IFN-inducible (Uchil et al., 2008, Barr et al., 2008). However, there are also notable exceptions to this IFN-inducibility rule (Carthagena et al., 2009, Rajsbaum et al., 2008). IFNs are themselves the main mediators of innate immunity against viral infection, and they play a significant role by upregulating the expression of many antiviral effectors within the cell (Fensterl and Sen, 2009, Sadler and Williams, 2008). Understanding the IFN-1 regulation of TRIMs is complicated by the fact that IFN-α has 13 functional isoforms. It is unclear whether all these isoforms have the same effect on IFN-stimulated genes (Mogensen et al., 1999).

Here we investigated the expression of the IFN-1s, IFN-α (IFN-α2b isoform), IFN-β, a surrogate interferon inducible gene (myxovirus resistance protein A, MxA) and TRIM22 in a longitudinal cohort of black African females at high risk for HIV-1 infection in which we have previously demonstrated that enhanced TRIM5α mRNA expression is associated with reduced susceptibility to HIV infection (Sewram et al., 2009). TRIM22 was selected for this analysis because in addition to TRIM5α, it is one of the relatively well characterized TRIM E3 ligase, has been shown to be IFN-1-inducible *in vitro* and appears to possess anti-HIV-1 activity (Barr et al., 2008, Obad et al., 2007, Carthagena et al., 2009). Specifically, we tested the hypotheses that increased expression of IFN-1 and TRIM22 is associated with significantly lower likelihood of HIV-1 acquisition, and lower viral loads or higher $CD4^+$ T cell counts

during primary HIV-1 infection. We tested whether there are differences in mRNA levels of select IFN-1s, huTRIM5α and TRIM22 in peripheral blood mononuclear cells from HIV negative versus positive individuals. We used mutivariate analysis models in order to better understand the kinetics and antiviral implications of expression of these genes *in vivo*. Finally, we performed *in vitro* experiments to better understand the relationship between IFN-1, TRIM22 expression and antiviral activity.

3.1.3 Results

Relative expression levels of IFN-1 (IFN-α, IFN-β), MxA and huTRIM5α in PBMCs from HIV-1 uninfected versus infected subjects

We have previously shown that HIV negative patients have higher levels of huTRIM5α when compared to HIV-1 positive patients (Sewram et al., 2009). However in matched pre- and post-infection samples we did not see significant dysregulation of TRIM5α. This result was surprising as TRIM5α is an IFN-1 responsive gene and IFN-1 is dysregulated in primary HIV-1 infection (Kamga et al., 2005). Furthermore, we found that women at high risk for HIV-1 infection who did not seroconvert following two years of follow up had significantly higher huTRIM5α mRNA levels in PBMCs compared to seroconverters (Sewram et al., 2009). A possible explanation for this latter finding is that high-risk non seroconverter study participants have generally higher innate antiviral defence mechanisms perhaps mediated through IFN-1, thus providing an explanation for our observation of elevated huTRIM5α among non-seroconverters. We therefore sought to better understand the relationship between TRIM5α, IFN-α, IFN-β, and an IFN-1 inducible gene, MxA.

We compared mRNA levels of IFN-α, IFN-β, MxA and TRIM5α in PBMCs from HIV-1 negative versus HIV-1 infected samples collected within the first 12 months post-infection. There were 32 individual HIV-1 negative samples available and 28 HIV-1 infected samples. Samples from HIV-1 positive individuals were available at multiple time points post-infection and samples closest to the 12 months post infection time point were included in the analyses presented here. Only patients that remained HIV-1 negative on two years of follow up were used for this analysis (n=19). The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared using an unpaired student's t test. There were no significant differences in IFN-α expression between HIV-1 negative and positive participants (Fig 3.1.1A). HIV-1 positive participants had significantly higher levels of IFN-β ($p = 0.0005$) and MxA ($p = 0.007$) (Fig 3.1.1B, D). As we have previously reported, HIV-1 negative PBMCs had significantly higher levels of TRIM5α compared to HIV-1 positive PBMCs ($p < 0.0001$), (Fig 3.1.1C).

We next investigated the relationship between TRIM5α and IFN-1 expression in HIV-1 negative and positive samples. There was no correlation between TRIM5α and IFN-α or MxA, (Fig 3.1.1E, G) in both negative and positive time points. All HIV-1 negative (n=32) and HIV-1 positive (n=75) samples available at multiple time points were used for this analysis. We found a significant inverse correlation between IFN-β and TRIM5α in both HIV-1 negative ($r = -0.49$, $p=0.004$) and positive ($r=-0.39$, $p=0.0008$) samples, (Fig 3.1.1F). As expected our data also indicated that MxA is a suitable surrogate for IFN-1 induction because MxA mRNA levels showed significant

positive correlation with IFN-α in both HIV-1 negative (r = 0.8, p= 0.0001) and positive (r=0.81, p=0.0001) PBMCs. MxA mRNA levels also significantly correlated with IFN-β levels in both HIV-1 negative (r = 0.7, p= 0.0001) and positive (r=0.8, p=0.0001) samples (Fig 1H). Thus in this cohort of individuals at high risk for HIV-1 infection in a high prevalence setting, TRIM5α was higher in HIV-1 negative compared to HIV-1 PBMCs and surprisingly, there was inverse correlation between TRIM5α and IFN-β.

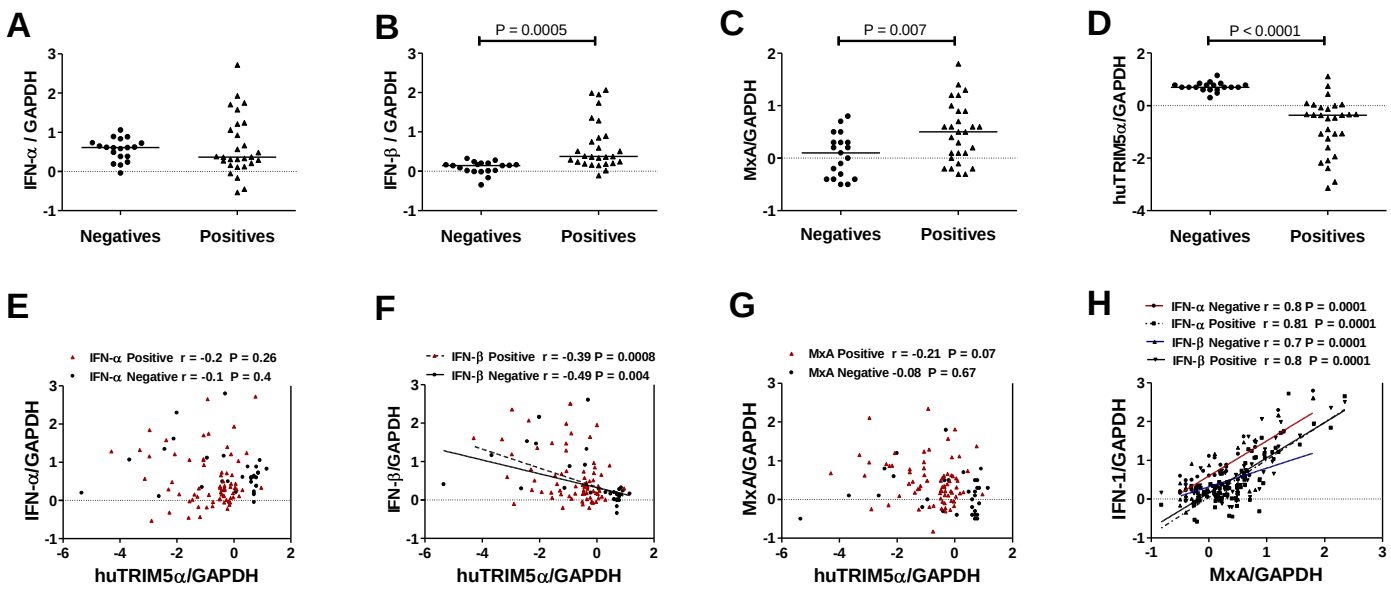


Figure 3.1.1 Expression of IFN-1 (IFN-α, IFN-β, MxA) and huTRIM5α, in PBMC from HIV-1 uninfected versus infected subjects and association between IFN-1 and huTRIM5α

The infected participant samples were all collected within 12 months of infection. At least 2 time points were available post-infection for the primary infection samples. For the HIV-1 positive group we compared one time point, closest to the set point of 12 months post infection (n=28). Only patients that remained HIV-1 negative on follow up where used for this analysis, (n=19). Data are depicted as normalized ratio of huTRIM5α or IFN-α or IFN-β versus GAPDH. The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared. The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant. Pearson correlations were performed for huTRIM5α and IFN-α or IFN-β or MxA for both negative and positive patients. Pearson correlations were also performed for MxA and IFN-α or IFN-β for both negative and positive patients.

Expression of TRIM22 in PBMC from HIV-1 uninfected versus infected subjects and association between IFN-1 and TRIM22

We next wished to evaluate the expression of the TRIM22 gene, which is located downstream of TRIM5α on chromosome #11, location 11p15 (Sawyer et al., 2005), because it has been shown to be IFN-1 inducible *in vitro*, and has known antiviral activity (Barr et al., 2008). HIV-1 positive participants had higher mRNA levels of TRIM22 when compared HIV negative patients ($p = 0.01$) (Fig 3.1.2A). IFN-α mRNA levels positively correlated with TRIM22 expression levels in both HIV negative ($r=0.91$, $p < 0.0001$) and HIV-1 positive subjects ($r=0.9$, $p < 0.0001$), (Fig 3.1.2B). IFN-β also showed significant positive correlation with TRIM22 in both HIV negative ($r=0.93$, $p < 0.0001$) and HIV-1 positive ($r=0.87$, $p < 0.0001$) subjects, (Fig 3.1.2C). Likewise, MxA mRNA levels correlated positively with TRIM22 mRNA levels in both HIV-1 negative ($r=0.81$, $p < 0.0001$) and HIV-1 positive ($r=0.92$, $p < 0.0001$) subjects, (Figure 3.1.2D). Thus TRIM22 positively correlates with IFN-1 expression in both HIV-1 negative and HIV-1 positive PBMCs *in vivo*.

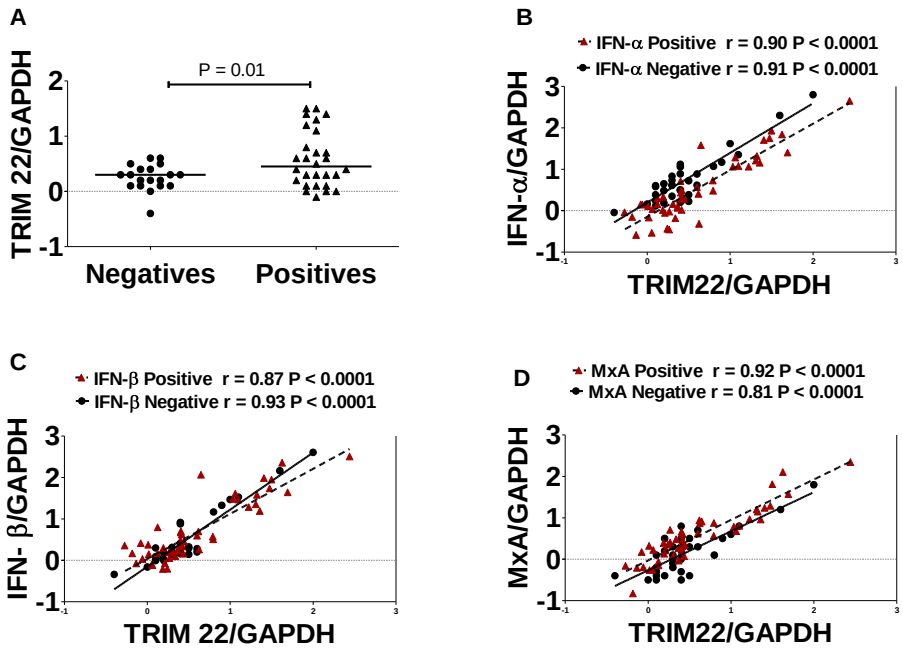


Figure 3.1.2 Expression of TRIM22 in PBMC from HIV-1 uninfected versus infected subjects and association between IFN-1 and TRIM22.

The infected participant samples were all collected within 12 months of infection (primary infection phase). At least 2 timepoints were available post-infection for the primary infection samples and for these. For the HIV-1 positive group we compared one time point, closest to the set point of 12 months post infection (n=28). Only patients that remained HIV-1 negative on follow up where used for this analysis, (n=19). Data are depicted as normalized ratio of TRIM22 versus GAPDH. The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared, (A). The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant. Pearson correlations were performed for TRIM22 and IFN-α, (B) or IFN-β, (C) or MxA, (D) for both negative and positive patients.

Expression of IFN-1 (IFN-α and IFN-β), MxA, and TRIM22 mRNA in PBMC at baseline (study enrollment) for non-seroconverters versus seroconverters.

We next addressed whether preinfection samples from seroconverters differed from those of non-seroconverters in IFN-α, IFN-β, MxA and TRIM22 expression levels. Although seroconverters showed generally higher mRNA levels of IFN-α and MxA than non-seroconverters, the differences between the groups did not reach statistical significance (Fig 3.1.3A, 3.1.3C). Individuals who became HIV-1 positive (n = 13) during the two year study follow-up period had significantly higher IFN-β and TRIM22 mRNA levels pre-infection compared to those who remained HIV-1 negative (n = 19), ($p < 0.0001$, $p = 0.0022$), (Fig 3.1.3B, D).

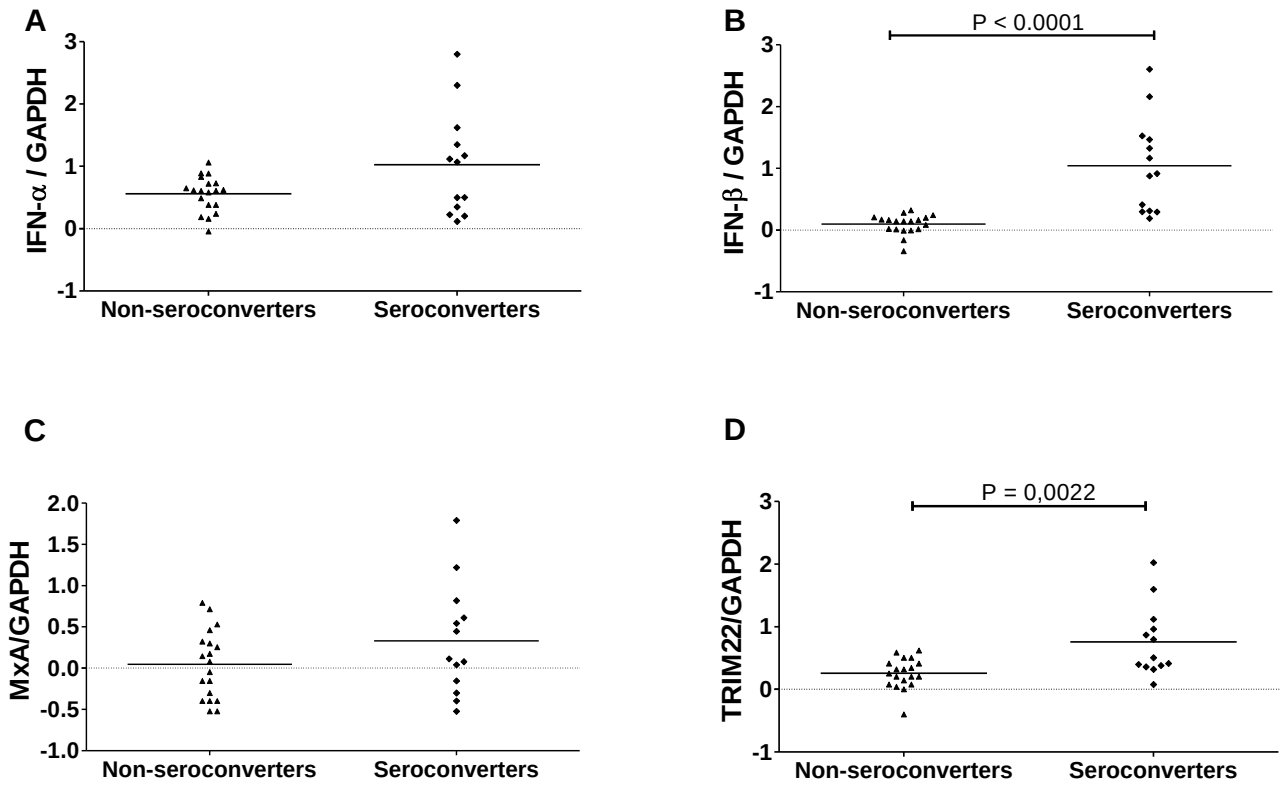


Figure 3.1.3 Expression of IFN-1 (MxA, IFN-α and IFN-β) and TRIM22 mRNA in PBMC at baseline (study enrollment) for non-seroconverters versus seroconverters.

Participants included in this analysis were all enrolled as high risk HIV-1 uninfected individuals and were longitudinally followed for at least 36 months each at the time of analysis. Data are depicted as normalized ratio of IFN-α, (A) or IFN-β, (B) or MxA, (C) or TRIM22, (D) versus GAPDH. The expression values were log-transformed to ensure normality. The horizontal line represents the median. The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant.

Kinetics of IFN-1, (IFN-α and IFN-β), MxA, and TRIM22 expression in seroconverters

Our analysis of the dynamics of expression of antiviral factors IFN-α, IFN-β, MxA and TRIM22 was based on unmatched HIV-1 negative and HIV-1 positive samples. We next wanted to investigate directly what the effect of HIV-1 infection was on these antiviral factors in matched HIV-1 negative and positive samples from the 13 individuals who acquired HIV-1 infection during follow-up. PBMCs were available from these individuals at study enrolment (pre-infection), within the first 4 months post-infection (acute phase), and at between 4 and 12 months post-infection (early chronic phase). Levels of IFN-α, IFN-β, MxA and TRIM22 pre-infection, at the acute phase and at early chronic phase for the 13 seroconverters are shown (Figure 3.1.4A, B, C and D). Median IFN-α, IFN-β, MxA and TRIM22 levels did not significantly differ between any of the 3 phases (pre-infection, acute and early chronic) analyzed even though for individual study participants there were fluctuations.

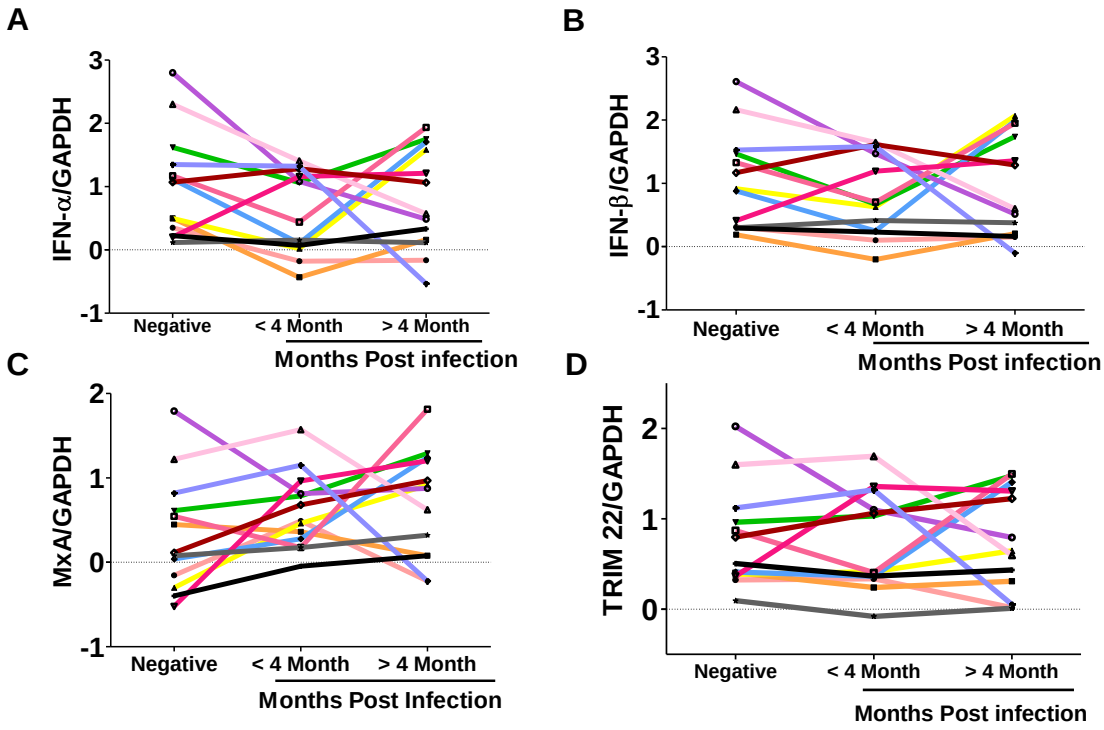


Figure 3.1.4 Kinetics of IFN-1, (MxA, IFN-α and IFN-β) and TRIM22 expression in the 13 seroconverters for whom pre- and post infection PBMCs were available

Kinetics of IFN-α or IFN-β or MxA or TRIM22 expression in the 13 seroconverters for whom pre- and post infection PBMCs were available. Samples were analyzed pre-infection, at < 4 months post-infection (acute phase) and at > 4 months post-infection (early chronic phase). One expression value is depicted for each timepoint. The expression values were log-transformed to ensure normality. There were no significant differences in median IFN-α or IFN-β or MXA or TRIM22 mRNA levels between the 3 phases. The differences between groups were evaluated by using the Dunn's Multiple Comparison Test. A P-value of < 0.05 was considered as statistically significant.

Association between antiviral gene expression and viral load and CD4 T cell counts

To determine if IFN-α, IFN-β, MxA, huTRIM5α and TRIM22 gene expression had functional implications for viral control during primary infection we used a Generalized Estimating Equation (GEE) model to evaluate viral load or $CD4^+$ T cell counts, adjusting for repeated measurements within the same individual. In the univariate models, MxA displayed a statistically significant association with HIV-1 plasma viral load. For every log increase in MxA mRNA levels, viral load increases by 0.29 log copies/ml (p=0.0444). IFN-β also showed a positive association with viral load, however, this was not statistically significant (p=0.0995).

Following adjustment for the other antiviral factors included in this study, MxA and TRIM22 maintained statistically significant association with viral load. The association between MxA and HIV-1 viral load increased after adjusting for the other antiviral factor expression variables with every log increase in MxA increasing viral load by 0.85 log copies/ml (p=0.0418). On the other hand, for every log increase in TRIM22, viral load decreased by 0.98 log copies/ml (p=0.0307) (Table 3.1.1).

Table 3.1.1 Association between Gene Expression on Viral load A Generalized Estimating Equation (GEE) model was fitted to viral load, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on viral load. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. Viral load and expression level was log-transformed to ensure normality.

	Unadjusted Models		**Adjusted Models**	
Gene/GAPDH	**Effect estimate (Std Err)**	**p-value**	**Effect estimate (Std Err)**	**p-value**
MxA	0.2887 (0.1436)	**0.0444**	0.8539 (0.4195)	**0.0418**
IFN-α	0.2163 (0.1363)	0.1126	0.2512 (0.6476)	0.6981
IFN-β	0.2216 (0.1345)	0.0995	0.1693 (0.4987)	0.7343
huTRIM5α	0.0368 (0.1308)	0.7785	0.1539 (0.1439)	0.2851
TRIM22	0.2154 (0.1532)	0.1597	-0.9807 (0.4539)	**0.0307**

Table 3.1.2 Association between Gene Expressions on CD4+ T cell counts after HIV infection A Generalized Estimating Equation (GEE) model was fitted to $CD4^+$ T cell counts, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on $CD4^+$ T cell counts. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. A square root transformation was applied to $CD4^+$ T cell counts to ensure normality. Expression level was log-transformed.

	Unadjusted Models		**Adjusted Models**	
Gene/GAPDH	**Effect estimate (Std Err)**	**p-value**	**Effect estimate (Std Err)**	**p-value**
MxA	-2.1227 (0.8552)	0.0131	-4.1024 (2.3833)	0.0852
IFN-α	-1.7526 (0.7378)	0.0175	-2.9797 (2.4699)	0.2277
IFN-β	-2.0405 (0.6065)	0.0008	-0.8828 (2.3954)	0.7125
huTRIM5α	-2.0405 (0.6065)	0.0008	-0.3175 (0.5683)	0.5764
TRIM22	-1.7936 (0.8945)	0.0450	6.0974 (2.7761)	**0.0281**

$CD4^+$ T cell counts are an important correlate of disease progression rate and outcome in HIV-1 infection. We therefore investigated whether IFN-α, IFN-β, MxA,

huTRIM5α and TRIM22 had any association with $CD4^+$ T cell counts during primary HIV-1 infection. A Generalized Estimating Equation (GEE) model was fitted to $CD4^+$ T cell counts, adjusting for repeated measurements within the same individual. In the univariate models, MxA, TRIM22, INF-α and INF-β have all significant negative association with $CD4^+$ T cell counts, thus as the expression increases with 1 log, $CD4^+$ T cell counts decreases by 2.12, 1.79, 1.75 and 2.04 square root $CD4^+$ T cells/μL for these factors respectively. However, in the multivariate model only TRIM22 remained statistically significant (p=0.0281), showing a positive association with $CD4^+$ T cell counts (Table 3.1.2).

TRIM22 expression is induced in HIV-negative PBMCs by infection with HIV-1

We next sought to determine whether infection of PBMCs isolated from HIV-negative donors could induce TRIM22 expression *in vitro*. Infection of PBMCs in the presence or absence of stimulating bi-specific antibody CD3.8 (Harrer et al., 1996) resulted in upregulation of TRIM22 protein in comparison to the uninfected controls (Fig 3.1.5A, B). Stimulation of PBMCs with CD3.8 antibody with no infection also resulted in a slight increase in TRIM22 expression, indicating that activation alone was enough to alter TRIM22 expression. Protein expression was quantified using densitometric analysis.

To validate the role of IFN- α in TRIM22 induction, we stimulated a number of cell lines used in HIV infection assays (CEM, Jurkat, THP-1) with increasing amounts of IFN-α and examined TRIM22 and huTRIM5α expression by RT-PCR. IFN-α significantly upregulated TRIM22 and huTRIM5α expression in a dose-dependent manner in all three cell lines tested (Fig 3.1.5C, D).

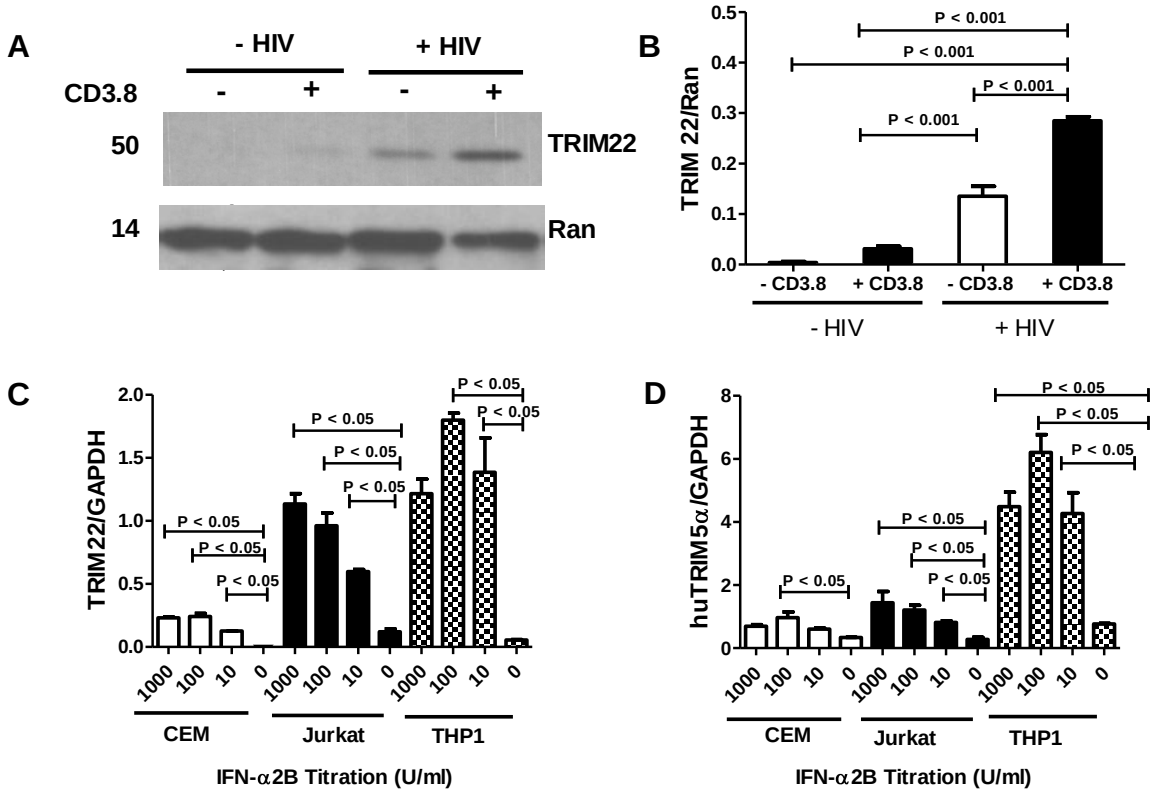


Figure 3.1.5 Induction of TRIM22 Expression by HIV and IFN-α Representative example of TRIM22 induction in PBMCs from a healthy HIV-negative donor following infection with HIV IIIB in the presence or absence of stimulating CD3.8 antibody. Expression of TRIM22 following HIV infection was assessed by western blotting (A). Ran levels are shown as a loading control. Relative fold induction of TRIM22 by HIV averaged over 3 HIV-negative donors as determined by densitometric analysis (NIH Image). Values were normalized to TRIM22 expression following CD3.8 stimulation and HIV infection in each donor. (B) Dose- dependent increases TRIM22 and huTRIM5α (C, D) in various immune cell lines as determined by RT-PCR.

Silencing of TRIM22 increases HIV infection and virus release in the presence of IFN-α

To determine its functional role in HIV infection, we tested the role of TRIM22 in HIV infection. JLTRG reporter cells, were stably transduced with empty pLKO vector (Vector), control scrambled shRNA (Control), or a shRNA directed against the 3'UTR of TRIM22 (3'UTR), and then challenged with HIV-IIIB (a fully infectious lab strain), with or without stimulation by IFN-α. At day 7, the percentage of HIV infected cells (GFP positive staining) in the 3' untranslated region of gene (3'UTR) cells treated with IFN-1 exhibited a significantly higher percentage of infected cells (51.2%), compared to vector (22%) and control (24.5%) cells in the presence of IFN-α (Fig 3.1.6A). Furthermore, the percentage of infected TRIM22-depleted cells was nearly equivalent regardless of whether cells had been treated with IFN-α or not, strongly demonstrating a significant functional role for TRIM22 in the anti-HIV IFN-α response. Culture supernatants collected on day 7 were consistent with these observations when assessed for p24 level by ELISA (Fig 3.1.6B). The knockdown of TRIM22 by the 3'UTR shRNA in the presence of IFN-α was validated by both RT-PCR and Western blot (Fig 3.1.6C. D).

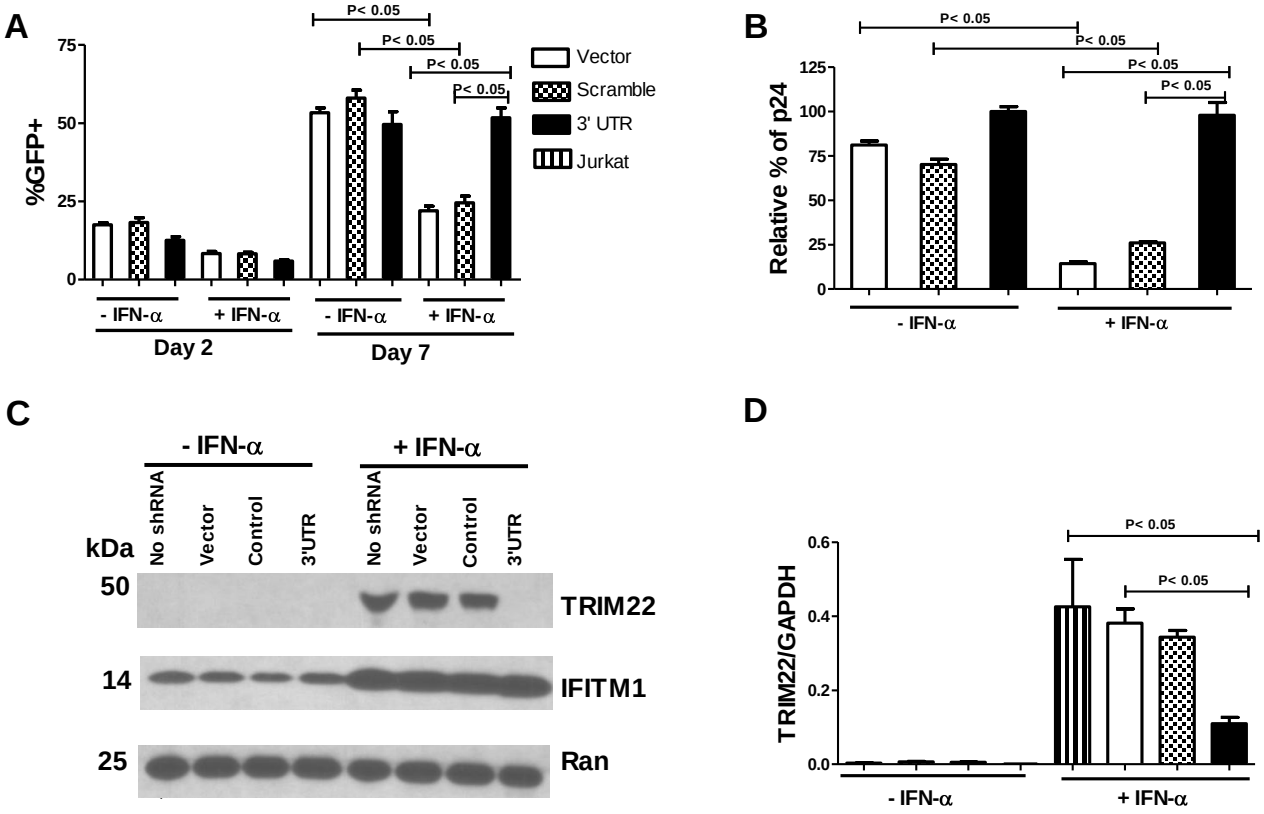


Figure 3.1.6 TRIM22 Silencing Increases HIV infection and Virus Accumulation in Jurkat reporter cells (JLTRG) transduced with the empty pLKO vector (Vector; empty), control shRNA (Control; checkered), or an anti-TRIM22 shRNA targeting the 3'UTR (3'UTR; filled) were infected with HIV IIIB following a one day stimulation with IFN-α (A). Infection of JLTRG cells (on day 7 post-infection) was assessed by GFP expression using flow cytometry. (B) TRIM22 silencing enhances accumulation of HIV particles into culture supernatant as determined by p24 ELISA on day 7 post-infection. (C, D) JLTRG cells transduced with the indicated lentivirus were assessed for TRIM22 expression by RT-PCR and Western blotting. IFITM1 was used as a control for IFN induction (Brass et al., 2009), while Ran was used as a loading control.

3.1.4 Discussion

In species other than humans, it has been demonstrated that restriction factors can completely block or partially restrict retroviral infection (Nisole et al., 2005, Stremlau et al., 2004, Goff, 2004a, Harris and Liddament, 2004, Gao et al., 2002). In contrast, little is known about the *in vivo* regulation of restriction factors or their possible role in protecting or controlling retroviral infections in humans. In this study, we used a well characterized clinical cohort of high risk seronegative and acute or primary infection samples to investigate the association of expression of select IFN-1 isoforms, two well characterized TRIM E3 ligases (TRIM5α and TRIM22) and the impact on HIV-1 susceptibility and viral control during primary HIV-1 infection.

Our earlier study revealed that lower huTRIM5α mRNA expression levels were associated with increased susceptibility to HIV-1 infection in a cohort of high risk black African females (Sewram et al., 2009). In addition, we found that in matched samples of HIV-1 negative individuals who later became HIV-1 positive, huTRIM5α levels were not dysregulated following infection.

Here we sought to understand the relationship between huTRIM5α and IFN-1 in HIV-1 negative and positive donor PBMCs, in part because huTRIM5α is IFN-1 inducible (Sakuma et al., 2007, Carthagena et al., 2009). Similarly, we also assessed the expression and activity of the related TRIM22 protein- focusing on this protein because like TRIM5α, it is IFN-inducible and has been demonstrated to have anti-HIV-1 activity (Barr et al., 2008, Obad et al., 2007, Carthagena et al., 2009). We found that PBMCs from HIV-1 positive study subjects had higher levels of IFN-β and MxA suggesting that these antiviral proteins are actively upregulated following HIV-1

infection. This result is consistent with findings from other groups (von Wussow et al., 1990, Woelk et al., 2004). We did not see significant differences in IFN-α expression between HIV-1 negative and positive PBMCs, suggesting that there may be differences in the mobilization of the varied IFN-1 isoforms (Mogensen et al., 1999, Hardy et al., 2009) following HIV-1 infection. However, overall in both HIV-1 negative and positive PBMCs, we found strong positive correlation between the two IFN-1 isoforms tested and the IFN-inducible gene, MxA, as previously described (Lehmann et al., 2008). Interestingly, huTRIM5α showed a significant inverse correlation with IFN-β and has no association with IFN-α or MxA, even though huTRIM5α has been shown to be an IFN-α inducible gene *in vitro* (Sakuma et al., 2007). These results suggest that there are differences in IFN-1 isoforms in the regulation of huTRIM5α expression. Further studies are needed to comprehensively investigate how different IFN-1 isoforms function in the regulation of huTRIM5α in different cellular environments.

Unlike huTRIM5α, TRIM22 mRNA levels were higher in HIV-1 positive participants compared to HIV-1 negative ones. We also found that TRIM22 correlated with IFN-α, IFN-β and MxA in both HIV-1 negative and positive samples. These results show an association between TRIM22 and IFN-1 expression *in vivo*, suggesting that TRIM22 is an IFN-1 responsive gene *in vivo* as has been shown *in vitro* experiments (Bouazzaoui et al., 2006, Barr et al., 2008). Higher levels of both IFN-α, IFN-β, MxA and TRIM22 were detected in seroconverters compared to nonseroconverters at baseline, with differences reaching significance for IFN-β and TRIM22. These results suggest that there is immune dysfunction or activation before

these persons become HIV-1 positive and this may have contributed to increased susceptibility to infection for these study subjects. It was previously demonstrated in a cohort of individuals at high risk of HIV-1 acquisition that participants who remained seronegative had lower levels of $CD4^+$ T cell activation at baseline (when the both groups where HIV-1 negative) (Koning et al., 2005). Similarly, IFN-1s are elevated during immune activation, which in turn has been associated with increased HIV-1/AIDS pathology (Mandl et al., 2008, Lehmann et al., 2009). However, in the present study we did not see significant differences in IFN-α, IFN-β, MxA and TRIM22 in matched pre- and post- infection samples. A possible explanation for these results is the difficulty in estimating the time of infection in human subjects. If significant changes had occurred within the first days of acute infection, the sampling intervals may not have been frequent enough to capture changes in our participants. Furthermore, it is also conceivable that we did not have a large enough size of matched pre- and post-infection samples to detect subtle differences in IFN-1 pathway response genes following HIV-1 infection.

We also investigated the association of antiviral gene expression and viral load and CD4 T cell counts, two commonly used markers of disease progression. We found that MxA mRNA levels showed a positive association with plasma viral load. For every log increase in MxA, viral load increased by 0.85 log copies/ml. This is consistent with previous studies that have demonstrated that IFN-1 increases as the viral load increases (Lehmann et al., 2008). Interestingly, we also observed that TRIM22 has a negative association with plasma viral load, and positive correlation with $CD4^+$ T cell counts. We found that for every log increase in TRIM22 mRNA

levels there is an associated viral load decrease of 0.98 log copies/ml (p=0.0307). TRIM22 also showed a positive association with $CD4^+$ T cell counts as every log increase in TRIM22 expression was associated with a 6.09 square root increase in $CD4^+$ cells/μL (p=0.0281).

The new findings of paradoxical elevation of TRIM22 in HIV+ versus HIV- PBMCs and favourable association of TRIM22 expression with markers of disease outcome prompted us to investigate whether TRIM22 could be induced in by HIV-1 infection of HIV-negative donor PBMCs. Our experiments confirmed that TRIM22 was induced by HIV (Fig 3.1.5A, B), and provided an *in vitro* corroboration of the results demonstrating that HIV-1 positive subjects have higher levels of TRIM22 compared to HIV-negative donors. This was similar to the work of Wang et al. (Wang et al., 2009) who showed that pseudotyped HIV-1 infection of could induce APOBEC3G expression. Since PBMC population are composed of a number of immune cell types (T cells, monocytes, etc), we also demonstrated that IFN-α exerts an enhancing effect on TRIM22 expression in a dose-dependent manner in CEM and Jurkat T cell lines and the monocyte cell line THP1 (Fig 3.1.5C).

In addition, we found that silencing TRIM22 in a T cell line nearly completely abrogated the IFN-mediated restriction of HIV-1 (Fig 3.1.6A). TRIM22 silencing also resulted in increased accumulation of HIV particles in culture supernatant (Fig 3.1.6B), suggesting a role for TRIM22 in late viral replication activities, such as viral release or budding. Overall therefore, our results are in agreement with several studies that have suggested that TRIM22 is induced by IFN-1 and that TRIM22 can potently

inhibit HIV replication and release (Barr et al., 2008, Bouazzaoui et al., 2006, Tissot et al., 1996).

Together these data are suggestive of both *in vivo* and *in vitro* anti-HIV role for TRIM22 although it is difficult to prove a cause-effect relationship between TRIM22 expression levels and viral load or CD4 T cell count variables. Based on our findings, we speculate that targeted enhancement of the expression of TRIM22 in HIV-1 infected individuals may be beneficial in reducing viral load and could be employed as a novel antiviral strategy.

In conclusion, we have demonstrated in a cohort of HIV-1 uninfected and infected individuals in a high prevalence setting that HIV-1 infection is associated with increased expression of the antiviral factor genes IFN-β and MxA, key components of the IFN-1 pathway. However, we did not find a correlation between IFN-α or MxA with huTRIM5α, a previously described IFN-1 responsive host restriction factor. Indeed, we found a significant negative correlation between IFN-β and huTRIM5α. In contrast, we found that TRIM22 levels strongly correlated with IFN-α, IFN-β and MxA expression in both HIV-1 negative and positive PBMCs, and were upregulated in HIV-1 positive study subjects. Intriguingly in matched pre- and post-infected samples from high risk women followed longitudinally, neither IFN-1 genes nor TRIM22 was upregulated raising the possibility of some immune dysfunction in this subset of individuals. Remarkably, TRIM22 was associated with lower plasma HIV viral load and higher CD4 T cell counts in multivariate models adjusted for multiple antiviral factors analyzed, suggesting that TRIM22 could have antiviral effects *in vivo*. We show *in vitro* that TRIM22 is induced by IFN-1 and HIV-1 infection. Furthermore, we

demonstrate that a TRIM22 plays a critical role in the IFN-1 induced anti-HIV-1 activity in tissue culture. This is the first study to provide evidence suggesting an *in vivo* anti-viral activity of TRIM22. Further studies will be needed to address what specific cell types in the PBMC milieu express TRIM22 and the other members of the TRIM family, to better define how expression of these proteins is regulated and to address whether these proteins can be harnessed as antiviral therapies or prophylactics.

3.2 TRIM11, TRIM19 and TRIM36 are induced by type 1 interferons but expression levels do not associate with viral control during primary HIV-1 infection

3.2.1 Abstract

Background:

An important part of the innate defense against viruses is the production of the type I interferons (IFN-1), interferon alpha and beta (IFN- α and IFN-β). INF-1 induces members of the Tri-partite interaction motif (TRIM) family of E3 ligases whose contribution to prevention of infection or control of HIV replication *in vivo* is not completely understood. Here we investigated the expression of TRIM11, TRIM19, TRIM36, IFN-α, IFN-β and MxA levels in a longitudinal cohort of black African females at high risk for HIV-1 infection. We hypothesized that increased expression of these antiviral proteins is associated with significantly lower likelihood of HIV-1 acquisition, and lower viral loads or higher $CD4^+$ T cell counts during primary HIV-1 infection. Samples were available from 28 infected subjects, all within one year of infection, and 32 uninfected persons.

Principal Findings: HIV-1 positive participants had higher levels of MxA (p=0.007), TRIM11 (p=0.015) and TRIM19 (p=0.0001) compared to HIV-1 negative participants (Student's t-test). Seroconverters had higher pre-infection TRIM19 levels compared to non-seroconverters ($P < 0.0001$). TRIM11, TRIM19 and TRIM36 correlated positively with IFN-1 (IFN-α, IFN-β and MxA) (all $p<0.0001$). In an adjusted

multivariate model TRIM11, TRIM19 and TRIM36 showed no association with plasma viral load or $CD4^+$ T cell counts.

Conclusions: HIV-1 positive patients have higher levels of TRIM11 and TRIM19 similar to that of MxA. We demonstrated that seroconverters have higher levels of immune activation with respect to TRIM19. TRIM E3 ligases levels correlated with type-1 IFN-1 levels suggesting that these proteins are IFN-1inducible *in vivo*. Further studies are urgently needed to understand the transcriptional regulation of TRIM E3 ligases, interaction with other arms of the the immune and to determine whether TRIM E3 ligases can be harnessed as antiviral therapies or prophylactics.

3.2.2. Introduction

The tripartite motif (TRIM) family of proteins consists of 70 TRIM genes and is characterized by the presence of a RING domain, one or two B-boxes and a coiled coil domain (Uchil et al., 2008, Reymond et al., 2001, Meroni and Diez-Roux, 2005). The RING domain of many TRIM has been shown to have E3 ubiquitin ligase activity, however the B-boxes and coiled coil domains may be involved in protein interactions and homo/heterodimerization (Meroni and Diez-Roux, 2005). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (Reymond et al., 2001, Towers, 2005).

TRIM E3 ligases are involved in a broad range of important biological processes and their alterations results in diverse pathological conditions such as genetic diseases, impaired transcriptional regulation and cancer development (Kano et al., 2008, Miyajima et al., 2008, Quaderi et al., 1997). TRIM11 interacts with Pax6, a transcription factor and mediates Pax6 degradation via the ubiquitin-proteasome

system in neuronal cells (Tuoc and Stoykova, 2008). TRIM11 plays a role in the regulation of intracellular Humanin (a neuroprotective peptide that suppresses Alzheimer's disease) levels through ubiquitin-mediated protein degradation pathways (Niikura et al., 2003). Increased levels of TRIM11 reduce Humanin levels (Niikura et al., 2003). TRIM19 inhibits the growth of many RNA and DNA viruses (Everett and Chelbi-Alix, 2007, Bernardi and Pandolfi, 2007). ProMyelocytic Leukaemia protein (PML) or TRIM19 is expressed as a family of alternatively spliced isoforms that are associated with cellular processes of oncogenesis, DNA-damage and stress responses, apoptosis and resistance to viral infections (Bernardi and Pandolfi, 2007, Everett and Chelbi-Alix, 2007, Regad et al., 2001, Regad and Chelbi-Alix, 2001). It has been reported that TRIM36 is involved in fertilization, embryogenesis and is upregulated in prostate cancer (Balint et al., 2004, Kitamura et al., 2003, Yoshigai et al., 2009).

An important part of the innate immune system is the production of type-1 interferons in response to stimuli (Fensterl and Sen, 2009, Sadler and Williams, 2008). Type 1 interferons include interferon α and β. There are 13 functional isoforms of interferon-α and a single interferon β (Mogensen et al., 1999). IFNs-α/β have immune-regulatory properties (Pestka, 2000, Stark et al., 1998).

Many cellular proteins have been identified as mediators of the antiviral activity of interferons. These include protein kinase RNA-dependent, 2'5' oligoadenylate synthetase/RNase L, and certain myxovirus resistance proteins (Mx) (Stark et al., 1998). In addition to these well characterized IFN-inducible antiviral mediators, other proteins belonging to the TRIM protein family have also been involved in IFN-induced antiviral defense, such as TRIM5α, PML or TRIM19,

TRIM22, TRIM25 and TRIM28 (Asaoka et al., 2005, Regad and Chelbi-Alix, 2001, Everett and Chelbi-Alix, 2007, Tissot and Mechti, 1995, Barr et al., 2008, Gack et al., 2007, Stremlau et al., 2004, Wolf and Goff, 2007). TRIM proteins represent a new and widespread class of antiviral molecules involved in innate immunity (Nisole et al., 2005, Ozato et al., 2008).

In a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, we have recently shown that elevated expression of human TRIM5α (huTRIM5α) is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). Our group has recently shown that the expression of TRIM22, but not the expression of huTRIM5α, in the PBMC of HIV-1 primary infected patients positively correlated with type I IFN and $CD4^+$ T cell counts, whereas a negative correlation was shown between TRIM22 expression and plasma viral load (Singh et al., 2011). Type 1 interferons are dysregulated during HIV-1 infection (Hardy et al., 2009, Kamga et al., 2005) and TRIM5α is a well known type 1 interferon inducible gene (Sakuma et al., 2007, Asaoka et al., 2005) and therefore we would have expected to observe similar trends for human TRIM5α as has been reported for type 1 interferons. In addition to TRIM5α, several TRIM proteins with antiviral activity have recently been described and shown to be interferon-inducible (Uchil et al., 2008, Barr et al., 2008).

TRIM E3 ligases appear to differ significantly in their antiviral effects and IFN-1 responsiveness. Therefore, there is a need to study how they are regulated *in vivo* and whether they directly contribute to viral control *in vivo* and whether they are correlated with type 1 IFN levels. Here we investigated the expression of type 1 interferons IFN-α (IFN-α2b isoform), IFN-β, a surrogate interferon inducible gene

(myxovirus resistance protein A, MxA), TRIM11, TRIM19 and TRIM36 in a longitudinal cohort of black African females at high risk for HIV-1 infection. Specifically, we tested the hypotheses that increased expression of these antiviral proteins is associated with significantly lower likelihood of HIV-1 acquisition, and lower viral loads or higher $CD4^+$ T cell counts during primary HIV-1 infection. We tested whether there are differences in mRNA levels of select type 1 interferons, TRIM11, TRIM19 and TRIM36 in peripheral blood mononuclear cells from HIV negative versus positive individuals. All three TRIM E3 ligases screened, TRIM11, TRIM19 and TRIM36, have a coil-coil, RING and B-Box2 domains (Short and Cox, 2006, Carthagena et al., 2009, Ozato et al., 2008) and this could be attributing to their anti viral activity. Finally, we used multivariate analysis models in order to better understand the kinetics and antiviral implications of expression of these genes *in vivo.*

3.2.3 Results

Expression of MxA and TRIM E3 ligases in PBMC from HIV-1 uninfected versus infected subjects

Interferon-1 induces a wide range of TRIM E3 ligases and other anti-viral factors. However *in-vivo* studies showing this relationship are sparse. We looked at the mRNA expression of MxA, myxovirus resistance protein A, a correlate for IFN-1 induction, TRIM11, TRIM19 and TRIM36 in unmatched negative and positive samples.

We compared mRNA levels of MxA, TRIM11, TRIM19 and TRIM36 in PBMCs from HIV-1 negative versus HIV-1 infected samples collected during the first 12 months post-infection. A total of 32 HIV negative samples and 28 HIV-1 infected

samples were used in this study. Samples from HIV positive individuals were available at multiple time points post-infection. For the HIV-1 positive group we compared one time point, closest to the set point of 12 months post infection. HIV-1 negative samples (n=19) were selected fron individuals who remained HIV negative throughout the study follow up period. The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared using an unpaired student's t test.

HIV-1 positive patients had significantly higher levels of MxA, TRIM11 and TRIM19, ($p=0.007$, $p=0.015$ and $p<0.0001$), (fig. 3.2.1 A, B and C), respectively. Comparison of the TRIM36 mRNA levels between HIV-1 negative and HIV-1 positive individuals did not reveal any significant differences. MxA expression levels have been described in the previous section.

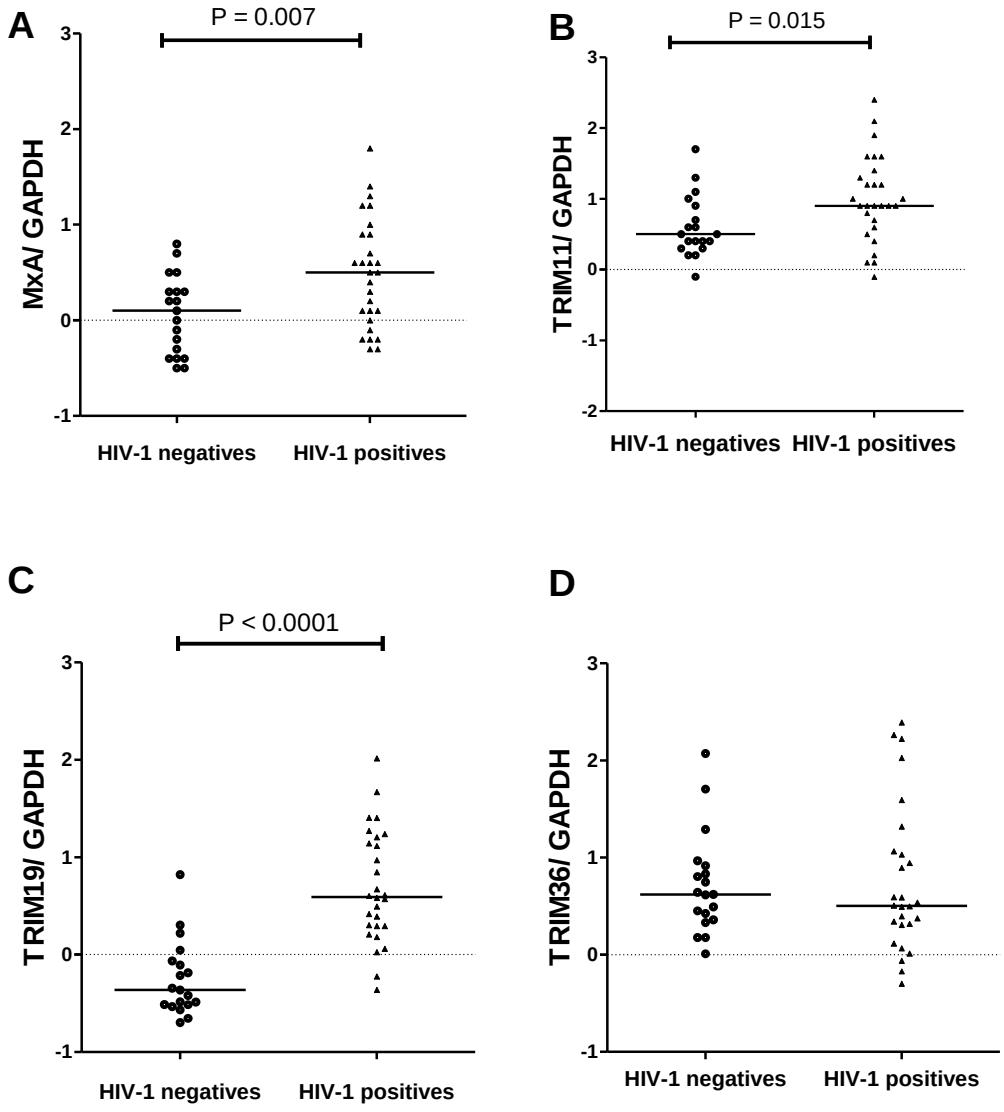


Figure 3.2.1 Expression of MxA and TRIME3 Ligases, in PBMC from HIV-1 uninfected versus infected subjects. The infected participant samples were all collected within 12 months of infection (primary infection phase). At least 2 timepoints were available post-infection for the primary infection samples and for these. For the HIV-1 positive group we compared one time point, closest to the set point of 12 months post infection (n=28). Only patients that remained HIV-1 negative on follow up where used for this analysis, (n=19). Data are depicted as normalized ratio of MxA,(A) or TRIM11,(B) or TRIM19, (C) or TRIM36, (D) versus GAPDH. The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared. The differences between groups were evaluated by using an unpaired Student's t test. A *P*-value of < 0.05 was considered as statistically significant.

Expression of MxA, TRIM11, TRIM19 and TRIM36 mRNA in PBMC at baseline for non-seroconverters versus seroconverters

We next addressed whether pre-infection samples from seroconverters differed from those of non-seroconverters in MxA, TRIM11, TRIM19 and TRIM36 expression levels. No significant differences in MxA, TRIM11 and TRIM36 mRNA levels were noted between the both groups, (fig.3.2.2A, B and D). Individuals seroconverted (n=13) during the study follow-up had significantly higher pre-infection TRIM19 mRNA levels when compared to HIV-1 negative individuals (n=19), ($p<0.0001$), (fig. 3.2.2C).

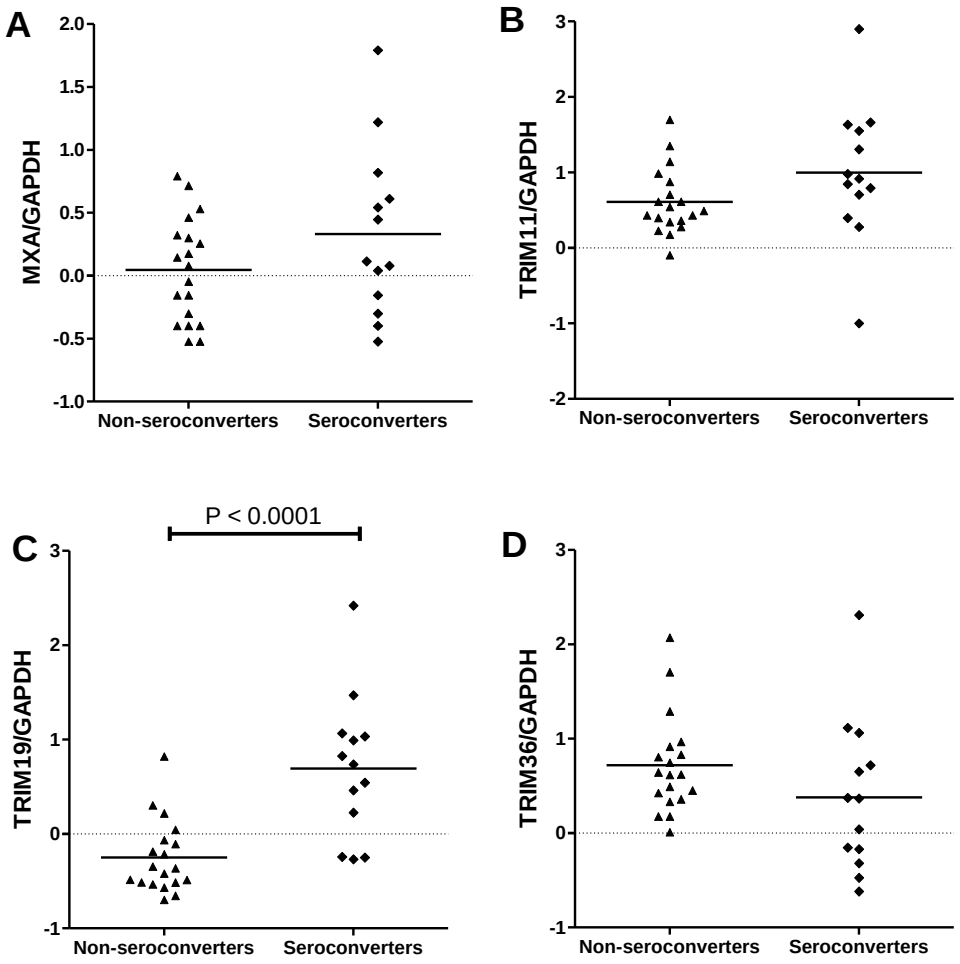


Figure 3.2.2 Expression of MxA and TRIME3 Ligases mRNA in PBMC at baseline for non-seroconverters versus seroconverters. Participants included in this analysis were all enrolled as high risk HIV-1 uninfected individuals and were longitudinally followed for at least 36 months each at the time of analysis. Data are depicted as normalized ratio of MxA, (A) or TRIM11, (B) or TRIM19, (C) or TRIM36, (D) versus GAPDH. The expression values were log-transformed to ensure normality. The horizontal line represents the median. The differences between groups were evaluated by using an unpaired Student's t test. A P-value of < 0.05 was considered as statistically significant.

Kinetics of gene expression in the 13 seroconverters for whom pre- and post infection PBMCs where available

HIV-1 infection could be associated with dysregulation of validated or potential intrinsic antiviral factors such as MxA, TRIM11, TRIM19 and TRIM36. We thus we examined the kinetics of MxA, TRIM11, TRIM19 and TRIM36 mRNA expression in matched HIV-1 negative and positive samples from the 13 individuals who acquired HIV-1 infection during follow-up. PBMCs were available from these individuals at study enrolment (pre-infection), within the first four months post-infection (acute phase), and at between 4 and 12 months post-infection (early chronic phase). Levels of MxA, TRIM11, TRIM19 and TRIM36 pre-infection, at the acute phase and at early chronic phase for the 13 seroconverters are shown (fig. 3.2.3A, B, C and D). Median MxA, TRIM11 and TRIM19 levels did not significantly differ between any of the three phases (pre-infection, acute and early chronic) analyzed. Also within individual patient level there were fluctuations in TRIM36 mRNA levels between different time points analyzed, both acutely and chronic time points have higher levels of TRIM36 when compared to the baseline levels of TRIM36 when this subset of patients where HIV-1 negative ($p = 0.0.021$), (fig 3.2.3 D).

HIV-1 positive patients had higher levels of MxA, TRIM11 and TRIM19 when compared to the HIV-1 negative group, but there was no difference in TRIM36 levels in both HIV-1 positive and negative patients (fig. 3.2.1). Similar results would be expected in matched pre and post infection samples. We did not see similar results in the matched pre and post HIV-1 infection samples. There were no significant changes in MxA, TRIM11 and TRIM19 levels between the different time points analysed. We

also saw that HIV-1 positive time points in matched pre and post infection samples had higher levels of TRIM36 when compared to the HIV-1 negative time points.

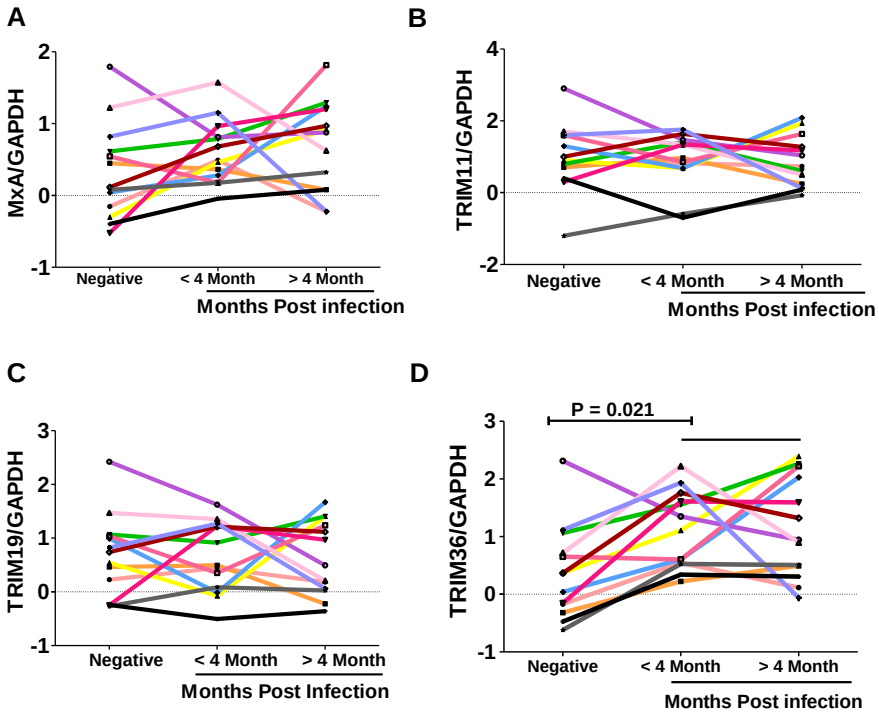


Figure 3.2.3 Kinetics of Gene expression in the 13 seroconverters for whom pre- and post infection PBMCs. Kinetics of MxA, (A) or TRIM11, (B) or TRIM19, (C) or TRIM36, (D) expression in the 13 seroconverters for whom pre- and post infection PBMCs were available. Samples were analyzed pre-infection, at < 4 months post-infection (acute phase) and at > 4 months post-infection (early chronic phase). One expression value is depicted for each time point. The expression values were log-transformed to ensure normality. There were no significant differences in median MxA or TRIM11 or TRIM19 mRNA levels between the 3 phases. However we saw that the post infection samples had higher levels of TRIM36. The differences between groups were evaluated by using the Dunn's multiple comparison Test. A *P*-value of < 0.05 was considered as statistically significant.

Association between IFN-1 and TRIM11, TRIM19 or TRIM36 in HIV-1 negative and positive patients

We investigated association between type 1 interferon and TRIM11, TRIM19 or TRIM36. Since there are 13 functional isoforms of interferon α (Hardy et al., 2009) and only one isoform of interferon-α was quantified in this study, we also extended the analysis to measurement of MxA which is a well characterized correlate for interferon-1 induction.

All TRIM E3 Ligases screened, (TRIM11, TRIM19 and TRIM36) significantly correlated with MxA or IFN-α or IFN-β in both HIV-1 negative and HIV-1 positive patients respectively as depicted in fig. 3.2.4.

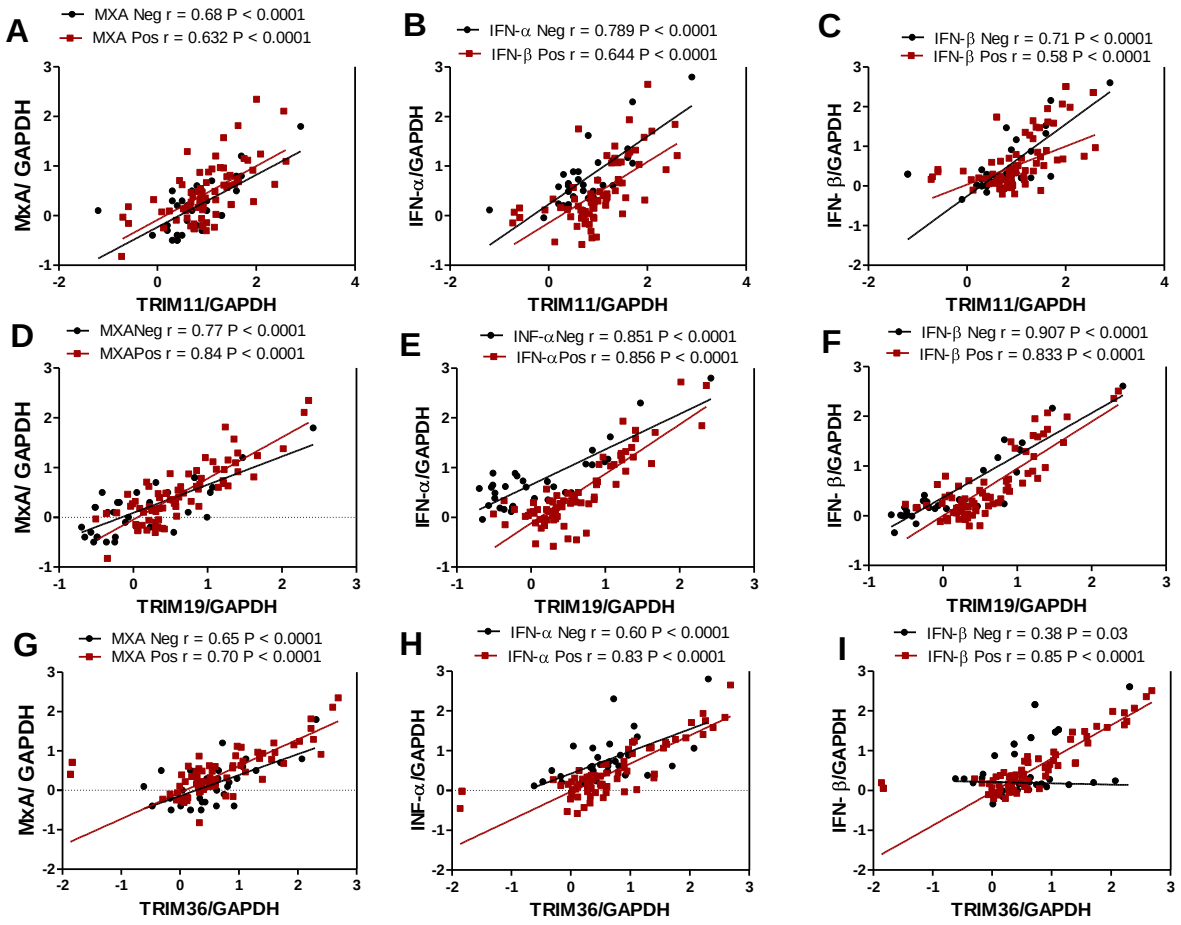


Figure 3.2.4 Association between IFN-1 and TRIM11 or TRIM19 or TRIM36 in HIV-1 negative and positive patients. Pearson correlations were performed for TRIM11 and MXA, (A) or IFN-α, (B) or IFN-β, (C) for both negative and positive patients. Pearson correlations were performed for TRIM19 and MxA, (D) or IFN-α, (E) or IFN-β, (F) for both negative and positive patients. Pearson correlations were performed for TRIM36 and MxA, (G) or IFN-α, (H) or IFN-β, (I) for both negative and positive patients. All expression values where included in the associations depicted.

Association between gene expressions on viral load

To determine if TRIM11, TRIM19 and TRIM36 gene expression had any functional implications for viral control during primary infection we used a Generalized Estimating Equation (GEE) model and fitted it to viral load, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on viral load. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels.

Viral load and expression level was log-transformed to ensure normality. No associations with viral load were noted for TRIM11, TRIM19 and TRIM36. In an adjusted model no significant associations was noted (table 3.2.1).

Table 3.2.1 Association between Gene Expression on Viral load A Generalized Estimating Equation (GEE) model was fitted to viral load, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on viral load. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. Viral load and expression level was log-transformed to ensure normality.

	Unadjusted Models		**Adjusted Models**	
Gene/GAPDH	**Effect estimate (Std Err)**	**p-value**	**Effect estimate (Std Err)**	**p-value**
TRIM11	0.0792 (0.1423)	0.5779	-0.0766 (0.1997)	0.7014
TRIM19	0.1176 (0.1436)	0.4128	-0.3965 (0.4026)	0.3247
TRIM36	0.2231 (0.1281)	0.0815	0.3462 (0.2695)	0.1989

Association between gene expression and $CD4^+$ T cell counts after HIV infection

To further examine the impact of gene expression on HIV-1 disease progression, the $CD4^+$ T cell counts was analyzed. The genes studied include; TRIM11, TRIM19 and TRIM36. A GEE model was fitted to $CD4^+$ T cell counts, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on $CD4^+$ T cell counts. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. A square root transformation was applied to CD4 data. In the unadjusted models, TRIM19 and TRIM36 have all significant negative effects on $CD4^+$ T cell counts, thus

as the expression increases with 1 log, $CD4^+$ T cell counts decreases by 2.14 and 1.57 square root $CD4^+$ T cells/uL respectively. No significant changes in gene expression were noted in adjusted models. Expression level was log-transformed (table 3.2.2.).

Table 3.2.2 Association between gene expression and CD4+ T cell counts after HIV infection A Generalized Estimating Equation (GEE) model was fitted to CD4+ T cell counts, adjusting for repeated measurements within the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on CD4+ T cell counts. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. A square root transformation was applied to CD4+ T cell counts to ensure normality. Expression level was log-transformed.

	Unadjusted Models		**Adjusted Models**	
Gene/GAPDH	**Effect estimate (Std Err)**	**p-value**	**Effect estimate (Std Err)**	**p-value**
TRIM11	-1.6101 (0.9749)	0.0986	0.5938 (1.2017)	0.6261
TRIM19	-2.1429 (0.8750)	0.0143	-2.5938 (1.8838)	0.1685
TRIM36	-1.5673 (0.4272)	0.0002	-1.2096 (0.897)	0.1776

3.2.4 Discussion

Little is known about the involvement of host restriction factors in the defence against or control of retroviral infections in humans. In other species, it has been demonstrated that host restriction factors can completely block or partially restrict infection (Nisole et al., 2005, Stremlau et al., 2004, Goff, 2004a, Harris and Liddament, 2004, Gao et al., 2002). TRIM E3 ligases are a recently discovered family of restriction factors with several members and isoforms (Ozato et al., 2008, Uchil et al., 2008) and their overall effects on retroviral restriction are incompletely understood. An extensively studied protein in this family is TRIM5α, which is responsible for species-specific post entry restriction of HIV-1 in primate cells and to a lesser extent in human cells (Sayah et al., 2004, Zhang et al., 2006, Yap et al., 2004). However, it is unclear what role the other members of the TRIM family play in anti-HIV immunity and there is little data available on how these diverse proteins are regulated in the human host.

To extend our earlier findings where we observed in a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, elevated expression of huTRIM5α is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). Results in the above mentioned study showed that in matched samples of HIV-1 negative individuals that seroconverted, the huTRIM5α levels were not dysregulated following HIV-1 infection (Sewram et al., 2009). Our group has also recently shown that the expression of TRIM22, but not the expression of huTRIM5α, in the PBMC of HIV-1 primary infected patients positively correlated with type I IFN

and $CD4^+$ T cell counts, whereas a negative correlation was shown between TRIM22 expression and plasma viral load (Singh et al., 2011).

Therefore this study examined if other TRIM E3 ligases have similar expression profiles as huTRIM5α or TRIM22 and whether there is a similar association between the other TRIM E3 ligases expression levels and reduced susceptibility to HIV-1 infection in a well characterized clinical cohort of high risk women. We investigated the relationship between TRIM11, TRIM19, TRIM36 and IFN-1 in HIV-1 negative and positive PBMCs, considering that TRIM19 and TRIM11 have previously been shown to be IFN-1 inducible and has anti-viral properties *in vitro* (Everett and Chelbi-Alix, 2007, Regad et al., 2001, Carthagena et al., 2009, Uchil et al., 2008). Little is known about the anti-viral activity of TRIM36.

In this study HIV-1 positive participants had higher levels of MxA, TRIM11 and TRIM19 when compared to HIV-1 negative participants however there were no significant differences in TRIM36 mRNA expression. This could be due to two possibilities. Firstly, HIV-1 is actively dysregulating MxA, TRIM11, TRIM19 or secondly, these participants who became HIV-1 positive had higher levels at baseline. These two possibilities were discriminated by analyzing samples from a longitudinal follow up cohort and comparing at pre-infection MxA, TRIM11, TRIM19 and TRIM36 mRNA levels for 13 individuals who subsequently became seropositive versus similar samples from 19 non-seroconverters from the same cohort. Interestingly, seroconverters had higher mRNA levels of MxA, TRIM11 and TRIM19 with only TRIM19 being significantly different.

I have recently shown that this particular group has high levels of TRIM22 and low levels of huTRIM5α (Singh et al., 2011, Sewram et al., 2009). There seems to be interplay between the different TRIM E3 ligases screened and it could be speculated that the hosts compensated for a decrease in huTRIM5α by increasing TRIM22 and TRIM19.

I investigated whether HIV-1 infection is associated with dysregulation of MxA, TRIM11, TRIM19 and TRIM36. The results showed that in the matched pre and post-infection samples there was no significant difference in median MxA, TRIM11 and TRIM19 expression. Median MxA, TRIM11 and TRIM19 mRNA levels remained relatively steady throughout primary HIV-1 infection, although levels did fluctuate from one time point to another within individuals. MxA, TRIM11 and TRIM19 have previously shown to be an interferon-1 regulated (Carthagena et al., 2009) and IFN-α is transiently dysregulated during primary HIV-1 infection (Kamga et al., 2005). The possible explanation for this result is that these matched samples, emerging from a cohort of high risk women longitudinally followed over time to identify acute infections, may have already reached an optimal threshold of immune activation. This may be due to the result of exposure to the virus or other infections, such that they were unable to further mobilize antiviral type 1 interferon related immune responses beyond this threshold. Therefore, it is possible that these 13 individuals represent a unique population whose immune IFN-1 responses are not altered upon acquisition of HIV-1, as they had similar expression levels as the HIV-1 positive patients analyzed in the unmatched subset.

Surprisingly, TRIM36 was upregulated in matched pre- and post-infection samples, with HIV-1 positive time points having higher median levels of TRIM36 mRNA levels. There was no presence of dysregulation of TRIM36 in unmatched negative and positive patients as TRIM36 levels were similar. We speculate that these 13 individuals represent a unique population whose TRIM36 expression are altered upon acquisition of HIV-1, as they had high levels of TRIM36 in matched HIV-1 positive patients. Very little is known about TRIM36 regulation *in vivo*. There have been no studies demonstrating that TRIM36 has anti-viral activities however studies have shown that TRIM36 is a protein localized in the cytoplasm (Reymond et al., 2001) and involved in prostate tumorigenesis (Balint et al., 2004).

Since TRIM E3 ligases have been shown to be IFN-1 inducible *in vitro* (Barr et al., 2008), we next investigated association between IFN-1 and TRIM11, TRIM19 and TRIM36. All TRIM E3 ligases screened correlated with IFN-1(MxA, IFN-α and IFN-β) in both HIV-1 negative and HIV-1 positive participants. TRIM11 and TRIM36 have a SPRY domain (Carthagena et al., 2009) which has shown to have antiviral activity (Uchil et al., 2008). These results suggest that the TRIM E3 ligases screened, (TRIM11, TRIM19 and TRIM36) are IFN-1 inducible *in vivo*, supporting *in vitro* data obtained by other groups (Martinez et al., 2006, Rajsbaum et al., 2008, Carthagena et al., 2009). The RING domain of many TRIM proteins has been shown to have E3 ubiquitin ligase activity (Meroni and Diez-Roux, 2005). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (Reymond et al., 2001, Towers, 2005). All three TRIM E3 ligases screened, TRIM11, TRIM19 and TRIM36 have a coil-coil, RING and B-Box2 domains (Short

and Cox, 2006, Carthagena et al., 2009, Ozato et al., 2008) and this could be attributing to the antiviral activity seen in TRIM11 and TRIM19 which has previously shown to be interferon 1 inducible *in vitro*. It could be speculated that since TRIM36 expression correlate with interferon 1 expression *in vivo* as seen in the current study that this gene may pocess anti viral properties and the exact mechanism for antiviral activity needs to be elucidated by further *in vitro* experiments such as interferon 1 titration experiments on some immortalized immune cell lines.

I looked at the association of antiviral gene expression and viral load or CD4 T cell counts, two commonly used markers of disease progression. In unadjusted models we observed that TRIM19 and TRIM36 showed a significant negative association with $CD4^+$ T cell counts, however, in adjusted models no association was noted.

The TRIM E3 ligases screened follow a similar expression profile as IFN-1. Since IFN-1 have 13 functional isoforms of IFN-α, we may not have fully elucidated its full impact in modulating the TRIM E3 ligases screened.

This is the first study to show that HIV-1 positive participants had higher levels of TRIM11 and TRIM19 compared to HIV-1 negative participants. TRIM11, TRIM19 and TRIM36 correlate with IFN-1 (IFN-α, IFN-β and MxA) in both HIV-1 positive and negative patients. The study of TRIM E3 ligases as possible anti-viral factors is still in its infancy. TRIM E3 ligase offers a new avenue of research as novel targets to HIV-1, for drug development. More mechanistic studies are clearly warranted on the regulation of TRIM E3 ligases in cell lines and other patient populations. Further studies will be needed to address what specific cell types in the PBMC milieu express TRIM11, TRIM19, TRIM36 and the other members of the TRIM family, to better

define how expression of these proteins is regulated and to address whether these proteins can be harnessed for antiviral therapies or prophylactics.

3.3 Regulation of huTRIM5α, TRIM22 and IFN-1 in chronic HIV-1 infection

3.3.1 Abstract

Background

I have recently demonstrated that huTRIM5α and TRIM22 which are induced by type 1 interferons and may be associated with reduced susceptibility to anti-HIV immune control during primary infection but the role of these factors is unknown in chronic infection. In the current study I investigated the expression of interferon-1 (myxovirus resistance protein A, MxA), huTRIM5α and TRIM22 in a chronically infected cohort with chronic meningitis. I tested the hypothesis that the abundance of the above mentioned HIV-1 restriction factors decrease during chronic HIV-1 infection as the immune system is being dysregulated by HIV-1 and other infections. We further hypothesized that these HIV-1 restriction factors localize at a site of infection in the central nervous system (CNS).

Results

The chronically HIV-1 infection group have significantly higher levels of MxA ($p < 0.0001$) and TRIM22 ($p = 0.0171$) when compared to the HIV-1 negative group. Similar results were obtained for the primary HIV-1 infectction group and the HIV-1 negative group for MxA ($p < 0.05$) and TRIM22 ($p = 0.0052$) respectively. The chronically HIV-1 infected group had lower levels of huTRIM5α when compared to HIV-1 primary infection group, $p < 0.0001$ or HIV-1 negative group, $p < 0.0001$. In matched CNS-derived and peripheral blood mononuclear cell samples, higher levels of MxA ($p < 0.001$) was noted in the CNS when compared to the periphery, however

huTRIM5α levels were lower in the periphery when compared to the CNS compartment, ($p < 0.0001$). I also have shown that the different cell types such as CD4 and NK cells express higher levels of huTRIM5α when compared to monocytes ($p < 0.001$ respectively). Similar levels of TRIM22 were noted in the CNS compartment when compared to the periphery. There was weak but significant negative association between TRIM22 levels and viral load in the periphery, ($r = -0.397$, $p = 0.044$).

Conclusions

Both huTRIM5α and TRIM22 were expressed in PBMC and CNS-drived cells. TRIM22 but not huTRIM5α was associated with low viremia in PBMCs. However, TRIM22 was not associated with viral control in the CNS. There appears to be an intricate balance between the roles of TRIM22 as a marker of viral control versus immune activation, which warrants further investigation.

3.3.2 Background

HIV-1 infection disrupts certain elements of the innate immune system (Boasso and Shearer, 2008, Donaghy et al., 2001). The first response to a pathogen is the production of type I IFNs α and β which are produced by many cell types (Sen, 2001). The role of IFNα/β in HIV-1 infection is controversial, as some studies have shown protective roles of IFN-1 while others have highlighted the pathological roles of IFN-1 (Hardy et al., 2009). It has been demonstrated that the administration of recombinant human IFN-α to patients in the asymptomatic phase of HIV-1 infection is beneficial, with attenuated CD4 T cell decline and reduction in the incidence of AIDS defining events, although these effects were not observed in more advanced disease (Lane et al.,

1990, Kovacs et al., 1996). Transiently high levels of endogenous serum IFN-α have been described in primary HIV-1 infection (von Sydow et al., 1991, Khatissian et al., 1996).

Type 1 interferons induce the expression of some members of the antiviral tripartite motif (TRIM) E3 ligase family of proteins, which consists of approximately 70 distinct proteins characterized by the presence of a RING domain, one or two B-boxes and a coiled coil domain (Uchil et al., 2008, Reymond et al., 2001, Meroni and Diez-Roux, 2005). TRIM5α, the best characterized of these proteins blocks HIV-1 replication in Old World monkey cells through a direct interaction with the viral capsid (Sayah et al., 2004, Stremlau et al., 2004). TRIM5α is responsible for species-specific post entry restriction of retroviruses, such as murine leukemia virus (N-MLV) and HIV-1 in primate cells (Yap et al., 2004, Stremlau et al., 2004).

TRIM22 is also interferon-1 inducible and inhibits viral replication by interfering with viral gene transcription and virion assembly (Barr et al., 2008, Bouazzaoui et al., 2006, Singh et al., 2011, Tissot and Mechti, 1995, Kajaste-Rudnitski et al., 2011). Genetic association studies have demonstrated that polymorphic variants of the human TRIM5α gene are associated with reduced susceptibility to HIV infection or overrepresented among HIV negative individuals compared to HIV positive ones (Javanbakht et al., 2006, Speelmon et al., 2006), suggesting that huTRIM5α may have some protective role against HIV-1 infection. It has also been reported that huTRIM5α genetic variants can influence the rate of disease progression, although the effects appear to be phase of infection dependent or modest (Goldschmidt et al., 2006, van Manen et al., 2008). It has also been suggested that human TRIM5α may select

for escape mutants after a prolonged duration of HIV-1 infection (Kootstra et al., 2007). In a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, we have recently shown that elevated expression of human TRIM5α (huTRIM5α) is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). We subsequently found that TRIM22 but not huTRIM5α, IFN-α, IFN-β or MxA expression showed a negative correlation with plasma viral load ($p=0.0307$) and positive correlation with $CD4^+$ T cell counts ($p=0.0281$) in primary HIV-1 infection, suggesting a protective, antiviral role *in vivo* (Singh et al., 2011).

The role of TRIM E3 ligases as an important component of innate defense against HIV-1 is therefore now well established. However, little is known about whether TRIM E3 ligases have significant antiviral activity *in vivo*, how they may be regulated and affected by HIV infection. We sought to understand whether these antiviral factors are differentially regulated according to the phase of HIV-1 infection and if there are compartmental differences in expression. We hypothesized that TRIM E3 ligases may play more significant in antiviral immunity during the early phases of infection as has been demonstrated with other components of antiviral innate immune mechanisms (Jefferies et al., 2011). Alternatively, the antiviral impact of these factors may become more pronounced as adaptive immune mechanisms become progressively dysfunctional in chronic HIV infection. Furthermore, innate defenses such as TRIM E3 ligases may be more important in remote or immune privileged sites without well-developed adaptive immune systems. We therefore studied here the relationships between the expression of IFN-1 and IFN-1-inducible genes, particularly huTRIM5α and TRIM22 in acute and chronic HIV-1 infection and in peripheral blood and central

nervous derived cells and investigated the impact on biomarkers of disease progression.

3.3.3 Results

Expression of IFN-1 (MxA), huTRIM5α and TRIM22 in PBMC from HIV-1 uninfected versus infected subjects and expression of MxA, huTRIM5α and TRIM22 in periphery and CSF-derived cells

We have previously shown that HIV negative individuals have higher levels of huTRIM5α when compared to HIV-1 positive patients (Sewram et al., 2009). In contrast, HIV-1 infected patients had higher levels of TRIM22 compared to HIV negative individuals and whereas there was no correlation between huTRIM5α and markers of disease progression, TRIM22 expression levels correlated negatively with viral load during early HIV-1 infection (Singh et al., 2011). These data suggested that despite being IFN-α-inducible, these TRIM E3 ligases may have different expression dynamics *in vivo* and play contrasting antiviral roles at preventing HIV-1 infection establishment or replication. Here, we sought to better understand the relationship between the expression of huTRIM5α, TRIM22 and a correlate for IFN-1 induction, myxovirus resistance protein A (MxA), in primary (or early) versus chronic HIV-1 infection. Furthermore, it is unknown whether TRIM E3 ligases are expressed in cells that reside or home into the CNS during inflammatory processes and whether these proteins may contribute to antiviral immunity in these sites. mRNA expression levels were compared in 19 HIV-1 negative individuals, 28 HIV-1 infected samples in primary infection and .in 26 matched cerebrospinal fluid **(**CSF) and PBMCs samples

obtained during the chronic phase of HIV-1 infection. HIV-1 chronically positive participants had significantly higher levels of MxA ($p < 0.0001$) and TRIM22 ($p = 0.0052$), (Fig3.3.1 A, C) in the periphery when compared to the HIV-1 negative group. Similarly, the primary infected patients also had higher levels of MxA ($p < 0.05$) and TRIM22, ($p = 0.0171$), (Fig3.3.1 A, C). Both the primary and chronically infected patients had lower levels of huTRIM5α ($p < 0.0001$) when compared to the HIV-1 negative group, (Fig3.3.1. B).

We analysed the mRNA levels of MxA, huTRIM5α and TRIM22 both in the periphery and the site of infection in chronically HIV-1 infected group. We show that there are higher levels of MxA ($p = 0.0022$) and huTRIM5α ($p < 0.0001$) at the site of infection compared to the periphery, (Fig3.3.1. D, E) however there are no significant changes in TRIM22 (Fig3.3.1 F).

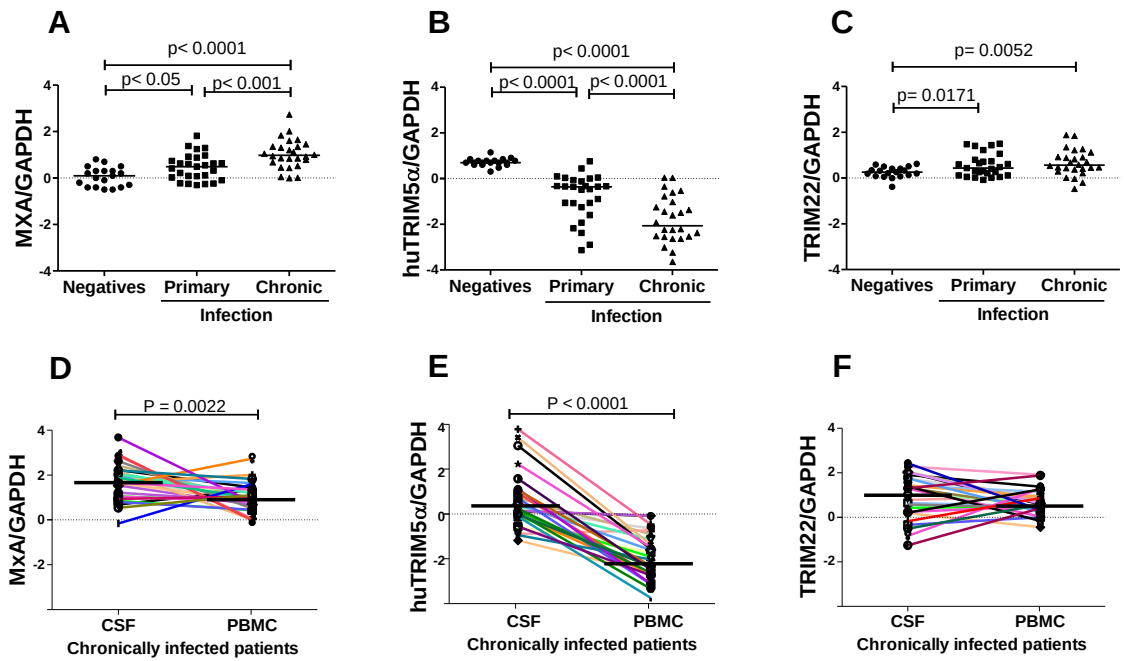


Figure 3.3.1 Expression of IFN-1 (MxA), (A), huTRIM5α, (B) and TRIM22, (C) in PBMC from HIV-1 uninfected versus infected subjects and expression of MxA, (D) huTRIM5α, (E) and TRIM22, (F) in periphery and in CSF-derived cells The primary infected participant samples were all collected within 12 months of infection and we compared one time point, closest to the set point of 12 months post infection (n=28). Only patients that remained HIV-1 negative on follow up where used for this analysis, (n=19). The chronically infected group consisted of participants presenting with chronic meningitis, (n=26). Matched CSF and PBMC's from the same patients were analysed, (n=26). Data are depicted as normalized ratio of MxA or huTRIM5α or TRIM22 versus GAPDH. The expression values were log-transformed to ensure normality. Median expression levels between HIV negative and HIV positive samples were compared. The differences between groups were evaluated by using the Kruskal-Wallis test and Dunn's multiple comparison tests. A *P*-value of < 0.05 was considered as statistically significant.

Cell populations in cerebrospinal fluid (CSF) and PBMCs in 9 matched patients & baseline expression of huTRIM5α and TRIM22 in immune cells

These results detailed in figure 3.3.1, showing higher levels of MxA and huTRIM5α at the site of infection led us to look at cell types found in the CSF. We reasoned that higher amounts of specific cell types may be driving the expression of these anti-viral factors. We found that this compartment has significantly higher amounts of T cells ($p = 0.0134$) and natural killer (NK) cells ($p = 0.0008$), (fig3.3.2A) when compared to the periphery of these patients with chronic HIV-1 infection and a meningitis. Lower levels of monocytes were noted in CSF when compared to periphery, ($p < 0.0001$), (fig 3.3.2A). The higher levels of these specific cell subsets in the CNS led us to investigate mRNA levels of huTRIM5α and TRIM22 in monocytes, CD4 and NK cells isolated from HIV-1 participants. Higher levels of huTRIM5α were noted in CD4 cells ($p < 0.0001$) and NK cells ($p < 0.0001$) when compared to monocytes (fig3.3.2B). No significant differences were noted for TRIM22 in these cell populations, (fig3.3.2C).

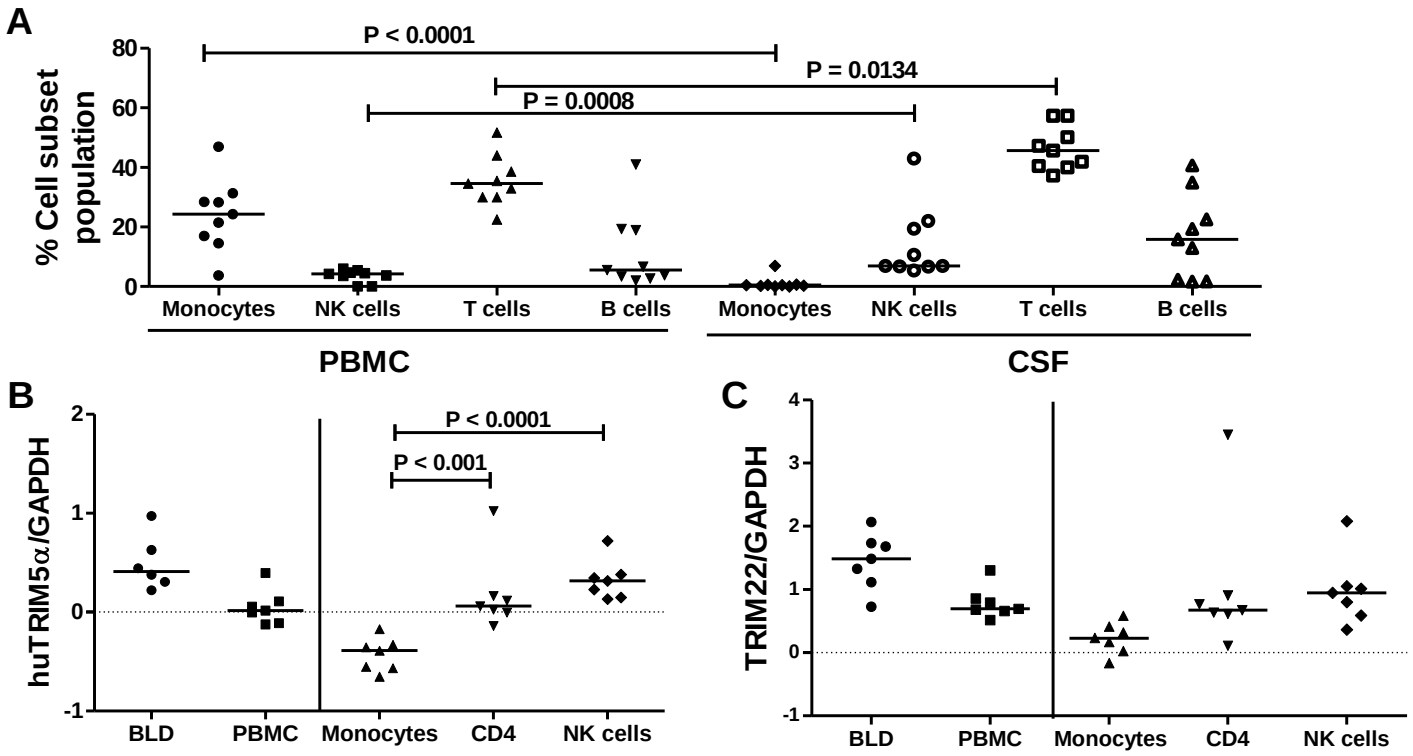


Figure 3.3.2 Cell populations in CSF & PBMCs in 9 matched patients & baseline expression of huTRIM5α and TRIM22 in immune cells. Percentage of monocytes, NK cells, T cells and B cells in CSF vs PBMC from the same patient. Individual points are shown with the horizontal bars indicating medians. Data was compared between each of the subsets of cells in CSF and PBMC only using the unpaired t test, A P-value of < 0.05 was considered as statistically significant, (fig3.3.2A). Magnetic cell sorting was employed to isolate CD4 cells and monocytes from 6 fresh HIV-1 negative PBMCs. Natural killer cells were isolated using the Easy Sep negative selection human NK cell enrichment kit (STEMCELL technologies). Different cell populations where analyzed for huTRIM5α expression, (fig3.3.2B) and TRIM22 expression, (fig3.3.2C). Data are depicted as normalized ratio of MxA or huTRIM5α or TRIM22 versus GAPDH. The differences between groups were evaluated by using the Kruskal-Wallis test and Dunn's multiple comparison tests. A P-value of < 0.05 was considered as statistically significant.

Association between MxA, huTRIM5α and TRIM22 in chronic HIV-1 infection

We next investigated the relationship between huTRIM5α, TRIM22 and IFN-1 (MxA) expression in the periphery and site of infection. There was no correlation between huTRIM5α and MxA in the site of infection or the periphery (fig3.3.3.A). We found significant positive correlation between TRIM22 and MxA in both the site of infection ($r = 0.75$, $p < 0.0001$) and periphery ($r = 0.39$, $p = 0.049$) (fig3.3.3.B). However, we found significant positive correlation between TRIM22 and huTRIM5α in the periphery ($r = 0.475$, $p = 0.014$), (fig3.3.3.C).

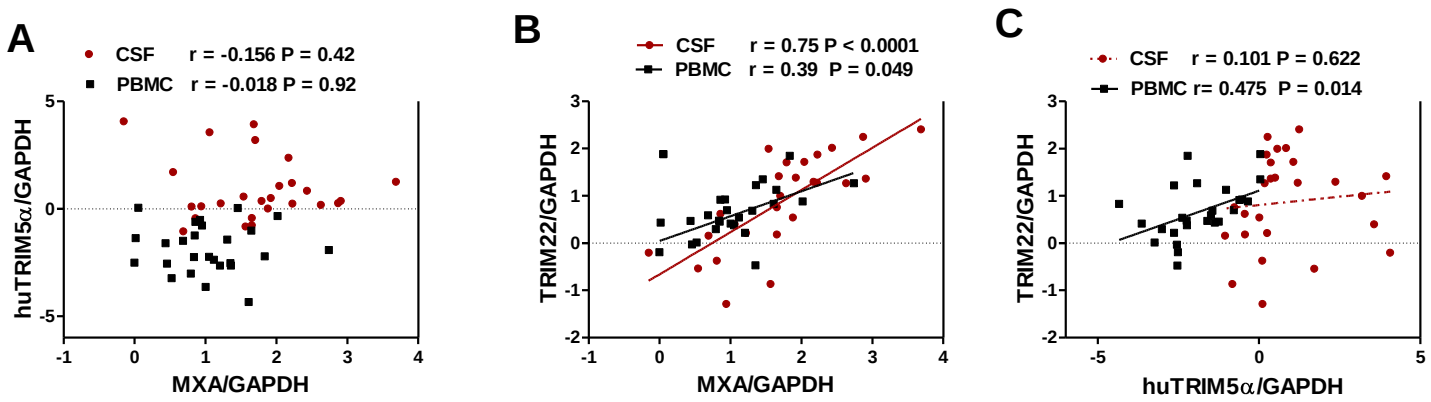


Figure 3.3.3 Association between MxA, huTRIM5α and TRIM22 in HIV-1 patients' with chronic meningitis

Pearson correlations were performed for MxA, huTRIM5α andTRIM22 for both CSF and PBMC from patients in the chronically HIV-1 infected group respectively, (fig3.3.3A, B and C). A *P*-value of < 0.05 was considered as statistically significant.

Markers of disease progression in chronic and primary HIV-1 infection and the association between gene expressions on viral load in chronic HIV-1 infection

We investigated the baseline characteristics of the two groups of samples being analyzed with respect to CD4 T cell counts and viral load. As expected, the chronically infected group had significantly lower levels of CD4 T cell counts when compared to the group in primary HIV-1 infection ($p < 0.0001$), (fig3.3.4A), while this group also has significantly higher levels of viral loads ($p < 0.0001$), (fig3.3.4B). We did not see significant differences in viral load in the chronically infected group between plasma and CSF (fig3.3.4C).

To determine if MxA, huTRIM5α and TRIM22 gene expression had any functional implications for viral control during chronic infection, we correlated viral loads to gene expression. No significant correlations were noted for MxA, huTRIM5α and viral load both in the periphery and the CNS (fig3.3.4D, E). There was negative correlation for TRIM22 and viral load in the periphery ($r=-0.397$, $p=0.044$) (fig3.3.4F).

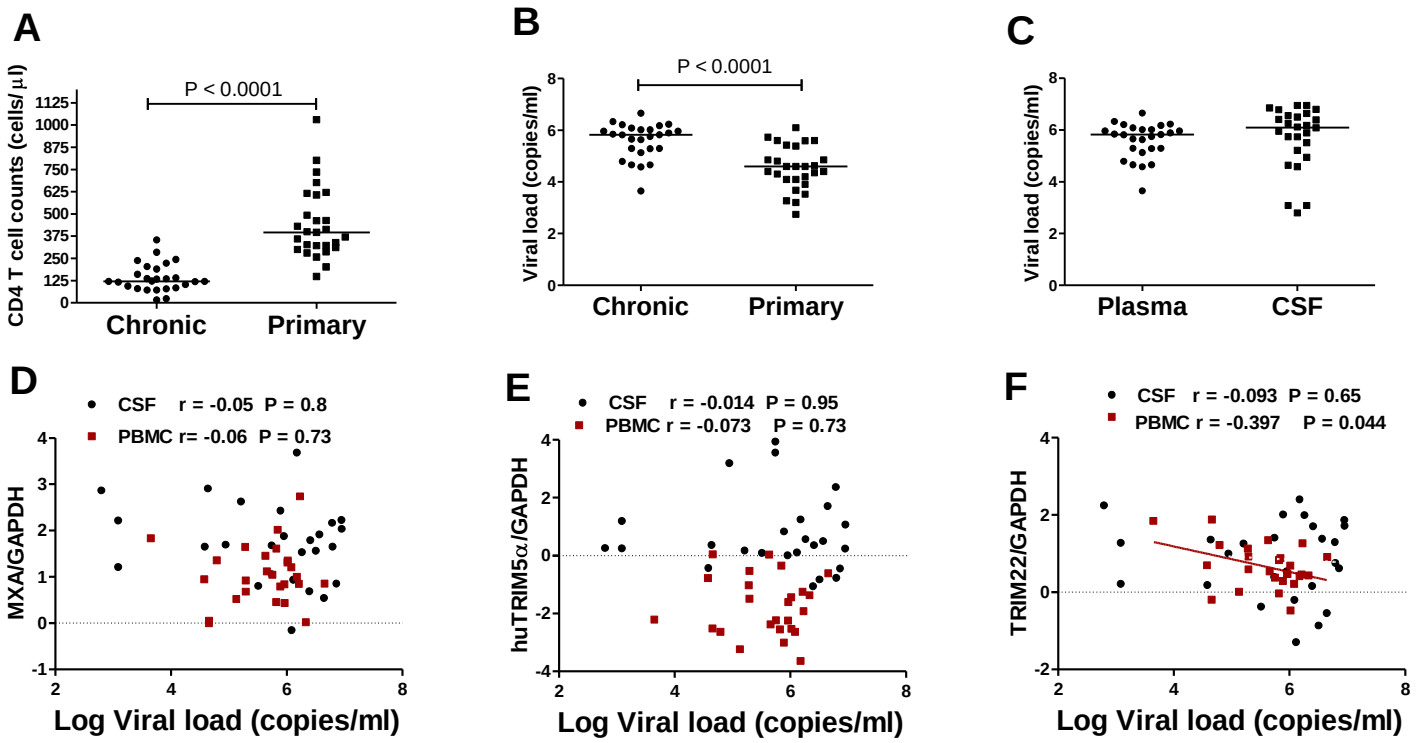


Figure 3.3.4 Markers of disease progression in chronic and primary HIV-1 infection and the association between gene expression on viral load in chronic HIV-1 infection The Primary infected participant samples were all collected within 12 months of infection and we compared one time point, closest to the set point of 12 months post infection (n=28). The chronically infected group consisted of participants presenting with a chronic meningitis, (n=26). The two groups where evaluated for differences in CD4 T cell counts and viral loads respectively, (A, B). Viral loads where compared in chronically infected group in plasma and CSF, (C). The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant. Pearson correlations were performed for MxA, huTRIM5α, TRIM22 and viral load for both CSF and PBMC patients in the chronically HIV-1 infected group respectively, (D, E and F).

In vitro investigation of the impact of immune stimulation and HIV-1 infection on huTRIM5α and TRIM22

The discordant expression levels of huTRIM5α and TRIM22 suggested to us that *in vivo* regulation of these TRIM E3 ligases may be determined by other immune factors or stimulants in addition to the IFN-α which is the best characterized inducer of TRIM E3 ligases expression. We therefore next investigated the direct impact of select pro and anti-inflammatory agents' stimulation on huTRIM5α and TRIM22 expression in the CEM-SS (a human T-cell line). CEM-SS cells were stimulated for 24 hours and cultured with or without HIV-1 over a period of seven days. RNA was isolated, and huTRIM5α and TRIM22 expression levels were analyzed by real-time PCR.

No significant changes in huTRIM5α in CEM-SS cell stimulated with IFN-α, IL-2, CD3/3/28 was noted on day 2 post stimulation however on day 7 post stimulation, significantly higher levels of huTRIM5α was noted, ($p< 0.05$, (fig 3.3.5.A). As noted in Fig 3.3.5A CEM-SS cells stimulated with IFN-α, IL-2 or CD2/3/28 and HIV-1 for 2 or 7 days yielded no significant changes in huTRIM5α. Significantly higher levels of TRIM22 were noted on day 2 post stimulation with IFN-α and day 7 post stimulation with CD2/3/28 and HIV-1, ($p < 0.05$, (fig 3.3.5.B), we did not see significant changes in other groups. Lower levels of p24 were noted in groups stimulated with IF-α or CD2/3/28, (fig 3.3.5.C).

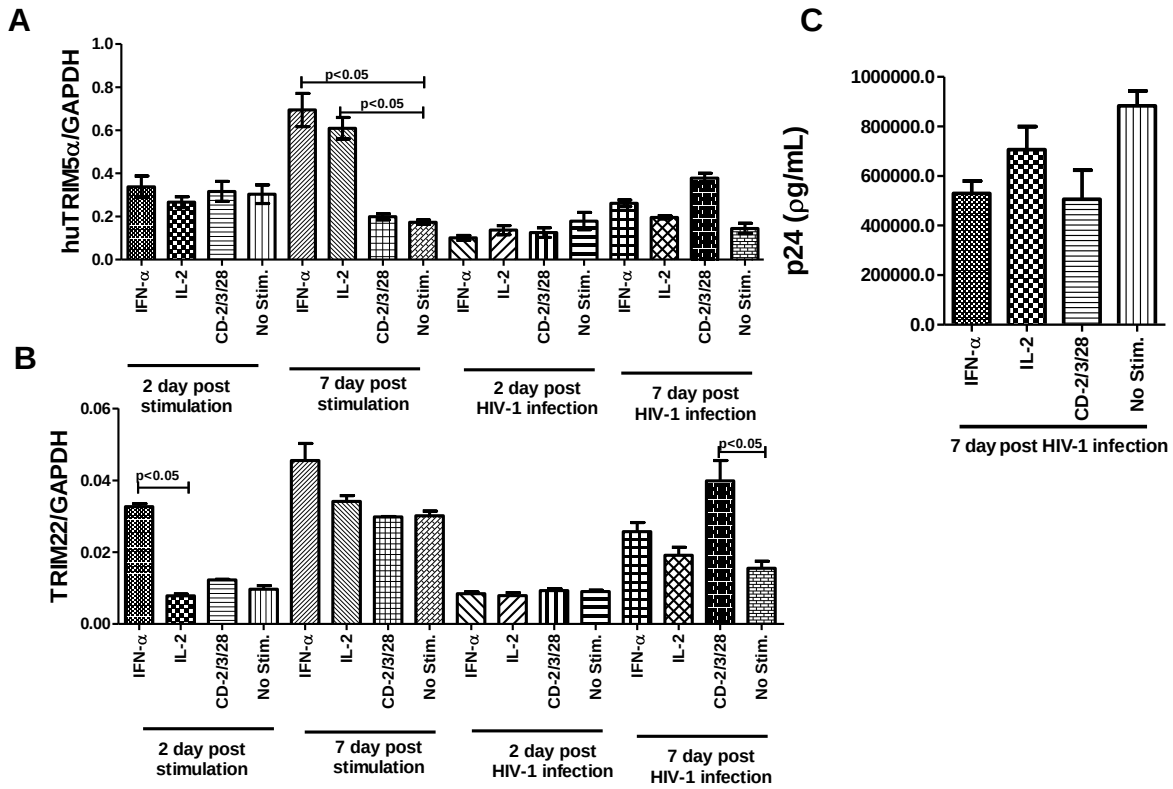


Figure 3.3.5 In vitro investigation of the impact of immune stimulation and HIV-1 infection on TRIM E3 ligases We investigate the direct connection between CEM cells stimulated with cytokines or antibodies, HIV-1 infection over a period of seven days and huTRIM5α or TRIM22 levels. RNA was isolated, and huTRIM5α expression was analyzed by real-time PCR. Results show the mean of three performed experiments (bars = range), each performed in duplicate, (Fig A and B). p24 Elisa were also performed on the above mentioned groups, Fig C. The differences between groups were evaluated by one-way ANOVA. A *P*-value of < 0.05 was considered as statistically significant.

3.3.4 Discussion

There have been numerous studies showing that TRIM22 and TRIM5 block HIV-1 infection *in vitro* (Barr et al., 2008, Stremlau et al., 2004), however their influence of HIV-1 infection *in vivo* has been largely unexplored with only two studies looking at huTRIM5α or TRIM22 expression in HIV-1 primary infection (Sewram et al., 2009, Singh et al., 2011). We have shown in a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, elevated expression of human TRIM5α (huTRIM5α) is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). We have subsequently shown that TRIM22 but not huTRIM5α, IFN-α, IFN-β or MxA showed a negative correlation with plasma viral load ($p = 0.0307$) and positive correlation with $CD4^+$ T cell counts ($p = 0.0281$),(Singh et al., 2011). Therefore it is important to extend these previous studies to chronic HIV-1 infection in order to understand whether TRIM ligases might play a role in all phases of HIV-1 infection. In this study we investigated the association of expression of MxA (a surrogate marker of interferon induction) and two well characterized TRIM E3 ligases (TRIM5α and TRIM22) with viral control in patients with chronic HIV-1 infection with an opportunistic infection (chronic meningitis).

Durring chronic HIV-1 infection there is a large heterogeneity in disease outcome as the time of infection is unknown. Here all patients studied in the chronic HIV-1 infection had an opportunistic infection. The induction of IFN-α/β in the CNS is the first line against viral spread or infections which has been demonstrated by high viral loads and mortality in mice deficient in the IFN-α/β receptor (Paul et al., 2007, Samuel and Diamond, 2005, Ireland et al., 2008, Cervantes-Barragan et al., 2007)

Both TRIM22 and huTRIM5α are interferon-1 inducible (Sakuma et al., 2007, Barr et al., 2008, Carthagena et al., 2009). The TRIM22 gene is located downstream of TRIM5 (Sawyer et al., 2005, Sawyer et al., 2007) and similar transcriptional regulation would be expected. Type 1 interferons are known to be elevated during increased generalized immune activation, which in turn has been associated with increased HIV/AIDS pathology (Mandl et al., 2008, Lehmann et al., 2009). We confirmed previous studies where we show higher levels of MxA in the chronically HIV-1 infected group when compared to primary infection and the HIV-1 negative group in PBMCs (Sedaghat et al., 2008).

Both in PBMCs of the primary and chronically infected groups had higher levels of TRIM22 compared to the HIV-1 negative group; however huTRIM5α is down-regulated during different states of HIV-1 disease pathogenesis where we noted that the chronically infected group has lower levels of huTRIM5α when compared to the primary HIV-1 infection phase and negative groups. This result was confusing as huTRIM5α has been shown to be an IFN-α inducible gene *in vitro* (Sakuma et al., 2007). We dissected this result further by studying the expression of these anti-viral factors in the cells found in the CNS compartment (CSF) of the HIV-1 chronically infected group with chronic meningitis. Moderately higher levels of IFN-1, MxA were noted in the CNS compartment when compared to the periphery as documented by other groups (Fink et al., 2007, Rouse and Sehrawat, Glimaker et al., 1994). We observed higher levels of huTRIM5α in the CNS compartment when compared to the periphery; however we did not see significant differences in TRIM22 between the periphery and CNS compartment.

The increase in IFN-1 in the chronically infected group with HIV-1 may be a result, rather than a cause, of disease progression and may reflect increasing pathologic immune activation, driven by HIV-1 itself, opportunistic pathogens, or other microbial stimuli (Brenchley et al., 2006). A plausible explanation for this result is that immune cells from the periphery translocated through the blood brain barrier into that CNS compartment where they get highly activated. CNS infections induce pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6 and IL-12 in astrocytes and microglia (Bergmann et al., 2006). Peripheral lymphocyte activation further up regulates adhesion molecules and chemokine receptors, which both facilitate entry of circulating lymphocytes into the CNS; compartment (Griffin, 2003, Bergmann et al., 2006, Chen et al., 2005).

The chronically infected group where characterized as being in the end stages of HIV-1 infection with high viral loads and low $CD4^{+}$ T cell counts with a co infection, meningitis. Co-infections with pathogens other than HIV also contributes to immune activation, and some pathogen gene products directly enhance HIV replication by trans-activation of HIV LTR (Lawn et al., 2001, Borkow and Bentwich, 2006). Higher immune activation brought about by the opportunistic infection may have elevated these huTRIM5α and MxA in the CNS compartment relative to periphery.

We further dissected this result by looking at the different cell types found in the CNS (CSF) and tested the hypothesis that higher proportions of immune cells found in the CNS, as consequence of an infection, is driving the expression seen in the CNS compartment when compared to the periphery. We found an enrichment of Natural Killer (NK) and Thymic cells found in the CNS compartment when compared

to matched samples from the periphery. This result is consistent with other studies looking at meningitis and cells found in CSF (Moench and Griffin, 1984, Cepok et al., 2001, Tan et al., 2008).

These data led me to further investigate which specific cells within the milieu of PBMCs express these anti-viral factors. I investigated the expression levels of huTRIM5α and TRIM22 in whole blood, PBMCs, monocytes, CD4 and NK cells from seven healthy HIV-1 negative individuals. I found that Natural Killer and $CD4^+$ T cell cells express higher mRNA levels of huTRIM5α when compared to monocytes.

Since both TRIM5α and TRIM22 have been shown to be interferon-1 inducible we looked at their association with interferon-1 and each other in both the periphery and the site of infection (Carthagena et al., 2009, Sakuma et al., 2007, Barr et al., 2008). I did not see any associations with huTRIM5α and interferon-1 in both the periphery and site of infection. I saw significant associations with interferon-1 and TRIM22 in the periphery and site of infection.

I also investigated the association of anti-viral gene expression and viral load, a commonly used marker of disease progression. I confirmed the results obtained in a previous study where we did not see an association between viral loads and huTRIM5α expression in primary HIV-1 infection, as I saw a similar result in chronic HIV-1 infection (Sewram et al., 2009). I was able to show that TRIM22 has a moderate negative association with viral loads in the periphery ($r=-0.397$, $p=0.044$).

In the current study I could not differentiate between the effects that chronic HIV-1 infection and an opportunistic infection had on the expression of huTRIM5α or TRIM22. I have noted that a higher level of IFN-1, (MxA) was found in the CNS

compartment when compared to the periphery which could be a surrogate marker for immune activation. I tried to differentiate between the effects of immune activation and HIV-1 by stimulating CEM-SS cells with IFN-α, IL-2 and CD2/3/28 followed by infecting these cells with HIV-1 and tracking the expression of huTRIM5α and TRIM22 over a seven day period.

The expression profile of huTRIM5α and TRIM22 is different, when cells are stimulated with IFN-α, IL-2 and CD2/3/28. The greatest induction of huTRIM5α by IFN-α or IL-2 was seen seven days post stimulation. However IFN-α induced TRIM22 from 24 hours post stimulation, by seven days post stimulation we did not significant differences between different stimuli on TRIM22 expression.

We confirmed previous results performed by Obad and colleagues where they showed that CD2/3/28 co-stimulation brings about modest upregulation of TRIM22 in purified NK and CD4 cells; we show similar results in CEM cell lines (Obad et al., 2007). We also confirmed previous studies where we show that IFN-α blocks HIV-1 replication, (Singh et al., Barr et al., 2008, Bouazzaoui et al., 2006). A block in HIV-1 replication was noted in groups stimulated with IFN-α and CD2/3/28. Significant induction in TRIM22 was noted for cells stimulated with CD2/3/28.

Collectively our results demonstrate that the modulation of huTRIM5α and TRIM22 is dependent on the activation state of the cell where different results is obtained at the time of sampling. I observed discordance between induction of TRIM22 or huTRIM5α and inhibition of HIV-1 replication. These results may explain the loss of association between viral load and gene expression seen in the CNS compartment where cells are highly activated.

Based on my findings, I speculate that targeted enhancement of the expression of TRIM22 in HIV-1 infected individuals may be beneficial in reducing viral load and could be employed as a novel anti-viral strategy. I can further speculate one of the approaches would be to reduce immune activation in chronically infected patients as this would enhance these anti-viral factors. Chronic HIV-1 infection is characterized by the dysregulation of the immune system, which in turn makes the individual susceptible to other secondary infections (Sedaghat et al., 2008). This report focuses on patients that are chronically infected with HIV-1 and have deteriorated to having chronic meningitis. This infection is characterized by high immune activation at the site of disease (Sedaghat et al., 2008, Appay and Sauce, 2008).

3.4 Expression and regulation of TRIM E3 ligases (TRIM5 and TRIM22) in human immune and neuronal cells

3.4.1 Abstract

Type 1 interferons (IFN-1) induce the expression of the Tri-partite interaction motif (TRIM) family of E3 ligases but the contribution of these antiviral factors to HIV pathogenesis in the CNS is not understood. We recently reported that TRIM5α expression did not correlate with IFN-1 expression in peripheral blood mononuclear cells *ex vivo*, contrary to findings in immortalized cell lines *in vitro*. Moreover, the impact of pro- and antinflammatory cytokines on TRIM E3 ligases expression is poorly understood. To better understand these relationships, we studied the modulation of TRIM5 (TRIM5α, TRIM5δ, and TRIM5γ isoforms) and TRIM22 in some well characterized T cell lines, B cell lines and neuroblastic cell lines under IFN-α and other cytokine/chemokine stimulation. The expression levels of huTRIM5α, TRIM5δ and TRIM5γ in the neuronal cells were comparable to the immune cells, however lower expression levels of TRIM22 were seen in neuronal cells. The modulation of these TRIM E3 ligases was cytokine/chemokine dependent and was also cell line type dependent. Neuroblast stimulated with TNF-α showed the highest level of huTRIM5α induction when compared to neuroblast stimulated with IFN-α, IL-2 and IL-10 and to other immune cell lines. huTRIM5α and TRIM22 expressed in neuronal cells had a biological function, as the suppression of huTRIM5α or TRIM22 by specific siRNA led to increased pseudotyped HIV-1 replication in these cells. This study shows that TRIM E3 ligases play a part in the innate defenses against HIV-1 in the CNS.

3.4.2 Background

TRIM E3 ligases are group of proteins with potent anti-viral properties. There are approximately 70 members in the group, characterized(Kajaste-Rudnitski et al., 2011) by the presence of the tri-partite motif, which consists of a RING domain, one or two B-box motifs and a coil-coil region (Uchil et al., 2008, Meroni and Diez-Roux, 2005, Reymond et al., 2001). The presence of the RING domain suggests that these proteins function as E3 ubiquitin ligases and mediate ubiquitylation events (Gack et al., 2007). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (Reymond et al., 2001, Towers, 2005). TRIM family ubiquitin ligase activity has been shown for the TRIM5δ isoform, TRIM18/MID1, TRIM25/EPF, TRIM32/HT2A and TRIM35/ARD1 (Meroni and Diez-Roux, 2005).

TRIM5α is responsible for the complete block of HIV-1 replication in Old World monkey cells, and this effect was shown to be mediated through the interaction of TRIM5αrh with the HIV-1 capsid (Sayah et al., 2004, Stremlau et al., 2004). Further studies have suggested that in addition to the effects of TRIM5αrh on HIV *via* binding to capsid, other mechanisms of viral inhibition are possible (Pertel et al., 2011, Perron et al., 2004, Sakuma et al., 2007). There are six isoforms of TRIM5, TRIM5α is the largest isoform coded by the TRIM5 gene and is the only one that encodes a C-terminal B30.2 domain and exhibits antiviral activity (Stoye, 1998, Stremlau et al., 2004). The smaller isoforms of TRIM5 such as TRIM5δ and TRIM5γ lack the C-terminus and when overexpressed act in a negative fashion on TRIM5α by promoting the rescue of restricted infection (Passerini et al., 2006, Stremlau et al., 2004). Pertel

et al showed that in addition to restricting retroviruses, TRIM5 has a general role in immunity through the activation of innate signaling pathways and this activity is amplified by retroviral infection and interaction with the capsid lattice (Pertel et al., 2011)

Other TRIM E3 ligases with antiviral activity have been described (Yap et al., 2004). TRIM family proteins affect specific steps in the HIV life cycle (Hatziioannou et al., 2004b, McNab et al., 2011). TRIM proteins appear to mediate their antiviral activities *via* diverse mechanisms; interference with uncoating of the viral pre-integration complex was noted for TRIM5α (Nisole et al., 2005), inhibition of viral budding has been described for TRIM22 (Ozato et al., 2008).

TRIM proteins have also been identified as IFN-inducible (TRIM19/PML, TRIM22/Staf50, TRIM5), which suggests involvement of TRIM proteins in viral defense (Lavau et al., 1995, Gongora et al., 2000, Asaoka et al., 2005, Barr et al., 2008). Apart from the IFN-inducible TRIM19, TRIM22 and TRIM5, other TRIM proteins have been found to interfere with viral replication, including TRIM1 and TRIM32 (Yap et al., 2004, Fridell et al., 1995, Barr et al., 2008). IFNs are themselves the main mediators of innate immunity against viral infection, and they play a significant role by upregulating the expression of many antiviral effectors within the cell (Fensterl and Sen, 2009, Sadler and Williams, 2008).

Bouazzaoui *et al* demonstrated that the IFN-inducible TRIM22 inhibits the HIV-1 replication in human monocyte-derived macrophages (Bouazzaoui et al., 2006). When determining endogenous expression of TRIM22 in different tissues, it was shown that, in the absence of exogenous IFN treatment, TRIM22 was mainly

expressed in peripheral blood leukocytes, in lymphoid tissue such as spleen and thymus, and in the ovary (Tissot and Mechti, 1995). Kajaste-Rudnitski et al have shown in U937 promonocytic cell lines that are either permissive or nonpermissive for HIV-1 replication, TRIM22 was the only factor constitutively expressed and absent in permissive U937 cells (Kajaste-Rudnitski et al., 2011). We have recently shown in a prospective cohort study of HIV-1 negative individuals at high risk for HIV-1 infection, that elevated expression of human TRIM5α (huTRIM5α) is associated with decreased susceptibility to HIV-1 infection (Sewram et al., 2009). We also demonstrated that TRIM22 but not huTRIM5α, IFN-α, IFN-β or MxA showed a negative correlation with plasma viral load ($p=0.0307$) and positive correlation with $CD4^+$ T cell counts ($p=0.0281$), (Singh et al., 2011).

Previous *in vivo* studies have focused on looking at the mRNA expression of huTRIM5α or TRIM22 in bulk PBMCs (Sewram et al., 2009, Singh et al., 2011). There is now a wealth of evidence, both *in-vivo* and *in-vitro* in well characterized cell lines that induction of TRIM22 inhibits HIV-1 replication (Kajaste-Rudnitski et al., 2011, Singh et al., 2011, Barr et al., 2008). Although it has been demonstrated that type 1 IFNs in lymphocytes, macrophages induce TRIM E3 ligases (Bouazzaoui et al., 2006) there is limited information about the expression and regulation of TRIM5 and TRIM22 in the human central nervous system (CNS), an important target for viral infections including HIV-1. There are no studies to our knowledge that have directly investigated the concomitant regulation of various TRIM5 isoforms and TRIM22 by IFN-1 on a range of cell lines, as both TRIM5 and TRIM22 lie next to each other on chromosome 11 (Sawyer et al., 2007, Tissot et al., 1996). In light of the above we

looked at the modulation of TRIM5, including TRIM5α, TRIM5δ, and TRIM5γ isoforms and TRIM22 in some well characterized T cell lines, B cell lines and neuroblastic cell lines under IFN-α and other cytokine/chemokine stimulation.

3.4.3 Results

Baseline huTRIM5α, TRIM5δ, TRIM5γ and TRIM22 expression in Immune and Neuronal cell lines

We first examined whether neuronal lineage (astrocytes, (U87.CD4 astrocytic cell lines expressing exclusively either CCR5 or CXCR4, NIH-4031) neuroblasts (cortical neurons, CRL-2137 and CRL-2142) cells express huTRIM5α, TRIM5δ, TRIM5γ and TRIM22 when compared to other immune cell lines such as Jurkat (Human T cell lymphoblast-like cell line, NIH-10095), CEM-SS (Human T4-lymphoblastoid cell line, NIH-776) and B lymphoblast (Daudi, CCL-213).

As shown in fig 3.4.1, huTRIM5α, TRIM5δ and TRIM5γ where detectable in neuronal cells. The levels of huTRIM5α, TRIM5δ and TRIM5γ in the neuronal cells where comparable to the immune cells. Neuroblasts (cortical neurons, CRL-2137) had elevated levels of huTRIM5α when compared to CEM-SS ($p < 0.05$) or B. cells, ($p < 0.001$). Jurkat cells had higher levels of huTRIM5α when compared to B cells ($p < 0.05$). Neuroblasts (cortical neurons, CRL-2137) had elevated levels of TRIM5δ when compared to Jurkats or CEM-SS cells, ($p < 0.05$). Neuroblasts (cortical neurons, CRL-2137) had elevated levels of TRIM5γ when compared to Astrocytes, ($p < 0.05$), Jurkat ($p<0.05$) or CEM-SS cells, ($p < 0.001$).

As shown in fig 3.4.1D, TRIM22 was detectable in B cells, CEM-SS, Jurkat, astrocytes and neuronal cell lines. Elevated levels of TRIM22 were noted in B cells when compared to CEM-SS, Jurkat, astrocytes and both neuroblastic cell lines, (cortical neuron, CRL-2137, CRL-2142), $p < 0.0001$.

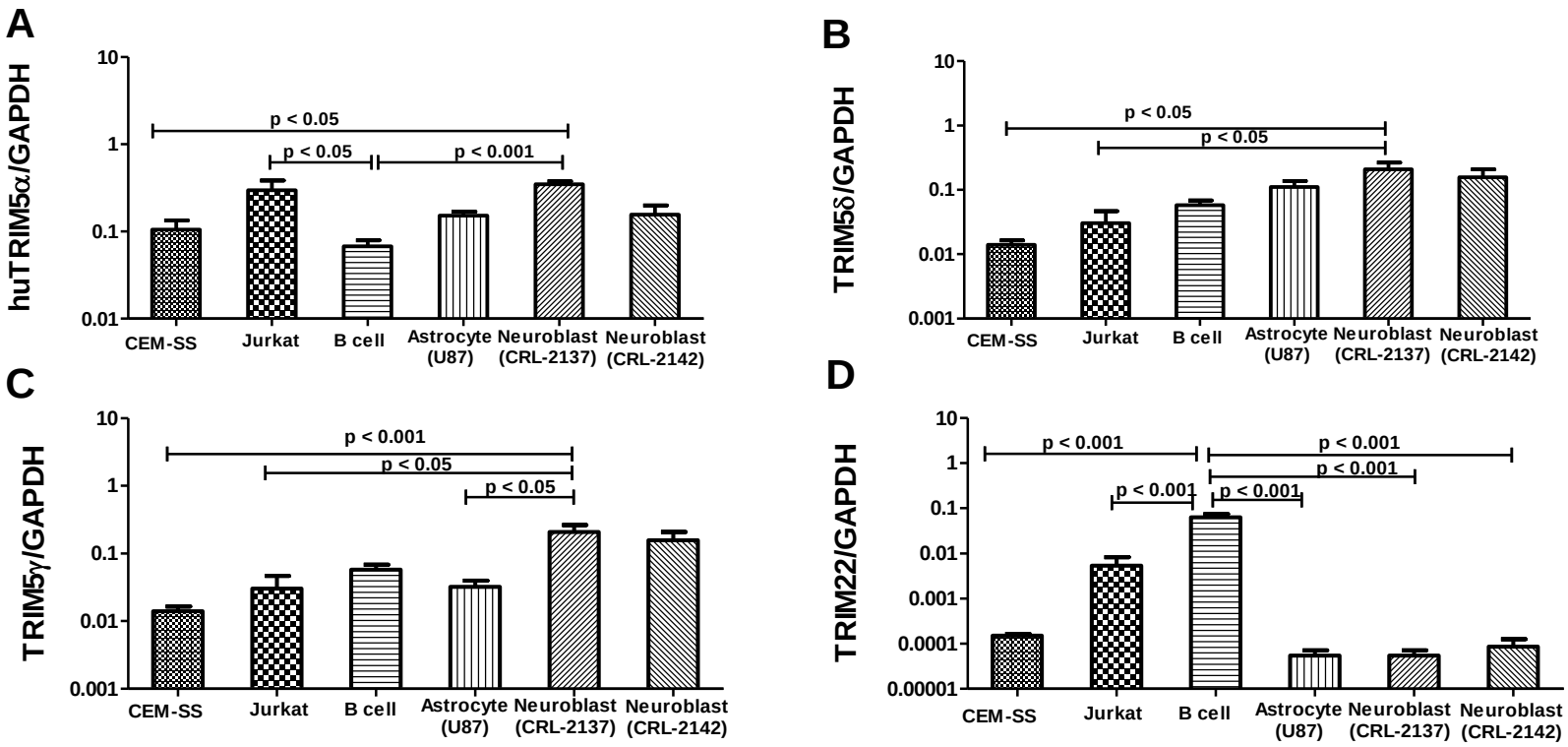


Figure 3.4.1 Baseline huTRIM5α, TRIM5δ, TRIM5γ and TRIM22 expression in Immune and Neuronal cell lines
Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for huTRIM5α, TRIM5δ, TRIM5γ and TRIM22 and GAPDH, (A,B,C and D). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

Regulation of huTRIM5α and TRIM5δ by IFN-α in neuronal and immune cell lines

To investigate the role of IFN-α in the transcriptional induction of huTRIM5α and TRIM5δ in neuronal and immune lineage cells, with increasing amounts of IFN-α (0, 10, 100 and 1000U) and examined huTRIM5α and TRIM5δ expression by RT-PCR. IFN-α upregulated huTRIM5α and TRIM5δ expression in a dose-dependent manner in all six cell lines tested (fig 3.4.2. A, B).

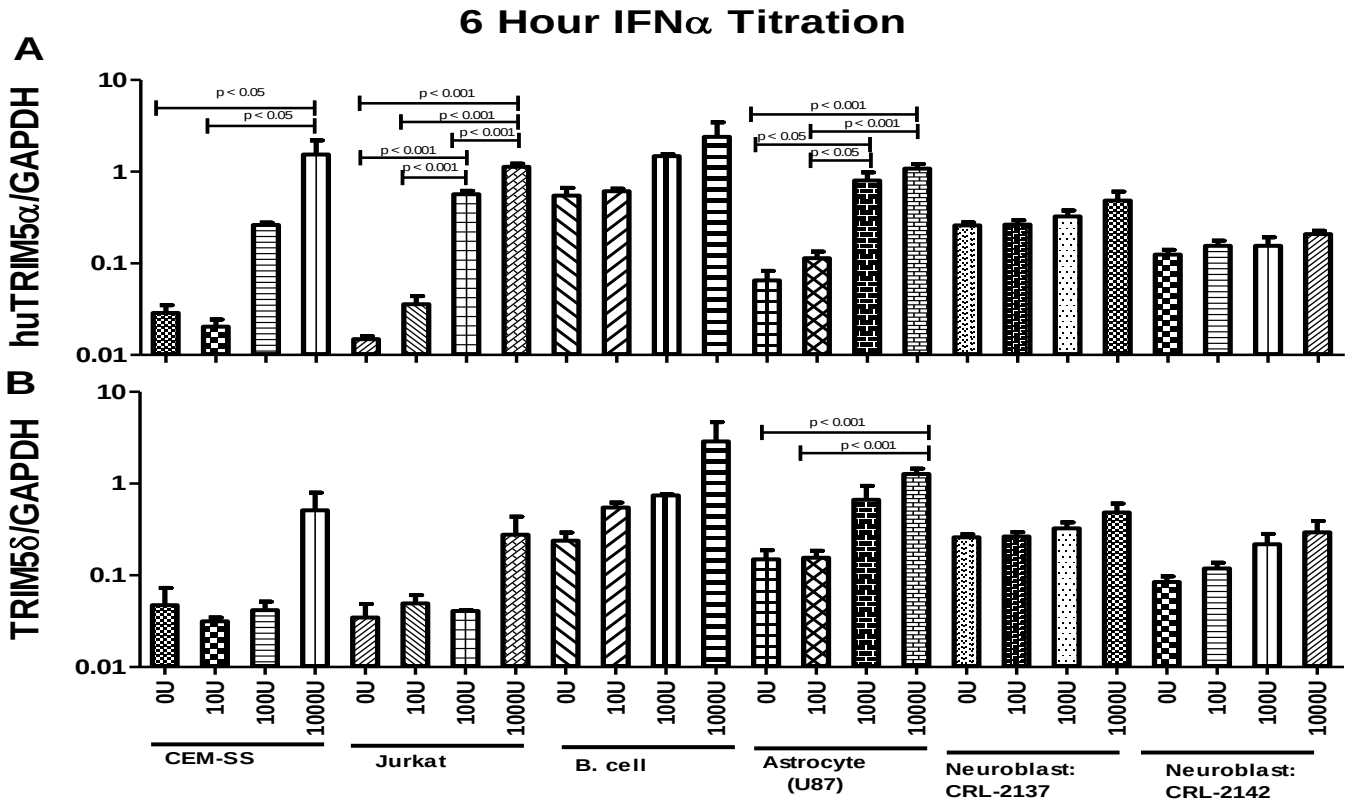


Figure 3.4.2 Regulation of huTRIM5α and TRIM5δ in immune and neuronal cells by 6 hour IFN-α stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for huTRIM5α, TRIM5δ and GAPDH, (A and B). Dose- dependent increases huTRIM5α and TRIM5δ in various immune and neuronal cell lines as determined by RT-PCR. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

Regulation of TRIM5γ and TRIM22 by IFN-α in neuronal and immune cell lines

To investigate the role of IFN-α in the transcriptional induction of TRIM5γ and TRIM22 in neuronal and immune lineage cells, with increasing amounts of IFN-α (0, 10, 100 and 1000U) and examined TRIM5γ and TRIM22 expression by RT-PCR. IFN-α upregulated TRIM5γ and TRIM22 expression in a dose-dependent manner in all six cell lines tested (fig3.4.3 A, B.).

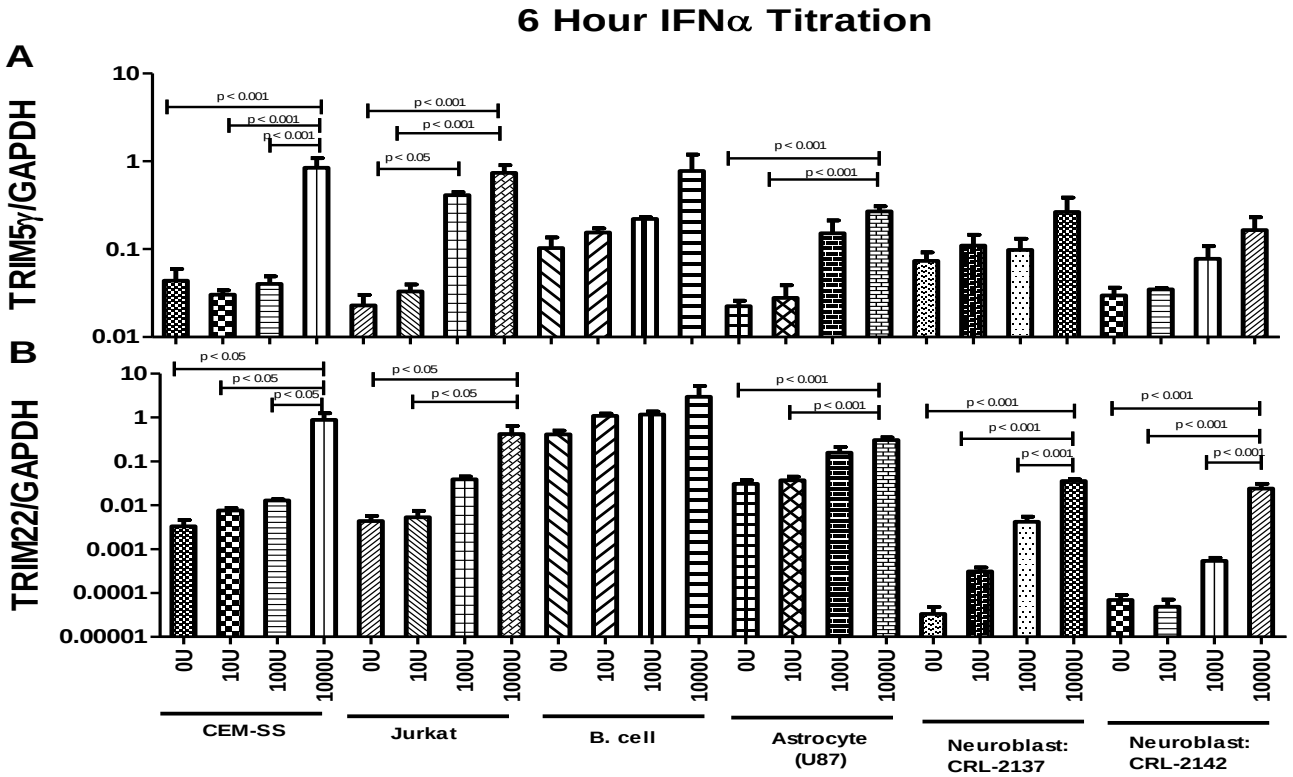


Figure 3.4.3 Regulation of TRIM5γ and TRIM22, IFN-α and IFN-β in immune and neuronal cells by 6 hour IFN-α stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for TRIM5γ and TRIM22 and GAPDH, (A and B). Dose- dependent increases TRIM5γ and TRIM22 in various immune and neuronal cell lines as determined by RT-PCR. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

Regulation of IFN-α and IFN-β by IFN-α in neuronal and immune cell lines

To investigate the role of IFN-α in the transcriptional induction of IFN-α and IFN-β in neuronal and immune lineage cells, with increasing amounts of IFN-α (0, 10, 100 and 1000U) and IFN-α and IFN-β expression by RT-PCR. IFN-α upregulated IFN-α and IFN-β expression in a dose-dependent manner in all six cell lines tested (fig 3.4.4 A and B).

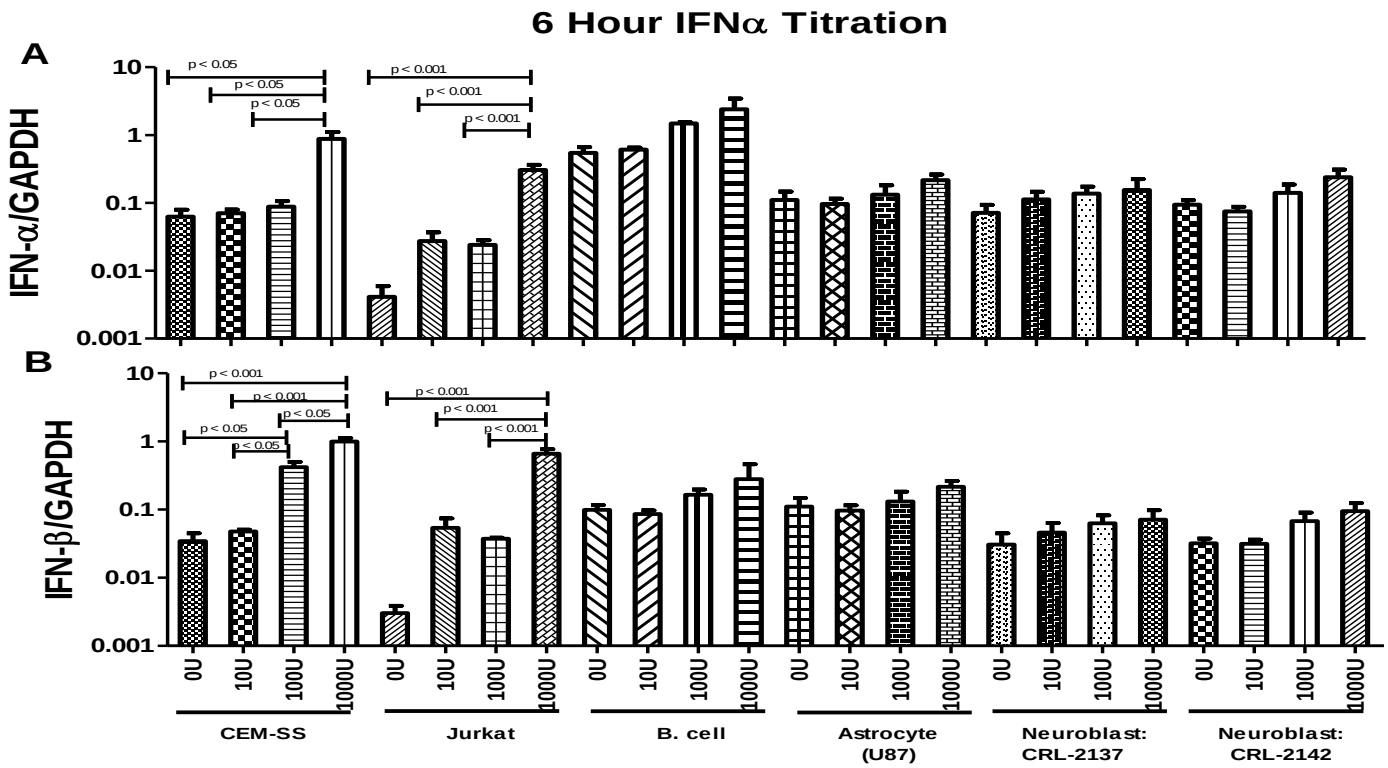


Figure 3.4.4 Regulation of IFN-α and IFN-β in immune and neuronal cell lines by 6 hour IFN-α stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for IFN-α and IFN-β (A and B). Dose- dependent increases in IFN-α and IFN-β in various immune and neuronal cell lines as determined by RT-PCR. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

Cytokine regulation of huTRIM5α, TRIM5δ in neuronal and immune cells

Little is known about the effects of these pro and anti-inflammatory cytokines on the expression of TRIM E3 ligases in immune and neuronal cells. We therefore next investigated the effects of particular cytokines on the expression of huTRIM5α and TRIM5δ in stable cell lines. We tested the hypothesis that the above mentioned cytokines may modulate huTRIM5α and TRIMδ, in immune and neuronal cell lines. As a positive control for the experiment we used IFN-α, since we and others have shown that IFN-α can induce these TRIM E3 ligases. We found that the modulation of these TRIM E3 ligases was specific cytokine and cell line dependant. Both the neuronal cell lines stimulated with IL-2 had lower levels of huTRIM5α when compared to unstimulated control cells (cortical neuron, CRL-2137 and CRL-2142) (fig 3.4.5 A). Neuroblasts, (cortical neuron, CRL-2142) stimulated with IL-2 had higher levels of TRIM5δ when compared to cells stimulated with IFN-α (fig 3.4.5 B).

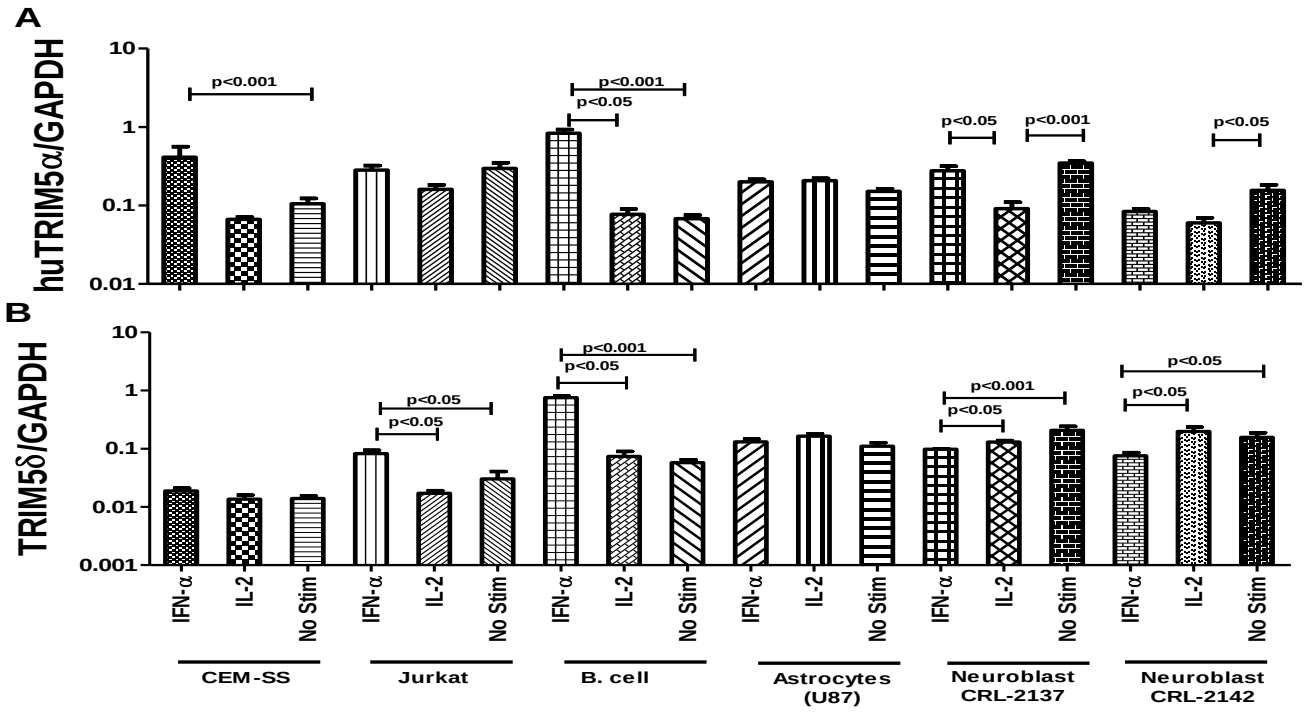


Figure 3.4.5 Regulation of huTRIM5α, TRIM5δ, in immune and neuronal cells by cytokines/chemokines, (IFN-α and IL-2) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for huTRIM5α and TRIM5δ and GAPDH, (A and B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Cytokine regulation of huTRIM5α and TRIM5δ in neuronal and immune cells

I found that the modulation of these TRIM E3 ligases was specific cytokine and cell line dependant. Neuroblasts (cortical neuron, CRL-2142) cells stimulated with IL-10 had higher levels of huTRIM5α when compared cells stimulated with IFN-α, (fig 3.4.6.A). CEM-SS and Jurkat cells stimulated with IL-10 had higher levels of TRIM5δ when compared to unstimulated control cells (fig 3.4.6 B). Both neuroblastic cell lines (cortical neuron, CRL-2137 and CRL-2142) had higher levels of TRIM5δ when compared to cells stimulated with IFN-α (fig 3.4.6 B).

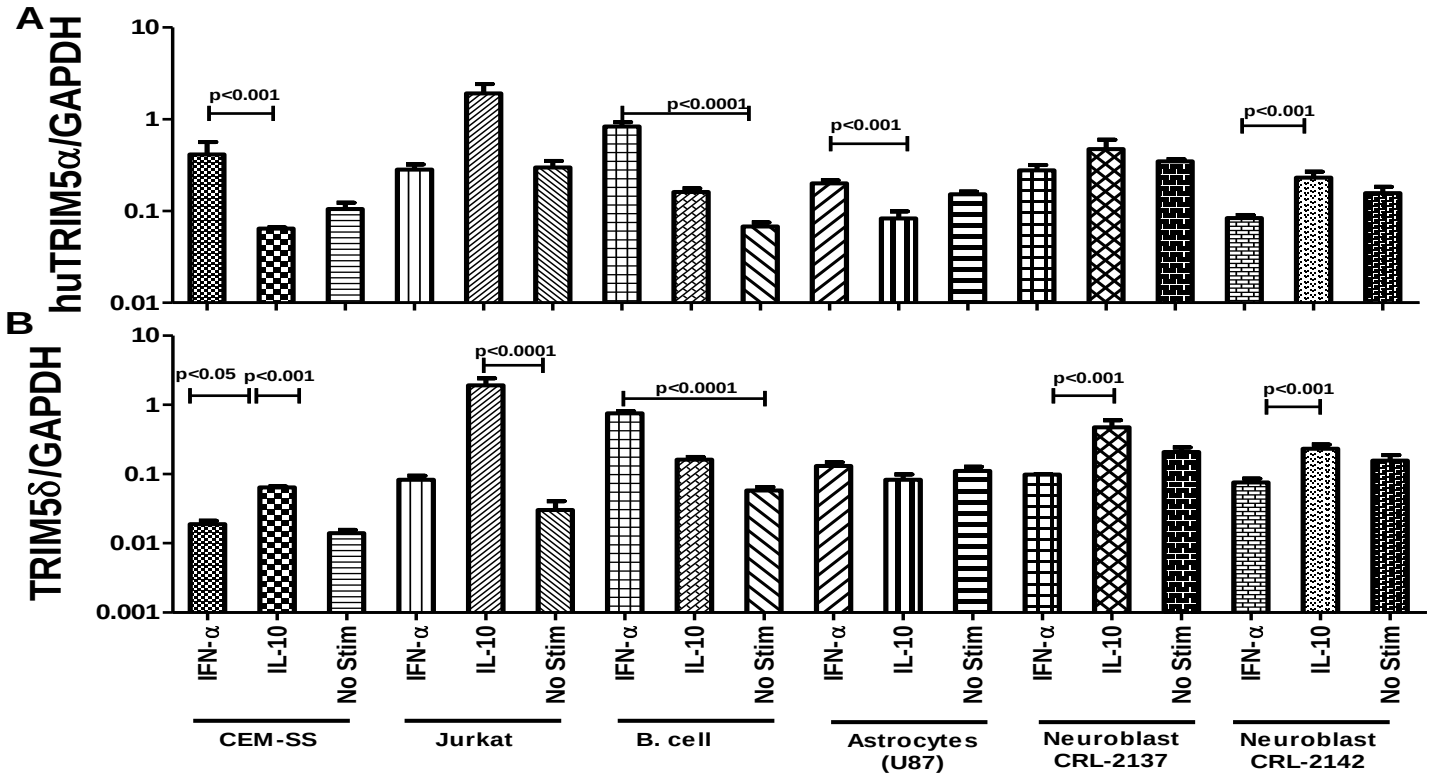


Figure 3.4.6 Regulation of huTRIM5α and TRIM5δ in immune and neuronal cells by cytokines/chemokines, (IFN-α and IL-2) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for huTRIM5α, TRIM5δ and GAPDH, (A and B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Cytokine regulation of huTRIM5α, TRIM5δ in neuronal and immune cells

I found that the modulation of these TRIM E3 ligases was specific cytokine and cell line dependant. Neuroblasts (cortical neuron, CRL-2137) had higher levels of huTRIM5α when compared to cells stimulated with IFN-α, (fig 3.4.7 A). CEM-SS, Jurkat and neuroblasts (cortical neuron, CRL-2137 stimulated with TNF-α had higher levels of TRIM5δ when compared to control unstimulated cells (fig 3.4.7 B).

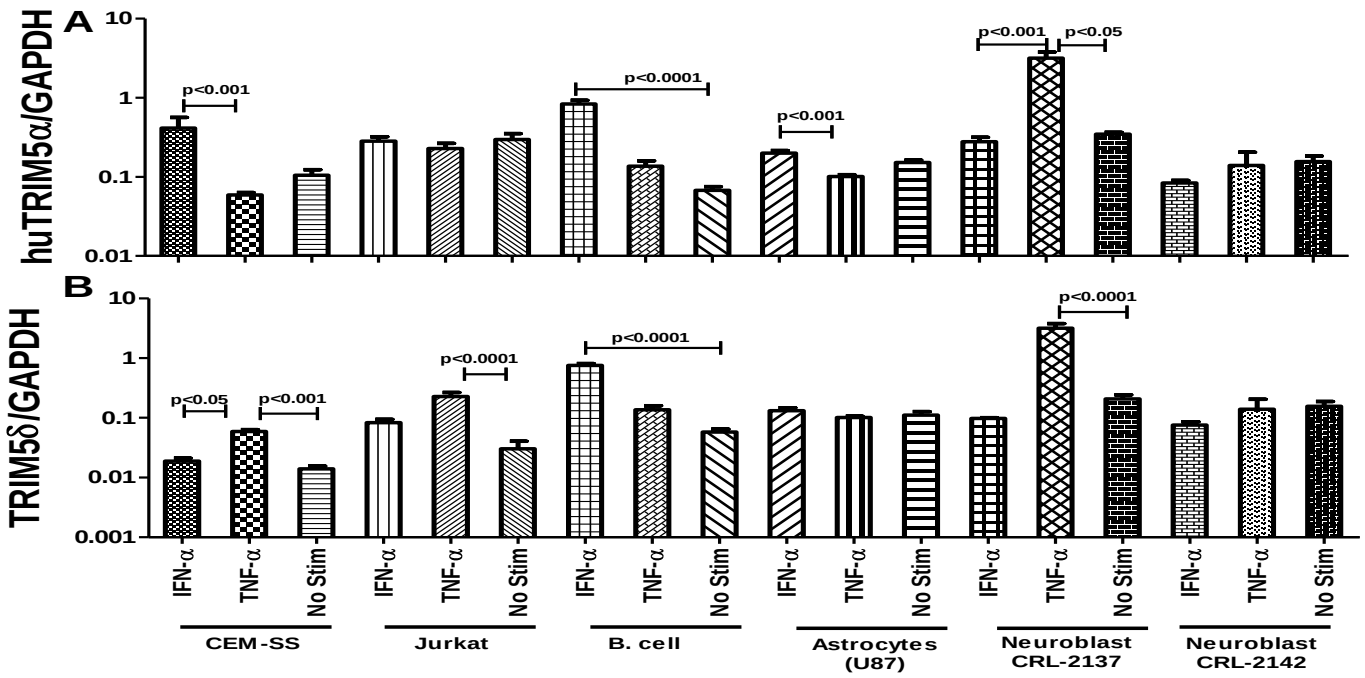


Figure 3.4.7 Regulation of huTRIM5α and TRIM5δ, in immune and neuronal cells by cytokines/chemokines, (IFN-α and TNF-α) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for huTRIM5α and TRIM5δ and GAPDH, (A and B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Cytokine regulation of TRIM5γ and TRIM22 in neuronal and immune cells

Higher levels of TRIM5γ was noted in astrocytes, after stimulation with IL-2 when compared to control cells (fig 3.4.8 A). Astrocytes stimulated with IL-2 had higher levels of TRIM22 when compared to control cells (fig 3.4.8 B). IFN-α induced both TRIM5γ and TRIM22 in all cell lines screened (fig 3.4.8 A, B).

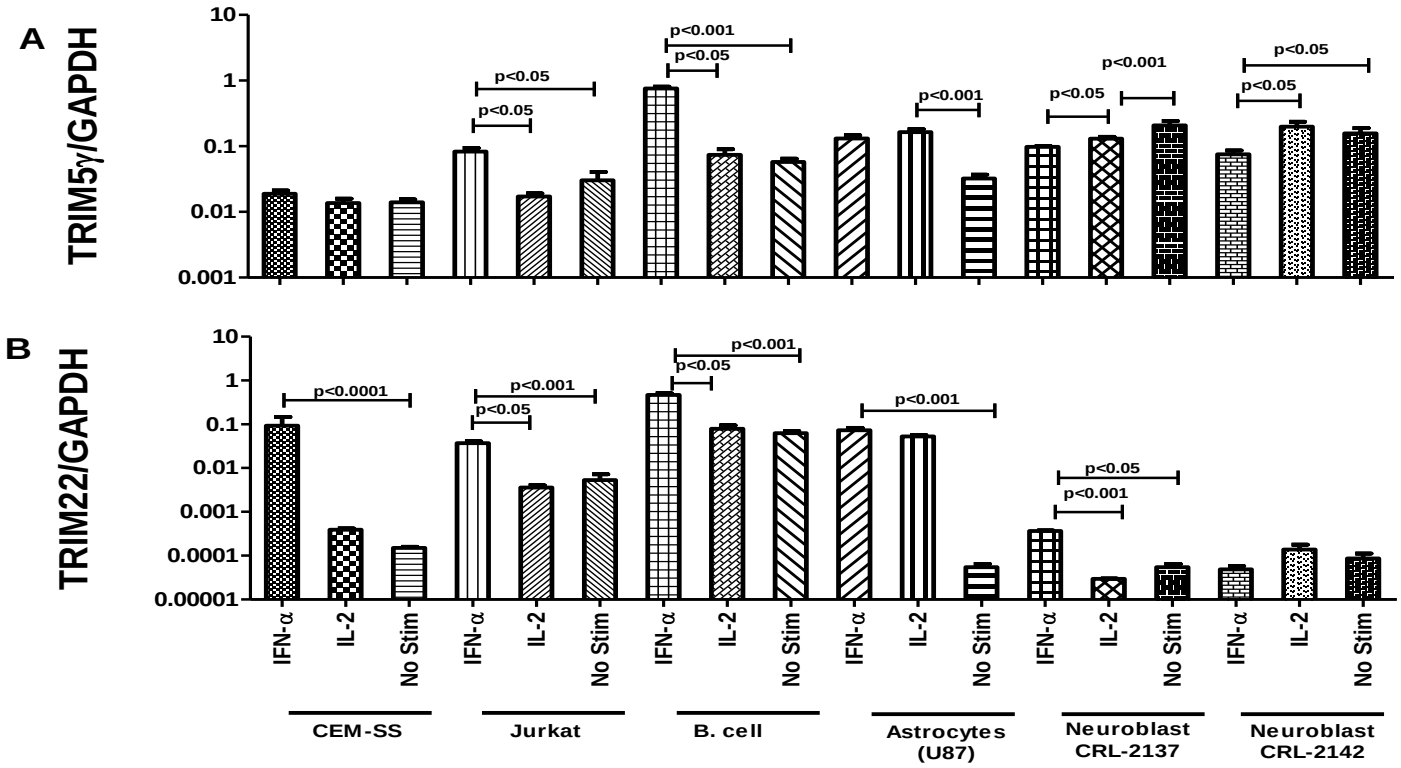


Figure 3.4.8 Regulation of TRIM5γ and TRIM22 in immune and neuronal cells by cytokines/chemokines, (IFN-α and IL-2) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for TRIM5γ, TRIM22 and GAPDH, (A and B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Cytokine regulation of TRIM5γ and TRIM22 in neuronal and immune cells

Lower levels of TRIM5γ were noted in CEM-SS stimulated with IL-10 when compared to control cells, (fig 3.4.9 A). Higher levels of TRIM5γ were noted in Jurkat cells stimulated with IL-10 when compared to control cells, (fig 3.4.9 A). Higher levels of TRIM5γ were noted in Jurkat and neuroblasts stimulated with TNF-α when compared to control cells (fig 3.4.9 A). Astrocytes stimulated with IL-10 had higher levels of TRIM22 when compared to control cells (B).). IFN-α induced both TRIM5γ and TRIM22 in all cell lines screened (fig 3.4.9 A, B).

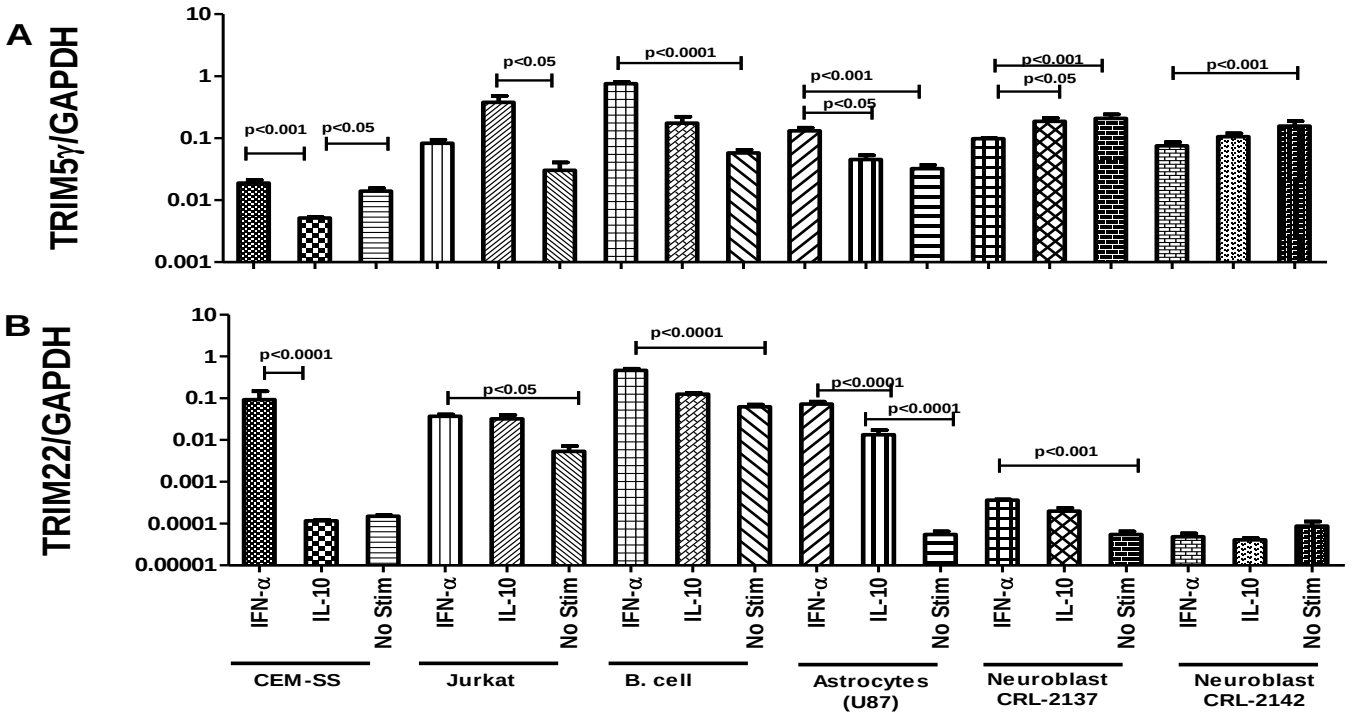


Figure 3.4.9 Regulation of TRIM5γ and TRIM22 in immune and neuronal cells by cytokines/chemokines, (IFN-α and IL-10) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for TRIM5γ, TRIM22 and GAPDH, (Aand B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Cytokine regulation of TRIM5γ and TRIM22 in neuronal and immune cells

Higher levels of TRIM5γ were noted in Jurkat and neuroblasts stimulated with TNF-α when compared to control cells (fig 3.4.10 A). Higher levels of TRIM22 was noted in neuroblasts, (cortical neuron CRL-2137) cells stimulated with TNF-α when compared to control cells (fig 3.4.10 B). IFN-α induced both TRIM5γ and TRIM22 in all cell lines screened (fig 3.4.10 A, B).

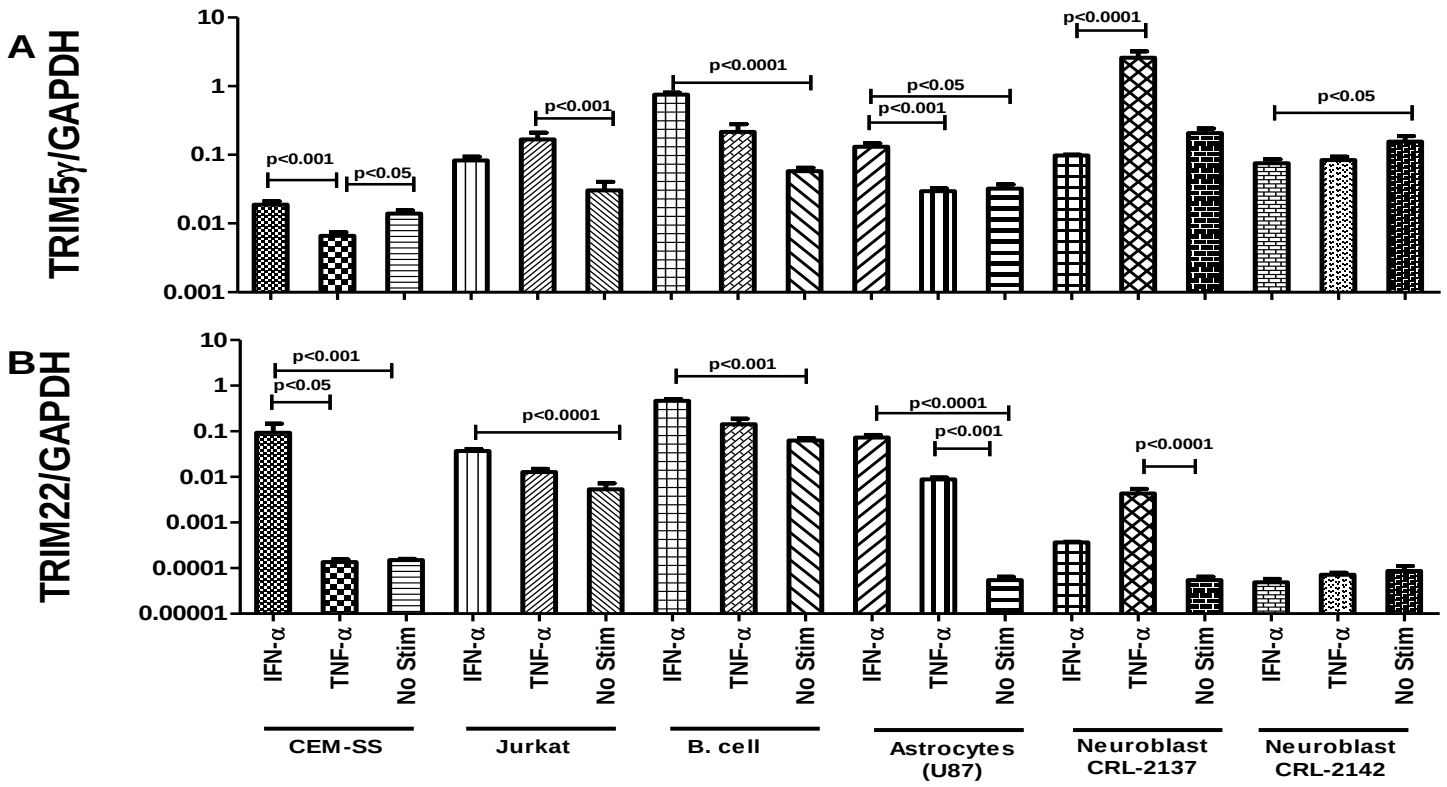


Figure 3.4.10 Regulation of TRIM5γ and TRIM22 in immune and neuronal cells by cytokines/chemokines, (IFN-α and TNF-α) after 24 hour stimulation Total cellular RNA from CEM-SS, Jurkat, B. cells, Astrocytes, Neuroblasts, (CRL-2137 and CRL-2142) was subjected to real-time RT-PCR using primers specific for TRIM5γ, TRIM22 and GAPDH, (A and B). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunns: Multiple comparison tests.

Effect of Transient knockdown of huTRIM5α and TRIM22 in Astrocytes and Neuroblast (cortical neuron, CRL-2137)

The knockdown of TRIM22 or huTRIM5α by siRNA in the presence of IFN-α was validated by RT-PCR in astrocytes (U87.CD4 astrocytic cell lines expressing exclusively CCR5 or CXCR4, NIH-4031) and neuroblasts (cortical neuron, CRL-2137). Significantly lower levels of TRIM22 where noted in the TRIM22 knockdown cell lines when compared to the control cell lines where GAPDH or non targeting siRNA was transfected into the above mentioned cell lines ($p < 0.001$, $p < 0.0001$), (fig 3.4.11.A and B). Significantly low levels of TRIM22 was noted in knock down TRIM5α Astrocytes cell line ($p < 0.0001$), (fig 3.4.11 A). Significantly low levels of TRIM22 was noted in knock down TRIM5α Astrocytes cell line ($p < 0.0001$), (fig 3.4.11 A). Significantly lower levels of huTRIM5α where noted in the huTRIM5α knockdown cell lines when compared to the control cell line where GAPDH and non targeting siRNA was transfected into the above mentioned cell lines, ($p < 0.001$, $p < 0.0001$), (fig 3.4.11 C and D).

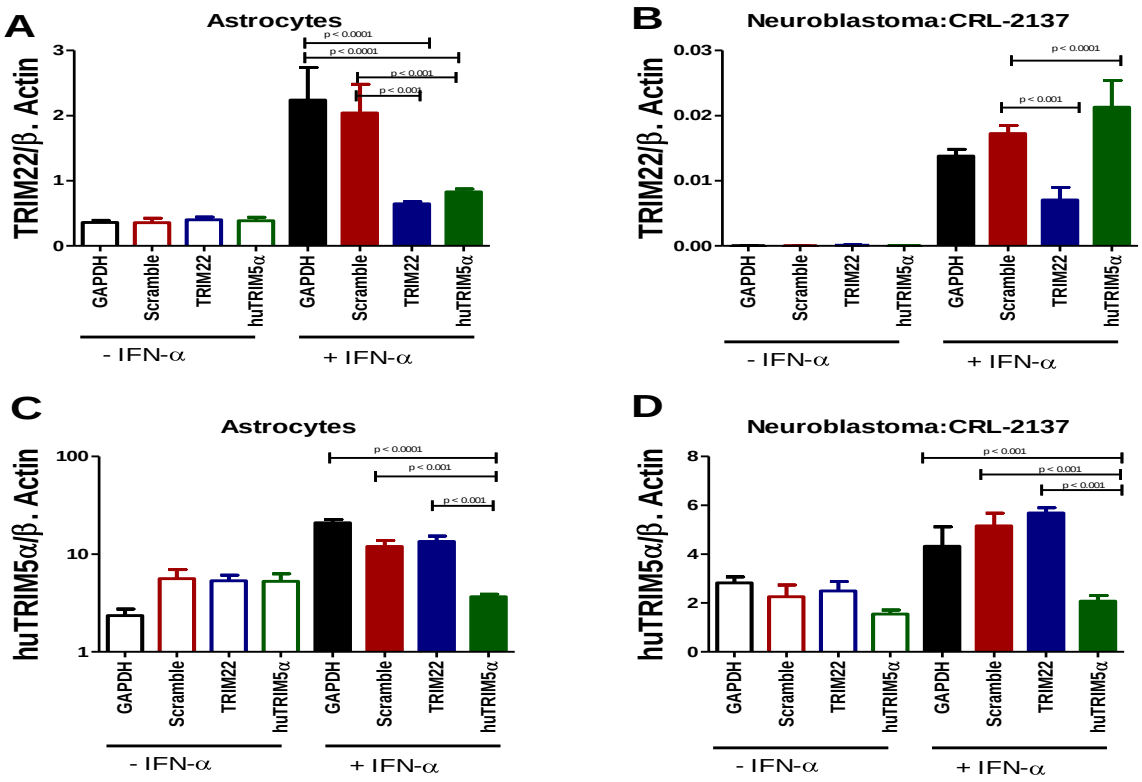


Figure 3.4.11 Effect of transient knockdown of huTRIM5α and TRIM22 in astrocytes and neuroblast (CRL-2137). Astrocytes and neuroblasts where transduced with control siRNA (GAPDH and Scramble) or huTRIM5α or TRIM22 specific siRNA. The knockdown of TRIM22 or huTRIM5α by siRNA in the presence of IFN-α was validated by RT-PCR in Astrocytes and Neuroblasts (CRL-2137), (A, B, C and D). Values are expressed as medians. Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

Silencing of TRIM22 or huTRIM5α increase HIV-1 infection in the presence of IFN-α

To determine their functional role in HIV-1 infection, we tested the role of TRIM22 or huTRIM5α knockdown in HIV-1 infection. Neuroblasts, (cortical neuron, CRL-2137) or astrocytes, (U87.CD4 astrocytic cell lines expressing exclusively either CCR5 or CXCR4, NIH-4031) where transduced with siRNA against GAPDH, a non-targeting siRNA or scramble, TRIM22 and huTRIM5α stimulated with or without IFN-α for 24 hours. The above mentioned cell lines where then challenged with VSG-env-pseudotyped HIV-1 (20ng of p24/ml) for 48 hours. Culture lysates where collected and assessed for p24 levels by ELISA and RT-PCR, was also performed on lysates from experiments.

Both the TRIM22 and huTRIM5α knockdown cell lines showed higher levels of HIV-1 replication when compared to the control cell line (GAPDH, scramble), after IFN-α stimulation in both neuroblasts and astrocytes, ($p < 0.0001$) (fig 3.4.12A, B). Higher levels of TRIM22 was noted in control cell lines (GAPDH and Scramble) when compared to the knockdown cell lines in both Astrocytes and Neuroblasts ($p < 0.001$, $p<0.0001$), (fig 3.4.12 C, D). Low levels of TRIM22 was noted in knock down TRIM5α Astrocytes cell line (fig 3.4.12 C). There were no significant changes in huTRIM5α in both control and knockdown cell lines (fig3.4.12 E, F).

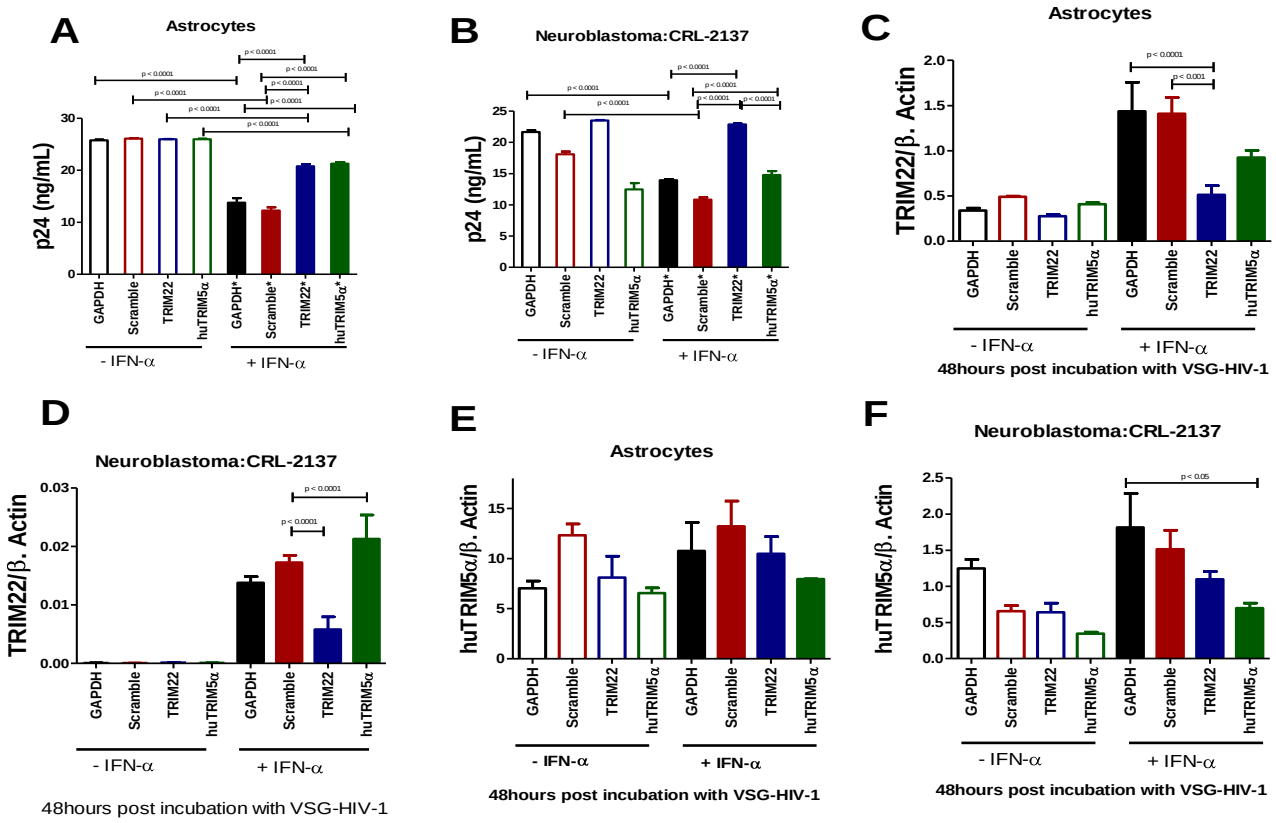


Figure 3.4.12 Silencing of TRIM22 or huTRIM5α increases HIV-1 infection in the presence of IFN-α. To determine its functional role in HIV-1 infection, we tested the role of TRIM22 or huTRIM5α knockdown in HIV-1 infection. Neuroblasts or Astrocytes cells, where transduced with siRNA against GAPDH, a non-targeting siRNA or scramble, TRIM22 and huTRIM5α stimulated with or without IFN-α for 24 hours. The above mentioned cell lines where then challenged with VSG-env-pseudotyped HIV-1 (20ng of p24/ml) for 48 hours. Culture lysates where collected and assessed for p24 levels by ELISA and RT-PCR was also performed on lysates from experiments, (A, B, C, D, E and F).

3.4.4 Discussion

TRIM5α and TRIM22 have been identified as antiviral proteins that can inhibit HV-1, MLV, SIV, FIV (Stremlau et al., 2004, Yap et al., 2004, Tissot and Mechti, 1995, Barr et al., 2008, Keckesova et al., 2004). Other HIV-1 restriction factors such as APOBEC3G has been detected in neuronal cells and shown to have some effects against HIV-1 in these cells (Wang et al., 2009). In light of these findings performed similar experiment however focusing on huTRIM5α and TRIM22.

We investigated the baseline levels of huTRIM5α, TRIM5δ, TRIM5γ and TRIM22 in T cell lines, (CEM-SS (Human T4-lymphoblastoid cell line, NIH-776), Jurkat (Human T cell lymphoblast-like cell line, NIH-10095)), B lymphoblast (Daudi, CCL-213), and neuronal cell lines (astrocytes, (U87.CD4 astrocytic cell lines expressing exclusively either CCR5 or CXCR4, NIH-4031), neuroblasts, (cortical neuron, CRL-2137, CRL-2142). We found that all isoforms of TRIM5 and TRIM22 were detectable by RT-PCR in all the cell lines screened; however neuronal cell lines such as neuroblasts and astrocytes compared to all T and B cell lines screened expressed similar levels of TRIM5 isoforms with not more than a 10 fold variation. However neuroblasts and astrocytes expressed low levels of TRIM22 when compared to B cells and Jurkats, Gongora et al. 2000 showed that the levels of TRIM22 in neuronal tissue is low when compared to compared to other immune cells (Gongora et al., 2000) (fig3.4.1).

I next investigated the effects of IFN-α stimulation of neuronal cell lines, T and B cell lines on the expression of TRIM5 isoforms and TRIM22. I demonstrated that the CNS cell lines where inducible by IFN-α stimulation for TRIM5 isoforms and

TRIM22 expression. We have previously shown that TRIM22 and huTRIMα is highly inducible by IFN-α in CEM, Jurkat and THP1 cell lines (Singh et al., 2011).

We next examined the hypothesis that cytokines have the ability to modulate TRIM5 isoforms and TRIM22 expression in neuronal cells, much of the recent reports have focused on the effects of IFN-1 in immune cells where it has been shown that TRIM22 or TRIM5 is highly inducible by IFN-1 (Sakuma et al., 2007, Barr et al., 2008, Carthagena et al., 2009).

The pattern of expression of TRIM5 isoforms or TRIM22 was dependent on the cell line screened and the cytokine/chemokine used. In the Jurkat T, (Human T cell lymphoblast-like cell line, NIH-10095) cell line stimulation with IFN-α and IL-10 induced TRIM5 isoforms and TRIM22, in B lymphoblast (Daudi, CCL-213) the greatest response is brought about by IFN-α. However in neuroblast (cortical neuron CRL-2137), TNF-α stimulation brings about the biggest effect on TRIM5 isoforms or TRIM22 expression. Human neuronal cells express multiple cytokine receptors and these cytokine receptors interacting with specific cytokines are involved in neuroimmunoregulation (Coughlan et al., 2000, Hopkins and Rothwell, 1995, Wang et al., 2009). The neuroblasts (cortical neuron CRL-2137, CRL-2142) used for these experiments express the TNF-α receptors (Gaetano et al., 1991, El-Badry et al., 1989, Iavarone et al., 1993, Goillot et al., 1992).

One of the main features of AIDS is the dysregulation of cytokine production (Clerici and Shearer, 1993). Chronic activation of the immune system may explain the increased levels of proinflammatory cytokines detected in plasma and tissues of patients with AIDS (Fauci, 1993). Tumor necrosis factor alpha (TNF-α) is one of the

major proinflammatory cytokines detected during the progression of the disease and seems to play a central role (Fauci, 1993). TNF-α enhances HIV-1 replication in chronically infected promonocytic and T-lymphoid cell lines by activation of the nuclear factor NF-kB, which stimulates the long terminal repeat (LTR) of the provirus (Duh et al., 1989, Osborn et al., 1989). However IFN-γ is an important enhancer of TNF-α production by macrophages which inhibits HIV-1 growth in primary tissue culture-differentiated macrophages (Meylan et al., 1993). HIV-1 is known to productively replicate in macrophages and microglia of the CNS, but it also infects, via a CD4-independent pathway, astrocytes and brain microvascular endothelial cells (BMVECs), which represent the major component of the blood brain barrier (BBB) (Argyris et al., 2003, Bagasra et al., 1996, Bissel and Wiley, 2004, Bobardt et al., 2004, Liu et al., 2002, Ludwig et al., 1999, Tornatore et al., 1994). Here we report that TNF-α can induce these restriction factors. It could be speculated that the IFN-α induction in the neuronal cell lines tested eventually induces restriction factors via the TNF-α receptors.

The above mentioned findings prompted us to look at if these host restriction factors namely huTRIM5α and TRIM22 had any functional effects on HIV-1 replication in neuronal cells. We firstly knocked down huTRIM5α and TRIM22 in neuroblasts (cortical neuron CRL-2137) and astrocytes using siRNA against huTRIM5α and TRIM22, successful knock down of TRIM22 and huTRIM5α is depicted in fig3.4.11. Briefly even after IFN-α induction both knock down cell lines for huTRIM5α and TRIM22 showed lower levels of gene when compared to the

control cell lines, where GAPDH and a non targeting siRNA was used. This is the first report to focus on huTRIM5α and TRIM22 in the CNS.

We further demonstrated that huTRIM5α and TRIM22 produced by astrocytes and neuroblasts has anti-viral activity, as cells transfected with siRNA against huTRIM5α or TRIM22 had higher levels of pseudotyped HIV-1 replication when compare to the control cell lines as seen by p24Elisa performed on the lysates from the experiment, fig3.4.12 A and B. We next measure the levels of huTRIM5α and TRIM22 on the lysates, to look at the effects of HIV-1 replication on expression of these restriction factors. Higher levels of TRIM22 were noted in the control cell lines when compared to the knock down cell lines, fig 3.4.12 C, D. However we did not see significant changes in huTRIM5α when compared to the control cell lines, fig3.4.12 E and F. This result suggests that although we had knockdown huTRIM5α, huTRIM5α was still being induced durring HIV-1 replication. We have strong evidence that TRIM22 plays a part inhibiting HIV-1 in neuronal cells however suttle changes in huTRIM5α expression may have profound effects on HIV-1 replication as demonstrated by fig3.4.12 E, F.

In conclusion we have demonstrated that the modulation of huTRIM5α and TRIM22 is different in neuronal cells when compared to T cell lines and B cell lines. Further studies to understand the specific mechanisms governing the regulation of these restriction factors work in by cytokines in different cells this may help unravel how intrinsic immunity may be affected by adaptive immune responses.

3.5 Association between Cyclophilin A Gene (PPIA) expression, Viral Load and CD4 T cells Counts during Primary HIV-1 Infection

3.5.1 Abstract

Human cyclophilin A, or CypA, encoded by the gene peptidyl prolyl isomerase A (*PPIA*), is incorporated into the HIV type 1 (HIV-1) virion and promotes HIV-1 infectivity by facilitating virus uncoating, possibly by protecting the HIV-1 capsid from an unknown restriction factor. However, the dynamics of cyclophilin A expression in cell lines and *in vivo* are poorly understood and it is unclear how differences in cyclophilin A levels affect HIV-1 replication and disease progression. We hypothesized that increased levels of CypA may raise HIV-1 susceptibility risk or facilitate faster disease progression. CypA levels were measured in several cell lines used for HIV replication *in vitro* and in peripheral blood mononuclear cells of individuals at high risk for HIV infection and during primary HIV infection. There were two distinct patterns of CypA expression in cell lines with CEM, HOS and 293T cells expressing higher levels of CypA compared to HeLa, H9, SupT and Jurkat cells. LPS stimulation of the cell lines did not induce CypA expression. The HIV negative individuals showed differential expression of CypA mRNA levels. CypA levels did not significantly change during HIV-1 primary infection. There was no association between CypA and viral load or CD4 T cell counts. The A1650G single nucleotide polymorphism in the *PPIA* promoter region was significantly associated with higher CypA expression in PBMCs from HIV-1 infected individuals but not HIV-1-uninfected PBMCs and PBMCs from individuals with this polymorphism associated

with higher HIV-1 replication *in vitro*. These data suggest that CypA a HIV-1 cellular cofactor plays a part in HIV-1 replication *in vivo* and that expression patterns differ in different cell types. We speculate that blocking CypA may be advantageous as an antiviral strategy.

3.5.2 Introduction

The most abundant and first cellular protein ever found in HIV-1 virions, is the *cis*/*trans* peptidyl-prolyl isomerase (PPIase) cyclophilin A (CypA). CypA was found to be specifically incorporated into HIV-1 virions but not into virions of other retroviruses (Franke et al., 1994, Luban et al., 1993, Thali et al., 1994). CypA was originally described as the binding partner of cyclosporine A (CsA), an immunosuppressive cyclic undecapeptide used clinically to suppress organ rejection after allograft transplantation (Handschumacher et al., 1984).

Numerous possible functions of CypA in the HIV-1 replication cycle have been investigated and proposed, but the biological function of this interaction is still unclear (Luban, 2007, Sokolskaja et al., 2006). Cyclophilin A have been found to bind HIV-1 Gag, originally by a yeast-two hybrid screen (Luban et al., 1993). This interaction was then mapped to the CA portion of Gag, specifically to the G89-P90 peptide bond (Bosco et al., 2002).

It has been demonstrated that blocking this interaction CypA and HIV-1 by CsA, RNAi (RNA interference), or mutation of the binding site in CA, reduced the virus titer produced from human cells (Towers et al., 2003, Stremlau et al., 2004). CypA is thought to release HIV-1 from an anti-viral restriction activity in cells from non-human primates. Such studies on CypA have led to the hypothesis that CypA modulates HIV-1 susceptibility to restriction factors such as the tripartite interaction motif (TRIM) 5α (Stremlau et al., 2004, Towers et al., 2003).

The discovery of TRIM-CypA fusion proteins in the New World monkey species of owl monkeys and Old World monkeys, the long tailed macaques and pigtail macaques led investigators to look at a connection between these two proteins. TRIM5α-mediated HIV-1 restriction in non-human primates is CypA dependent (Sayah et al., 2004, Brennan et al., 2008). However, in human cells TRIM5α and CypA have been shown to independently regulate HIV-1 infection (Keckesova et al., 2006, Sokolskaja et al., 2006).

CypA promotes HIV-1 infectivity by facilitating virus uncoating as opposed to TRIM5α that has been shown to promote premature disassembly of HIV-1 capsid. I investigated associations between CypA and huTRIM5α expression levels in the same study cohort. I hypothesized that increased levels of CypA may raise ones HIV-1 susceptibility risk. I measured CypA messenger RNA (mRNA) levels in peripheral blood mononuclear cells (PBMCs) of high risk, HIV-1 uninfected participants, and HIV-1 positive study participants. I investigated the correlation of CypA expression with markers of disease progression such as viral load and CD4 T cell counts. I further evaluated the CypA mRNA Genetic polymorphisms in the regulatory region of *PPIA* gene have been implicated in HIV-1 infection and pathogenesis (An et al., 2007b, Bleiber et al., 2005, Rits et al., 2008). Bleiber *et al*. reported an association between the minor allele (G) of SNP A1650G (referred to as 1650A>G in their report) and rapid disease progression (Bleiber et al., 2005).

H9 and Jurkat cells have previously been shown to differ in CypA expression and differentially support HIV-1 replication. However, it is unknown whether how

much CypA other HIV permissive cells express and whether these levels can be altered by cell activation. Therefore in order to better understand the range and patterns of CypA expression and the effect of general immune activation baseline levels in various cells lines, CEM, HOS, 293T cells, Hela, SupT, H9 and Jurkat and following LPS stimulation were assessed.

3.5.3 Results

Relative expression of CypA in PBMCs from HIV-1 uninfected versus infected subjects

I compared mRNA levels of CypA in PBMCs from HIV-1 negative versus HIV-1 infected samples collected within the first 12 months post-infection. There were 32 individual HIV-1 negative samples available and 28 HIV-1 infected samples. Samples from HIV-1 positive individuals were available at multiple time points post-infection and samples closest to the 12 months post infection time point were included in the analyses presented here.

There were no significant differences in CypA expression between HIV-1 negative and positive participants (Fig 3.5.1A), ($p = 0.093$). However the HIV-1 positive group shows a trend of higher CypA expression. There was a wide range of CypA expression in the HIV-1 negative group with the minimum value being 0.093 and maximum being 222. I also investigated whether CypA expression differed over time in the HIV-1 positive group. I did not see significant changes in CypA expression during the first 12 months of HIV-1 infection in our unmatched groups, (Fig 3.5.1 B).

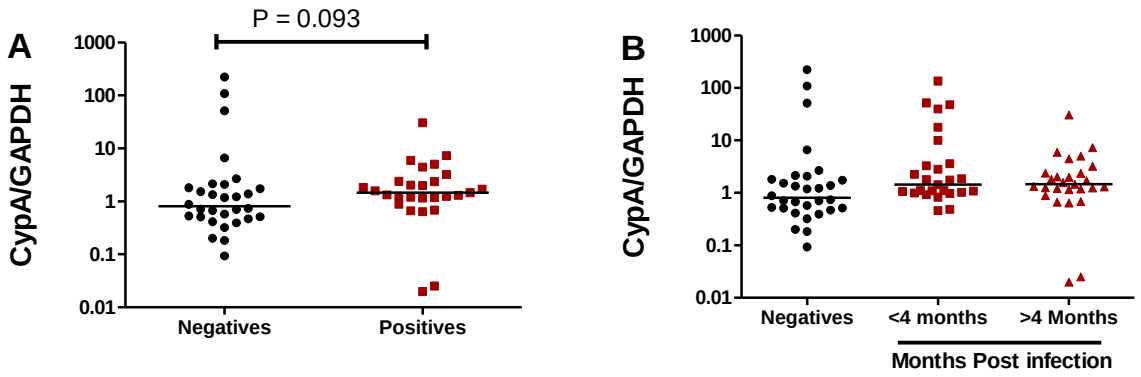


Figure 3.5.1 Expression of CypA, in PBMC from HIV-1 uninfected versus infected subjects. The infected participant samples were all collected within 12 months of infection. At least 2 time points were available post-infection for the primary infection samples and for these. For the HIV-1 positive group we compared one time point, closest to the set point of 12 months post infection (n=28). Only patients that remained HIV-1 negative on follow up where used for this analysis, (n=19), (fig 3.5.1A). Samples were analyzed before infection, at <4 months after infection (in the acute phase), and at >4 months after infection (in the early chronic phase), (fig 3.5.1B. Median expression levels between HIV negative and HIV positive samples were compared. The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant.

Expression of CypA mRNA in PBMC at baseline (study enrolment) for non-seroconverters versus seroconverters

I next addressed whether pre-infection samples from seroconverters differed from those of non-seroconverters in CypA expression levels. Although seroconverters showed generally higher mRNA levels of CypA than non-seroconverters, the differences between the groups did not reach statistical significance (Fig 3.5.2).

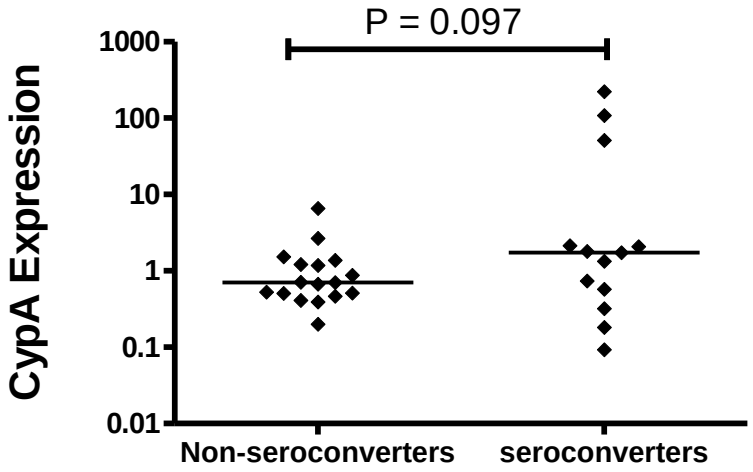


Figure 3.5.2 Expression of CypA mRNA in PBMC at baseline (study enrollment) for non-seroconverters versus seroconverters. Participants included in this analysis were all enrolled as high risk HIV-1 uninfected individuals and were longitudinally followed for at least 36 months each at the time of analysis. The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant.

Association between CypA gene expression and viral load and CD4 T cell counts

I performed Pearson correlations between CypA expression and viral load or CD4 T cell counts. I did not see significant associations between viral load and CypA expression ($r = -0.1$, $p = 0.25$), (Fig 3.5.3A). I also did not see significant associations between CD4 T cell counts and CypA expression, (Fig 3.5.3B), ($r = -0.041$, $p = 0.59$).

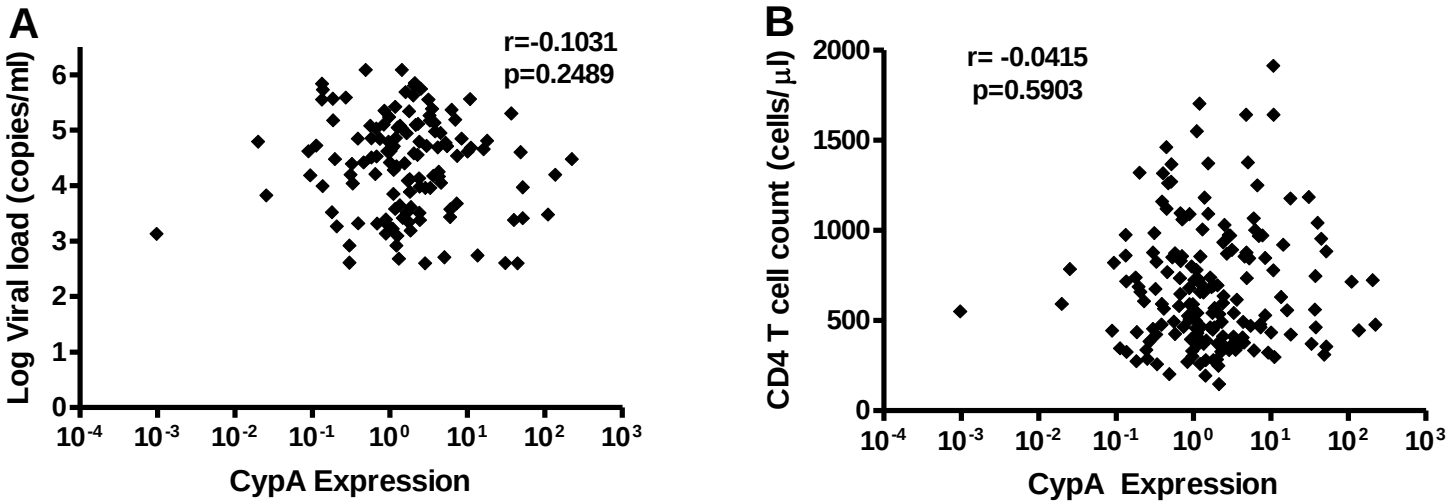


Figure 3.5.3 Analysis of the correlation between CypA messenger RNA levels and plasma human immunodeficiency virus type 1 (HIV-1) load (A) and CD4+ T cell counts (B)

Correlation between CypA gene expression and huTRIM5α gene expression

CypA is incorporated into the HIV type 1 (HIV-1) virion and promotes HIV-1 infectivity by facilitating virus uncoating as opposed to TRIM5α that has been shown to promote premature disassembly of HIV-1 capsid. I investigated correlation between CypA and huTRIM5α expression. I did not find significant correlation between huTRIM5α expression and CypA expression (r = -0.075, p= 0.45), (Fig 3.5.4).

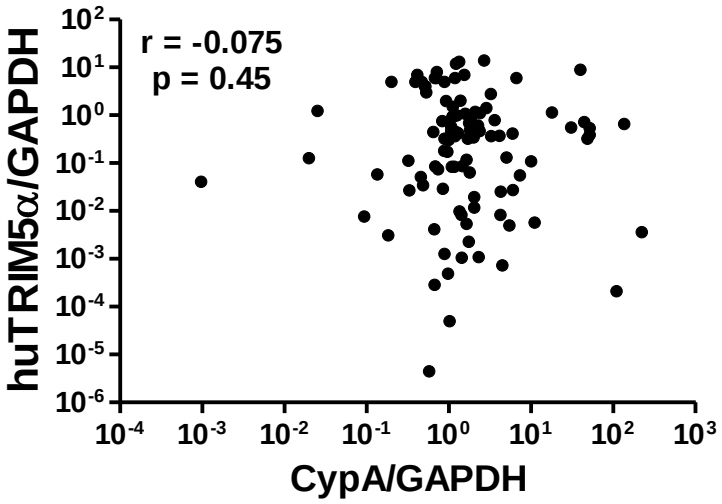


Figure 3.5.4 Analysis of the correlation between CypA messenger RNA levels and huTRIM5α

Effect of CypA genotype on CypA expression

In this study, two regulatory SNPS (A1650G and C1604G) of *PPIA* were chosen to be genotyped. However, 1604G occurred at a very low frequency (0.01) in this cohort and therefore, it was excluded from the analysis.

I analyzed whether SNP A1650G of *PPIA* gene influences CypA expression levels *in vivo.* CypA expression between 1650A and 1650G genotypes was not significantly different amongst the negative group (figure 3.5.5A). Interestingly, 1650G was significantly associated with elevated levels of CypA mRNA expression post infection ($p<0.01$) (figure 3.5.5 B).

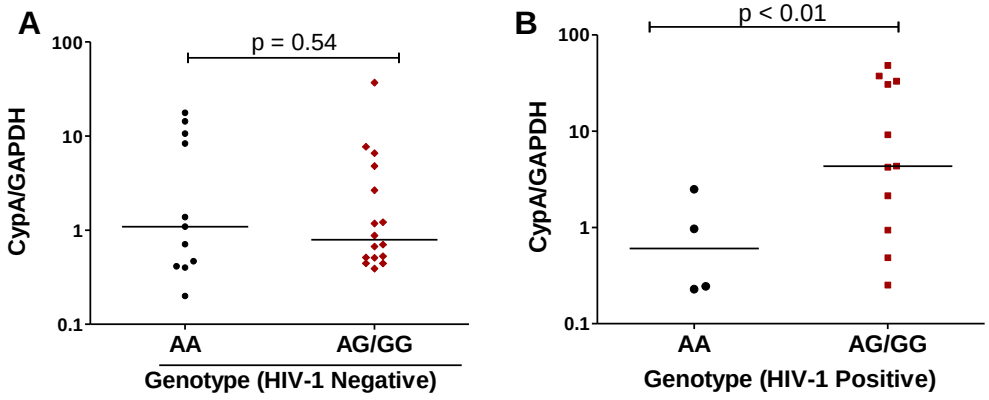


Figure 3.5.5 Expression of CypA mRNA in HIV-1 negative group as modulated by different genotypes of SNP A1650G, A. B, and Expression of CypA mRNA in HIV-1 positive group as modulated by different genotypes of SNP A1650G. The differences between groups were evaluated by using an unpaired student's t test. A P-value of < 0.05 was considered as statistically significant.

Effect of 1650G on CypA expression levels and HIV-1 replication *in vitro*

Next I analyzed whether the A1650G SNP of *PPIA* was associated with altered CypA expression levels *in vitro*. I performed quantitative RT-PCR on CypA mRNA levels in peripheral blood mononuclear cells (PBMC) of 30 highly exposed but persistently seronegative (HEPS) group. All PBMC samples were pre stimulated with IL-2 for 72hrs. Pre-HIV-1 infection expression levels of CypA were significantly higher in PBMC with 1650G genotyped compared to CypA expression levels in PBMC genotyped for 1650A ($p < 0.01$) (figure 3.5.6A). Post-HIV-1 infection expression levels of CypA were significantly higher in PBMC genotyped for 1650G compared to CypA expression levels in PBMC genotyped for 1650A ($p < 0.01$) (figure 3.5.6A).

In order to confirm that 1650G indeed promotes HIV-1 replication post cell entry, we analyzed the replication of HIV-1 in IL-2 stimulated PBMC from 28 SP individuals with known genotypes for SNP A1650G. Our results did not show significant differences in p24 production at any time point during the culture period, irrespective of the genotype of A1650G SNP (figure 3.5.6 B).

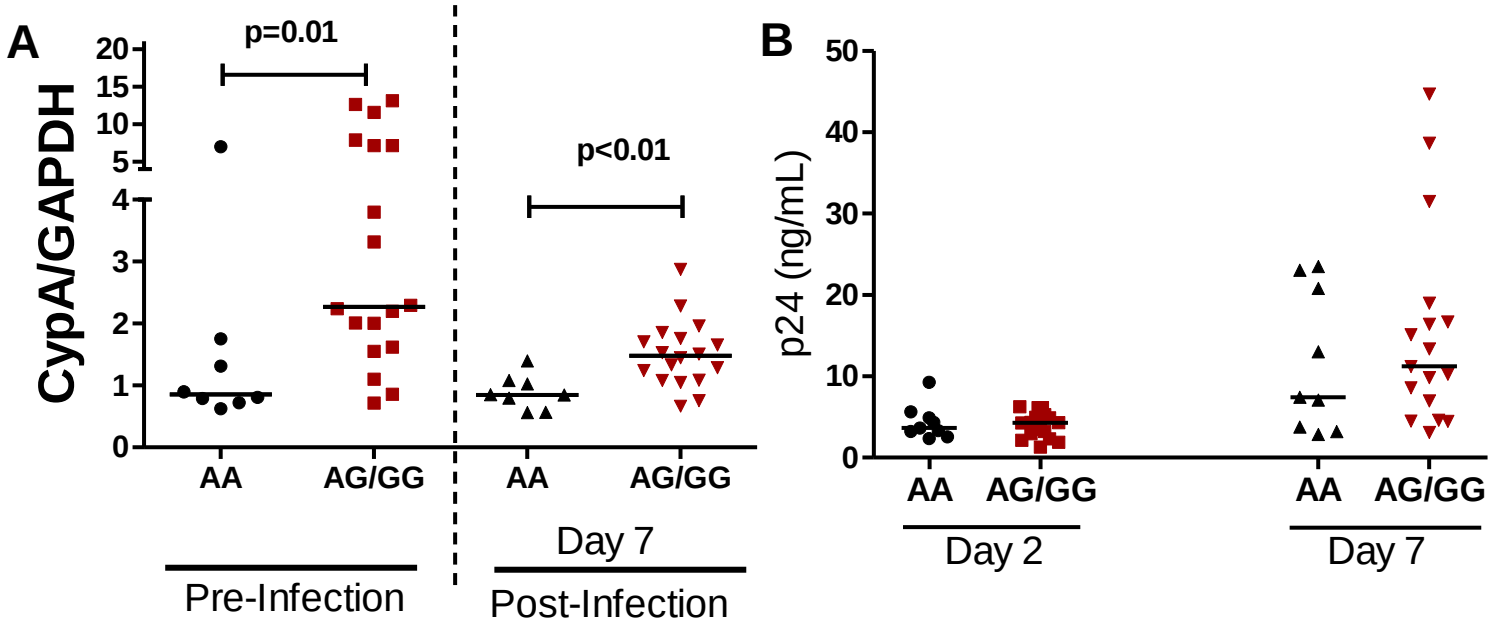


Figure 3.5.6 Analysis of HIV-1 replication and CypA expression levels in genotyped peripheral blood mononuclear cells (PBMCs). A. Analysis of CypA mRNA levels in PBMC of seroconverters that were either wild type (AA) or mutated (AG/GG) at position 1650 of *PPI* gene. CypA mRNA levels were normalized for GAPDH mRNA levels. **B,** Analysis of NL4.3 replication in IL-2 stimulated PBMC from highly exposed but persistently seronegative (HEPS) individuals wild type for A1650G (AA) and mutant for SNP A1650G (AG/GG). Virus replication was analyzed by measuring p24 production in culture supernatant. P24 production at day 2 and day 7 after infection is shown.

Baseline CypA expression in CEM, HOS, 293T, HeLa, H9, SupT, Jurkat and following LPS stimulation

H9 and Jurkat cells have previously been shown to differ in CypA expression and differentially support HIV-1 replication. However, it is unknown whether how much CypA other HIV permissive cells express and whether these levels can be altered by cell activation. Therefore in order to better understand the range and patterns of CypA expression and the effect of general immune activation baseline levels in various cells lines and following LPS stimulation were assessed. I found that H9 and Jurkat cell lines have significantly lower levels of CypA expression when compared to CEM, HOS, 293T, HeLa and SupT cell lines, ($p<0.0001$), (Fig 3.5.7A).

The results obtained from the above mentioned led me to next look at whether I could induce CypA using LPS a known stimulant that induces general immune activation. I performed titration curves of LPS on the above mentioned cell lines. I did not see significant changes in CypA expression under LPS stimulation, the differences in CypA expression were according to the cell line screened, (Fig3.5.7 B).

Baseline CypA expression in CEM, HOS, 293T, HeLa, H9, SupT, Jurkat and following LPS stimulation

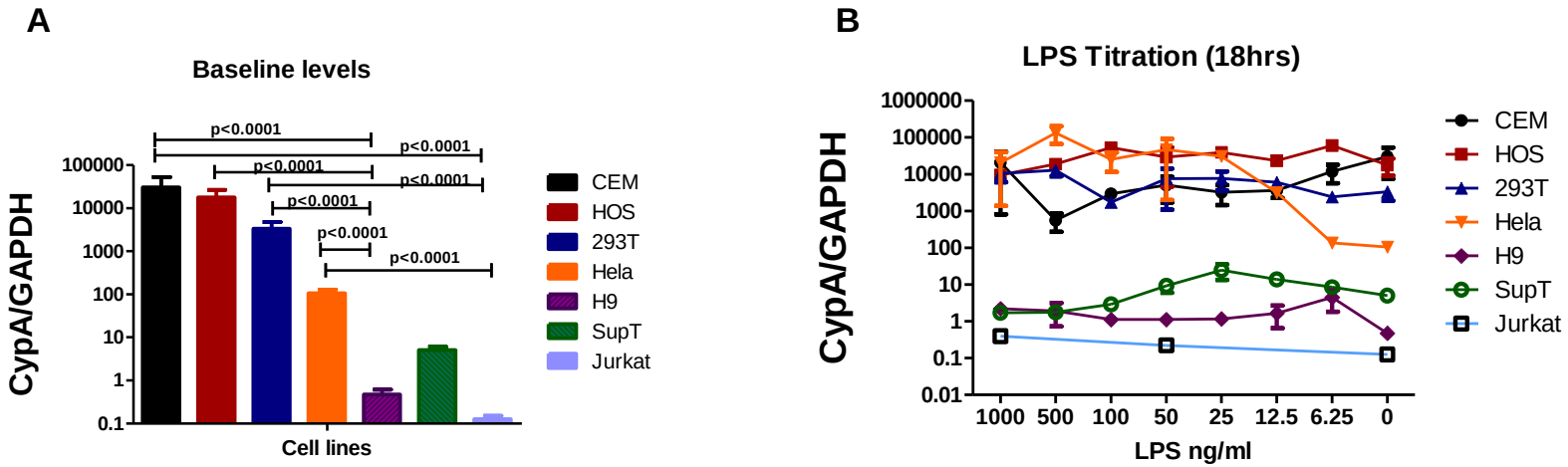


Figure 3.5.7 Baseline CypA expression in CEM, HOS, 293T, HeLa, H9, SupT, Jurkat cell lines (A) and following LPS (B) stimulation Total cellular RNA from CEM-SS, HOS, 293T, HeLa, H9, SupT and Jurkat was subjected to real-time RT-PCR using primers specific for CypA.

Differences between groups were evaluated by using a non parametric statistical test, Kruskal-Wallis test and differences between groups where evaluated using the Dunn's multiple comparison tests.

3.5.4 Discussion

CypA which is a ubiquitously expressed cellular protein that was identified as a cofactor for HIV-1 that leads to enhanced infection by an unknown mechanism (Luban et al., 1993). The discovery of Trim5-CypA fusion protein as a HIV restriction factor in rhesus macaques (Sayah et al., 2004, Nisole et al., 2004) and the dependency of Rhesus TRIM5α on CypA for HIV restriction has revived interest in this ubiquitously expressed cellular protein (Berthoux et al., 2005, Keckesova et al., 2006, Stremlau et al., 2006, Rits et al., 2007). Previous studies had suggested that HIV-1 is susceptible to restriction by huTRIM5α, but only when interaction between CA and CypA is disrupted (Towers et al., 2003). Other studies had shown that supranormal levels of huTRIM5α inhibit the titer of wild-type HIV-1, even in the presence of CypA (Hatziioannou et al., 2004b, Perron et al., 2004, Song et al., 2005c, Stremlau et al., 2005).

The *TRIM5* gene in the New World owl monkey (genus *Aotus*) is unusual in that it resulted from retrotransposition of the cyclophilin A (CypA) cDNA into intron 7 of the TRIM5 gene (Sayah et al., 2004, Nisole et al., 2004). CypA was found to bind CA, to facilitate the proper uncoating of the CA. The fusion protein of TRIM5 and CypA, which is referred to as *Aotus* T5Cyp fusion (AoT5Cyp) acts as a restriction factor preventing HIV-1 infection (Sayah et al., 2004, Nisole et al., 2004, Luban et al., 1993).

This evidence led me to investigate CypA and huTRIM5α expression levels in the same study cohort to better understand the expression of the transcripts of these factors independently and in relation to each other. My data demonstrated no

significant correlation between huTRIM5α and CypA. These results suggest that huTRIM5α and CypA are independently regulated *in vivo*.

I next investigated differences in CypA levels between HIV negative and HIV-1 positive participants and found no significant differences in CypA expression between HIV-1 negative and positive participants (Fig 3.5.1A), ($p = 0.093$). I did not see significant changes in CypA expression during the first 12 of HIV-1 infection in our unmatched group, (Fig 3.5.1B). I did not see significant associations between viral load or CD4 Tcell counts and CypA expression ($r = -0.1$, $p= 0.25$) (Fig 3.5.3A), ($r = -0.041$, $p = 0.59$), (Fig 3.5.3B).

I further investigated the differential expression of CypA in the PBMCs screened by looking at CypA mRNA expression in a range of T cell lines and immune cell lines. I show that H9 and Jurkat cell lines have significantly lower levels of CypA expression when compared to CEM, HOS, 293, Hela and SupT cell lines, ($p<0.0001$), (Fig3.5.7A). Hatziioannou and colleagues showed similar results to our study where they showed by western blot analysis of CypA in HeLa, HOS, H9 and Jurkat cell lines, the Jurkat line expressed the lowest levels of CypA while HeLa expressed the highest levels and HOS and H9 cell lines expressed intermediate levels (Hatziioannou et al., 2005). I extended this study to examine whether LPS a known stimulant that induces immune activation could induce CypA expression. I did not see significant changes in CypA expression under LPS stimulation, however the differences in CypA expression was due to the difference in cell line screened, (Fig3.5.7B).

I further examined the relationship between the CypA genotype and mRNA expression by analyzing whether SNP A1650G of *PPIA* gene influences CypA expression levels *in vivo.* CypA expression between 1650A and 1650G genotypes was not significantly different amongst the negative group (figure 3.5.5A). Interestingly, 1650G was significantly associated with elevated levels of CypA mRNA expression post infection ($p<0.01$) (figure 3.5.5B). These results were validated by performing an HIV-1 replication experiment. PBMC with AG or GG genotype had higher levels of CypA both in pre and post HIV-1 infection samples. Virus replication was assessed by measuring p24 antigen levels in the culture supernatant every other day for 7 days. The results demonstrate a 51% increase in p24 production among individual with the mutant genotype (AA/AG) vs the ones with the wild type genotype (AA), however this was not statistically significant (figure 3.5.6B). Similar results were obtained by Rits and colleagues where they had shown for the CypA 1604CC/AA genotype compared to CypA 1604 CG expressed higher levels of CypA mRNA but they did not find significantly higher p24 levels between these two genotypes when a viral replication experiment was performed and analyzed 12 days post infection (Rits et al., 2008).

Studies validated CypA as an important cellular cofactor of HIV-1 by identifying single nucleotide polymorphisms (SNPs) within *PPIA* gene that are associated with HIV-1 disease outcome (An et al., 2007b, Bleiber et al., 2005, Rits et al., 2008). Here we show that this SNP may effect mRNA expression of CypA *in vivo* and *in vitro*. Our results demonstrate that PBMCs genotyped for 1650G allele expresses higher levels of CypA mRNA than PBMCs genotyped for wild type allele,

1650A. We have demonstrated in this study that rather than the environment (LPS stimulation), a specific genotype effects CypA expression and viral control.

4 Discussion

Approximately 34 million people live with HIV globally, with 23.5 million people living in sub-Saharan Africa (UNAIDS, 2012). Although HIV-1 has been known as the causative agent for AIDS for approximately 30 years now, it continues to kill several million people each year.

The easiest way to eliminate the HIV-1 pandemic will be to develop an anti-HIV-1 vaccine. Initial vaccine approaches focused on the HIV-1 envelope protein (gp120), and aimed to induce an antibody response to gp120 (Flynn et al., 2005, Pitisuttithum et al., 2006). An alternative approach, aimed to induce a T-cell response to HIV-1 using a recombinant adenovirus vector expressing HIV-1 Gag, Pol and Nef proteins (Shiver et al., 2002). Unfortunately, results from both of these trials were disappointing, as neither approach provided protection from HIV-1 infection (Buchbinder et al., 2008, McElrath et al., 2008). A third trial was recently performed in Thailand, and aimed to induce both a T-cell and antibody response to HIV-1(Rerks-Ngarm et al., 2009). In this, 16,000 Thai men and women received either placebo or vaccine injections, and were subsequently monitored for HIV-1 infection over a 3 year period (Rerks-Ngarm et al., 2009). Results from this trial showed a modest benefit among vaccine recipients, with a vaccine efficacy of 26-30% (Rerks-Ngarm et al., 2009). However, vaccination did not affect the levels of viremia or CD4+ T cell counts of infected individuals, and many were disappointed with the results (Rerks-Ngarm et al., 2009).

There is a long way before we have an effective vaccine against HIV-1. In mean time we are currently treating HIV-1 infected patients with ARVs. Due to the high mutation rate of the virus, the virus has shown itself to be an elusive target to the immune system (Barouch, 2008, Medzhitov and Littman, 2008). The current antiviral therapy accepted by the scientific and medical community is known as the highly active antiretroviral therapy (HAART) or combination antiretroviral therapy (cART) (De Clercq, 2009). Although there have been major breakthroughs in the development of these antiviral drugs, the eradication of HIV and the cure of AIDS has still not been achieved (Mamo et al., 2010).

Due to the various limitations of current combination antiretroviral therapies, the field of antiretroviral drug research remains very active, where the HIV biology and the interaction between the virus and its host cells can provide new insights for the development of new antiviral therapeutic strategies (Mamo et al., 2010). HIV-1 restriction factors may give one a new avenue for creating new ARVs. Targeting or enhancing recently identified host innate host factors such as TRIM E3 ligases that restrict HIV replication in mammalian cells may be one such potential approach (Ndung'u, 2011).

Before new anti-HIV-1 drugs can be developed, one needs to identify possible targets and understand their relationship in a natural course of HIV-1 infection. In the current study I looked at a novel set of HIV-1 restriction factors, (TRIM E3 ligases) and a HIV-1 cellular cofactor (Cyclophilin A (CypA) in different phases of HIV-1 infection in some well characterized HIV-1 infected cohorts. Mechanistic studies where performed to validate or shed more light on the *in vivo* results. Lots of the

research in the TRIM E3 ligase family have been performed using transformed cell lines and overexpression assays, and this may not reflect actual physiologic circumstances (McNab et al., 2011). I looked at some well characterized members of TRIM E3 ligases namely huTRIM5α and TRIM22. I also included in my screen other members of the group that have shown to be interferon 1 inducible such as TRIM11, TRIM19 and TRIM36.

TRIM proteins are most studied in host defence as viral restriction factors (Ozato et al., 2008, Sardiello et al., 2008, Nisole et al., 2005). They have attracted particular attention for their ability to restrict retroviruses, including HIV (Ozato et al., 2008). They act at various stages of the viral replication cycle, including cell entry, viral transcription and viral release (Nisole et al., 2005). Both the ability of TRIM proteins to act as an E3 ubiquitin ligase and the presence of the C-terminal B30.2 domain is important for the TRIM protein anti-viral activity (Li et al., 2007b, Uchil et al., 2008, Barr et al., 2008, Li et al., 2007a).

The RING domain of many TRIM proteins has been shown to have E3 ubiquitin ligase activity (Meroni and Diez-Roux, 2005). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (Reymond et al., 2001, Towers, 2005). All five TRIM E3 ligases screened, TRIM5α, TRIM11, TRIM19, TRIM22 and TRIM36, have a coil-coil, RING and B-Box2 domains (Short and Cox, 2006, Carthagena et al., 2009, Ozato et al., 2008) and this could be attributing to their anti-viral activity.

Rhesus TRIM5α potently restricts HIV-1 in old world monkeys (Hatziioannou et al., 2004b, Keckesova et al., 2004, Perron et al., 2004, Stremlau et al., 2004). Rhesus monkey TRIM5α (rhTRIM5α) was shown to account for the previously described Ref-1 and Lv-1 anti-viral activities in a species-specific manner (Uchil et al., 2008). Restriction required the RING and SPRY domains and recent studies have looked at role of the B-Box domain (Li and Sodroski, 2008, Diaz-Griffero et al., 2009). Recent studies have shown that when rhTRIM5α has single point-mutation in the C terminal SPRY domain the anti HIV activity is reduced (Zhang et al., 2010, Li et al., 2006b, Yap et al., 2005). Human TRIM5α (huTRIM5α) has weak anti-HIV activity and is less potent than rhTRIM5α (Zhang et al., 2010). Although huTRIM5α has reduced activity against HIV-1 recent reports have shown polymorphisms in the TRIM5 gene, may influence susceptibility to HIV-1, indicating a physiological role for huTRIM5α in retro-viral restriction (Zhang et al., 2010, van Manen et al., 2008).

TRIM22 which lies next to TRIM5 has been shown to have potent viral restriction capacity (Li et al., 2007a, Zhang et al., 2006). TRIM22 is able to restrict Encephalomyocarditis virus (EMCV) and Hepatitis B, by targeting of the EMCV 3C protease, which is crucial for viral protein processing, for degradation and inhibition of the core promoter of HBV, which is necessary for viral pre-genomic RNA synthesis (Gao et al., 2009, Eldin et al., 2009). In both cases the E3 ligase activity of TRIM22 was required as well as the C-terminal domains, which are thought to be required for protein–protein interactions (Eldin et al., 2009, Gao et al., 2009).

TRIM22 has been shown to inhibit HIV through suppression of viral long terminal repeat transcription (Tissot and Mechti, 1995). Type I IFN treatment of

TRIM22 inhibits HIV particle budding by interfering with the trafficking of HIV-1 GAG proteins to the plasma membrane (Barr et al., 2008). This required the RING domain of TRIM22, again suggesting E3 ubiquitin ligase activity of TRIM22 is important (Barr et al., 2008).

TRIM19 is inhibits a variety of viruses, including vesicular stomatitis virus, influenza A virus, human cytomegalovirus (HCMV), herpes simplex virus-1 and HIV-1, although the exact mechanisms of restriction have not fully been elucidated (Geoffroy and Chelbi-Alix, 2011, Nisole et al., 2005). TRIM19 have been shown to mediate the conjugation of ubiquitin-related molecules such as small ubiquitin-like modifier (SUMO) and IFN-inducible ISG15 indicating it plays a crucial part in interferon modulation (Bernardi and Pandolfi, 2007). Uchil and colleagues showed TRIMs 11 enhanced entry of murine leukemia virus (MLV) by restricting TRIM5 activity (Uchil et al., 2008).

In primary HIV-1 infection I saw HIV positive patients had higher levels of IFN-β, MxA (a surrogate marker of interferon 1 induction), TRIM22, TRIM11 and TRIM19 when compared to the HIV-1 negative group. However HIV-1 negative participants had higher levels of huTRIM5α when compared to the HIV-1 positive participants. Our earlier study revealed that lower huTRIM5α mRNA expression levels were associated with increased susceptibility to HIV-1 infection in a cohort of high risk black African females (Sewram et al., 2009). In addition, we found that in matched samples of HIV-1 negative individuals who later became HIV-1 positive, huTRIM5α levels were not dysregulated following infection. This result was surprising as all TRIM E3 ligases screened are interferon 1 inducible *in vitro*

suggesting they possibly work in a similar manner (Everett and Chelbi-Alix, 2007, Regad et al., 2001, Carthagena et al., 2009, Uchil et al., 2008, Barr et al., 2008).

Expression of TRIM22, TRIM11, TRIM19 and TRIM36 in both the HIV-1 positive and negative groups showed a positive association with the levels IFN-1 (IFN-α, IFN-β and MxA). These results suggest that the TRIM E3 ligases screened, (TRIM22, TRIM11, TRIM19 and TRIM36) are IFN-1 inducible *in vivo*, supporting *in vitro* data obtained by other groups (Martinez et al., 2006, Rajsbaum et al., 2008, Carthagena et al., 2009). TRIM11 and TRIM36 have a SPRY domain (Carthagena et al., 2009) which has shown to have antiviral activity (Uchil et al., 2008).

huTRIM5α showed a negative association with IFN-β in both the negative and positive groups. Our group and other groups have showed that huTRIM5α and TRIM22 in immune cell lines (CEM, Jurkat and THP1) are IFN-1 inducible groups suggesting discordance between the *in vivo* data and *in vitro* data for huTRIM5α (Singh et al., 2011, Barr et al., 2008, Uchil et al., 2008).

I also investigated the association of antiviral gene expression and viral load and CD4 T cell counts, the two commonly used markers of disease progression. I found that MxA mRNA levels showed a positive association with plasma viral load. For every log increase in MxA, viral load increased by 0.85 log copies/ml. This is consistent with previous studies that have demonstrated that IFN-1 increases as the viral load increases (Lehmann et al., 2008).

An interesting and potentially important finding in this study was that TRIM22 was negatively associated with HIV plasma viral load during primary HIV-1 infection.

TRIM22 also showed a significant positive correlation with CD4 T cell counts. I found that for every log increase in TRIM22 mRNA levels there is an associated viral load decrease of 0.98 log copies/ml ($p=0.0307$). TRIM22 also showed a positive association with $CD4^+$ T cell counts as every log increase in TRIM22 expression was associated with a 6.09 square root increase in $CD4^+$ cells/μL ($p=0.0281$).

In addition, I found that gene silencing of TRIM22 in a T cell line nearly completely abrogated the IFN-mediated restriction of HIV-1. TRIM22 silencing also resulted in increased accumulation of HIV particles in culture supernatant. Overall therefore, my results are in agreement with several studies that have suggested that TRIM22 is induced by IFN-1 and that TRIM22 can potently inhibit HIV replication and release (Barr et al., 2008, Bouazzaoui et al., 2006, Tissot et al., 1996).

Together these data suggests that TRIM22 of both *in vivo* and *in vitro* anti-HIV role although it is difficult to prove a cause-effect relationship between TRIM22 expression levels and viral load or CD4 T cell count variables. Based on our findings, I speculate that targeted enhancement of the expression of TRIM22 in HIV-1 infected individuals may be beneficial in reducing viral load and could be employed as a novel antiviral strategy.

The landscape of a chronic HIV-1 infection is characterized by a progressive dysfunction of the host's immune system. Chronic HIV infection affects multiple components of the immune system and high levels of immune activation is noted in chronically HIV-1 infected patients (Pandrea et al., 2008). Immune activation is attributed to bystander activation in response to e.g. viral products, microbial products,

cell destruction and/or self-antigens (Grossman et al., 2006). There are numerous factors such as : direct effects of specific HIV-1 gene products, innate and the adaptive immune response to HIV-1, translocation of microbial products across damaged intestinal mucosa, co-infections with non-HIV-1 pathogens, bystander activation, depletion/dysregualtion of CD4 Tregs, viral evaluation that are considered as potential contributors to the chronic immune activation in HIV infection (Grossman et al., 2006, Sodora and Silvestri, 2008, Appay and Sauce, 2008, Bartha et al., 2008, Cadogan and Dalgleish, 2008, Munier and Kelleher, 2007). It was important to look at modulation of host restriction factors during a chronic HIV-1 infection. I looked at the levels of MxA, huTRIM5α and TRIM22 to extend previous results.

HIV-1 chronically infected patients have higher levels of MxA when compared to other groups. Type 1 interferons are known to be elevated during increased generalized immune activation, which in turn has been associated with increased HIV-1/AIDS pathology (Mandl et al., 2008, Lehmann et al., 2009). I confirmed previous studies that show higher levels of MxA in the chronically HIV-1 infected group when compared to primary infection and the HIV-1 negative group (Sedaghat et al., 2008).

Both the HIV-1 infected groups had higher levels of TRIM22 compared to the HIV-1 negative group; however huTRIM5α levels progressive decrease during different phases of HIV-1 infection when compared to the HIV-1 negative group. This result contradicts the literature on the induction of TRIM5. huTRIM5α has been shown to be an IFN-α inducible gene *in vitro* and we have shown that there is a marked interferon 1 responses in these samples, increases in MxA in HIV-1

chronically infected group was noted (Sakuma et al., 2007). The regulation of huTRIM5α *in vivo* is more complicated than just interferon 1 induction.

The chronically infected group where characterized as being in the end stages of HIV-1 infection with high viral loads and low $CD4^{+}$ T cell counts with a co-infection, meningitis. Co-infections with pathogens other than HIV also contributes to immune activation, and some pathogen gene products directly enhance HIV replication by trans-activation of HIV LTR (Lawn et al., 2001, Borkow and Bentwich, 2006).

I investigated the expression of these anti-viral factors in the cells found in the CNS compartment (CSF) of the HIV-1 chronically infected group. Moderately higher levels of IFN-1, MxA were noted in the CNS compartment when compared to the periphery as documented by other groups (Fink et al., 2007, Rouse and Sehrawat, 2010, Glimaker et al., 1994). I observed higher levels of huTRIM5α in the CNS compartment when compared to the periphery; however I did not see significant differences in TRIM22 between the periphery and CNS compartment. TRIM5α may potentially regulate inflammatory pathways differently depending on the stimuli given (Jefferies et al., 2011). Pertel and colleagues showed that TRIM5α acts as a pattern recognition receptor (PRR) for the retroviral capsid of HIV, promoting innate immune signalling and NF-κB activity (Pertel et al., 2011). TRIM5α has been shown to interact with and ubiquitylate TAK1-binding protein 2 (TAB2) and this results in the degradation of TAB2, disruption of the TRAF6–TAK1–TAB complex and inactivation of NF-κB-dependent pathways (Tareen and Emerman, 2011). In our setting huTRIM5α may be holming to site of infection brought about by the meningitis itself. The increase in IFN-1 in the chronically infected group with HIV-1 may be a result,

rather than a cause, of disease progression and may reflect increasing pathologic immune activation, driven by HIV-1 itself, opportunistic pathogens, or other microbial stimuli (Brenchley et al., 2006). CNS infections induce pro-inflammatory cytokines such as IL-1α, IL-1β, TNF-α, IL-6 and IL-12 in astrocytes and microglia (Bergmann et al., 2006).

I further dissected this result by looking at the different cell types found in the CNS (CSF) and tested the hypothesis that higher proportions of immune cells found in the CNS, as consequence of an infection, is driving the expression seen in the CNS compartment when compared to the periphery. I found an enrichment of NK and T cells found in the CNS compartment when compared to matched samples from the periphery. This result is consistent with other studies looking at meningitis and cells found in CSF (Moench and Griffin, 1984, Cepok et al., 2001, Tan et al., 2008).

I also investigated the association of anti-viral gene expression and viral load, a commonly used marker of disease progression. I confirmed the results obtained in a previous study where we did not show an association between viral loads and huTRIM5α expression in primary HIV-1 infection, as here we show a similar result in chronic HIV-1 infection (Sewram et al., 2009). I showed that TRIM22 has a moderate negative association with viral loads in the periphery ($r=-0.397$, $p=0.044$).

In the current study I could not differentiate between the effects that chronic HIV-1 infection and an opportunistic infection had on the expression of huTRIM5α or TRIM22. I have noted that a higher level of IFN-1, (MxA) was found in the CNS compartment when compared to the periphery which could be a surrogate marker for

immune activation. I tried to differentiate between the effects of immune activation and HIV-1 by stimulating CEM-SS cells with IFN-α, IL-2 and CD2/3/28 followed by infecting these cells with HIV-1 and tracking the expression of huTRIM5α and TRIM22 over a seven day period.

The expression profile of huTRIM5α and TRIM22 is different, when cells are stimulated with IFN-α, IL-2 and CD2/3/28. The greatest induction of huTRIM5α by IFN-α or IL-2 was seen seven days post stimulation. However IFN-α induced TRIM22 from 24 hours post stimulation, by seven days post stimulation we did not significant differences between different stimuli on TRIM22 expression.

I confirmed previous results performed by Obad and colleagues where they showed that CD2/3/28 co-stimulation brings about modest upregulation of TRIM22 in purified NK and CD4 cells; we show similar results in CEM cell lines (Obad et al., 2007). I also confirmed previous studies where we show that IFN-α blocks HIV-1 replication, (Singh et al., Barr et al., 2008, Bouazzaoui et al., 2006). A block in HIV-1 replication was noted in groups stimulated with IFN-α and CD2/3/28. Significant induction in TRIM22 was noted for cells stimulated with CD2/3/28.

Collectively my results demonstrate that the modulation of huTRIM5α and TRIM22 is dependent on the activation state of the cell where different results is obtained at the time of sampling. I observed discordance between induction of TRIM22 or huTRIM5α and inhibition of HIV-1 replication. These results may explain the loss of association between viral load and gene expression seen in the CNS compartment where cells are highly activated.

In the current study I looked at immune cells that translocated into the CNS compartment, however I did not look at the CNS cell types such as neuroblasts and astrocytes which make up the CNS (Leavitt et al., 1999). I tried to address this question by looking at the expression of huTRIM5α and TRIM22 in immune and neuronal cell under different pro and anti-inflammatory stimuli.

I looked at the effects of IFN-α stimulation on CNS cell lines and compared them to the T and B cell lines on huTRIM5α and TRIM22 expression. I demonstrated that the CNS cell lines where inducible by IFN-α stimulation but not as much as the T and B cell lines for huTRIM5α and TRIM22 expression. I have previously shown that TRIM22 and huTRIMα is highly inducible by IFN-α in CEM-SS, Jurkat and THP1 cell lines (Singh et al., 2011).

I next examined the hypothesis that cytokines/chemokines have the ability to modulate huTRIM5α and TRIM22 expression in neuronal cells, much of the recent reports have focused on the effects of IFN-1 in immune cells where it has been shown that TRIM22 or TRIM5 is highly inducible by IFN-1(Sakuma et al., 2007, Barr et al., 2008, Carthagena et al., 2009). The pattern of expression of huTRIM5α and TRIM22 is dependent on the cell line screened and the cytokine/chemokine used.

The above mentioned findings prompted me to look at if these host restriction factors namely huTRIM5α and TRIM22 had any functional effects on HIV-1 replication in neuronal cells. I firstly knocked down huTRIM5α and TRIM22 in neuroblasts (CRL-2137) and astrocytes using siRNA against huTRIM5α and TRIM22. Briefly even after IFN-α induction both knock down cell lines for huTRIM5α and

TRIM22 showed lower levels compared to the control cell lines where GAPDH and a non targeting siRNA was used. This is the first report to focus on huTRIM5α and TRIM22 in the CNS.

I further demonstrated that huTRIM5α and TRIM22 produced by astrocytes and neuroblasts has anti-viral activity, as cells transfected with siRNA against huTRIM5α or TRIM22 had higher levels of pseudotyped HIV-1 replication when compare to the control cell lines as seen by p24Elisa performed on the lysates from the experiment. I next measured the levels of huTRIM5α and TRIM22 on the lysates, to look at the effects of HIV-1 replication on expression of these restriction factors. Higher levels of TRIM22 were noted in the control cell lines when compared to the knock down cell lines. However I did not see significant changes in huTRIM5α on knock down cell lines when compared to the control cell lines. This result suggests that although we had knockdown huTRIM5α, huTRIM5α was still being induced by HIV-1 replication. I have strong evidence that TRIM22 plays a part inhibiting HIV-1 in neuronal cells however suttle changes in huTRIM5α expression may have profound effects on HIV-1 replication.

Based on my findings, I speculate that targeted enhancement of the expression of TRIM22 in HIV-1 infected individuals may be beneficial in reducing viral load and could be employed as a novel anti-viral strategy. I can further speculate one of the approaches would be to reduce immune activation in chronically infected patients as this would enhance these anti-viral factors.

Apart from enhancing the effect of HIV-1 restriction factors to combat HIV-1 replication one could inhibit the effects of a cellular co factors to HIV-1. I looked at a well characterized cellular co factor to HIV-1, Cyclophilin A (CypA) in the primary infected samples to understand its modulation in a natural course of HIV-1 infection. I investigated associations between CypA expression and huTRIM5α expression. TRIM5α promotes premature disassembly of the capsid of HIV-1 and CypA promotes proper disassembly of the capsid. CypA which is a ubiquitously expressed cellular protein that was identified as a cofactor for HIV-1, leading to enhanced infection by an unknown mechanism (Luban et al., 1993).

The discovery of Trim5-CypA fusion protein as a HIV restriction factor in rhesus macaques (Sayah et al., 2004, Nisole et al., 2004) and the dependency of rhesus TRIM5α on CypA for HIV restriction has revived interest in this ubiquitously expressed cellular protein (Berthoux et al., 2005, Keckesova et al., 2006, Stremlau et al., 2006, Rits et al., 2007). The owl monkey and pig-tailed macaque TRIM5 are fusion proteins with Cyclophilin A, where the C-terminal domain of TRIM5 is replaced by Cyclophilin A (Newman et al., 2008, Nisole et al., 2004, Sayah et al., 2004). In owl monkeys this fusion protein potently restricts HIV (Nisole et al., 2004, Sayah et al., 2004). Neugue and colleagues humanized this TRIM5-Cyclophilin fusion protein and showed that this fusion protein was more effective at restricting HIV1 than rhTRIM5α and may have potential as a novel therapeutic (Neagu et al., 2009, McNab et al., 2011).

Previous studies had suggested that HIV-1 is susceptible to restriction by huTRIM5α, but only when interaction between CA and CypA is disrupted (Towers,

2005). Other studies had shown that supranormal levels of huTRIM5α inhibit the titer of wild-type HIV-1, even in the presence of CypA (Hatziioannou et al., 2004b, Perron et al., 2004, Song et al., 2005c, Stremlau et al., 2005).

I investigated whether there is a relationship between CypA levels and huTRIM5α, by looking for associations between each other. I did not see association's between huTRIM5α and CypA levels. These results suggest the expression of huTRIM5α and CypA are independently regulated *in vivo*. Other groups have demonstrated that a decrease of CypA activity in human cells by siRNA mediated knock down or CsA treatment also decreases replication of HIV-1 and this appears to be in a TRIM5α independent manner (Sokolskaja et al., 2006, Keckesova et al., 2006).

I did not see a difference in CypA levels between HIV negative and HIV-1 positive participants and I did not see significant changes in CypA expression during the first 12 months of HIV-1 infection in our unmatched group. However I saw there was marked differential expression in CypA in the HIV-1 negative group. I also did not see significant associations between viral load or CD4 T cell counts and CypA expression.

It has been demonstrated that the target cell CypA levels rather than the virion associated CypA impacts on HIV-1 infectivity (Towers et al., 2003, Hatziioannou et al., 2005, Sokolskaja et al., 2004, Kootstra et al., 2003). I looked at the relationship between the CypA genotype and mRNA expression to try to shed some light on the differences in CypA levels seen. I looked at whether SNP A1650G of *PPIA* gene influence CypA expression levels *in vivo.*

CypA expression levels between 1650A and 1650G genotypes were not significantly different amongst the negative group. However the 1650G SNP was significantly associated with elevated levels of CypA mRNA expression post infection ($p < 0.01$). These results were validated by performing an HIV-1 replication experiment. Similar results were obtained by Rits and colleagues where they had shown for the CypA 1604CC/AA genotype compared to CypA 1604 CG expressed higher levels of CypA mRNA but they did not find significantly higher p24 levels between these two genotypes when a viral replication experiment was performed and analyzed 12 days post infection (Rits et al., 2008, Bleiber et al., 2005).

Studies validated CypA as an important cellular cofactor of HIV-1 by identifying single nucleotide polymorphisms (SNPs) within *PPIA* gene that are associated with HIV-1 disease outcome (An et al., 2007b, Bleiber et al., 2005, Rits et al., 2008). Here I showed that this SNP may effect mRNA expression of CypA *in vivo* and *in vitro*.

Although CypA expression levels are dysregulated by HIV-1 infection, my results demonstrate that PBMCs genotyped for 1650G allele expresses higher levels of CypA mRNA than PBMCs genotyped for wild type allele, 1650A. I have demonstrated in this study that rather than the environment (LPS stimulation), a specific genotype effects CypA expression and viral control.

Collectively it could be speculated that if a person expresses high levels of CypA or the 1650G/AG genotype, this may increase ones risk to become HIV-1

infected or a person expressing high levels of CypA 1650G/AG will have a faster disease progression to AIDS. New drugs could block this interaction.

The data generated in this study adds to our understanding of the mechanisms of HIV-1 pathogenesis and provides evidence that some HIV-1 restriction factors and replication cofactors to HIV-1 are plausible targets for the design of novel therapeutics to HIV-1.

One of these strategies could be to induce or increase *in vivo* endogenous TRIM22 and huTRIM5α expression by using a small molecule. Many TRIM E3 ligases are interferon inducible and it is likely that systemic administration of interferon will induce systemic deleterious effects and hence the need to explore the small molecules that can specifically enhance antiviral TRIM E3 ligases (such as TRIM22) expression *in vivo*. Another strategy may to design a small molecule that interacts with the CypA promoter region to reduce CypA expression which may be beneficial to patients with HIV-1. A strategy in which we could reduce immune activation, increase TRIM22 expression and block CypA overexpression using small molecules could be beneficial in combating HIV-1 pathogenesis.

Future studies will need to look at other HIV-1 restriction factors as plausible targets for novel therapies. This will extend the knowledge of how these HIV-1 restriction factors work as a network. Apart from looking at the expression profiles of these novel HIV-1 restriction factors during HIV-1 pathogenesis, one could look for novel SNPs that could be modulating the expression of these HIV-1 restriction factors.

Future studies will need to address the specific mechanisms of viral blockade and enhancement by these restriction and replication cofactors respectively, and to explore possible specific intervention strategies targeting these factors for novel anti-HIV-1 therapies or prophylactics.

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6 Appendix

Appendix I: Protocol and Ethics forms

UNIVERSITY OF
KWAZULU-NATAL

03 August 2010

Professor T Ndung'u
HPP
DDMRI

Dear Professor Ndung'u

PROTOCOL: " Regulation of TRIM E3 Ligases and Cyclophilin A and the Impact on HIV-1 Replication and Pathogenesis" R Singh 204519807 Molecular Biology

The Postgraduate Education Committee ratified the approval of the abovementioned study on 03 August 2010.

Please note:

- The Postgraduate Education Committee must review any changes made to this study.
- The study may not begin without the approval of the Biomedical Research Ethics Committee.

May I take this opportunity to wish the student every success with the study.

Yours sincerely

Professor SJ Botha
Chair Postgraduate Education Committee

CC. Mr R Singh

Biomedical Research Ethics Committee
Westville Campus

Postgraduate Education Administration
Medical School Campus

Postal Address: Private Bag 7, Congella, 4013, South Africa
Telephone: +27(0)31 260 4327 **Facsimile:** +27(0)31 260 4401 **Email:** heslopj@ukzn.ac.za **Website:** www.ukzn.ac.za
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

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BIOMEDICAL RESEARCH ETHICS ADMINISTRATION
Research Office, Westville Campus
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KwaZulu-Natal, SOUTH AFRICA
Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/ResearchEthics11415.aspx

04 November 2008

Professor S S Abdool Karim
CAPRISA
2nd Floor, DDMRI Building
Nelson R Mandela School of Medicine

Dear Professor Abdool Karim

PROTOCOL: CAPRISA 002 Viral set point and clinical progression in HIV-1 subtype C infection: The role of immunological and viral factors during acute and early infection, From 15th September 2005, Version 3.00 to Amendment#2 Version 4.00 dated 19 June 2007. S S Abdool Karim, CAPRISA. REF: E013/04

I wish to advise you that your letter dated 16 September 2008 was noted by the full sitting of the Committee at its meeting of **14 October 2008.**

The Committee agreed to grant permission to use the tissues and requested that the following information be provided :

1. Reference numbers
2. Proof of Postgraduate studies

Yours sincerely

Prof. D Wassenaar
Chair: Biomedical Research Ethics Committee
DW/pf

RECEIVED
2008 -11- 07
CAPRISA

CAPRISA

CENTRE FOR THE AIDS PROGRAMME OF RESEARCH IN SOUTH AFRICA

CAPRISA IS A UNAIDS COLLABORATING CENTRE FOR HIV PREVENTION RESEARCH

Doris Duke Medical Research Institute (2nd Floor),
Nelson R Mandela School of Medicine,
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Congella, 4013, Durban, South Africa
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E-mail: [illegible]

UNIVERSITY OF KWAZULU-NATAL

UNIVERSITY OF CAPE TOWN

National Institute for Communicable Diseases

UNIVERSITY of the WESTERN CAPE

2nd December 2008

Receipt of Documents

This form serves as confirmation of receipt of documents. Please check attached documents and sign confirmation of receipt. Please return to:

Francois
CAPRISA
2nd Floor DDMRI
Nelson R Mandela School of Medicine

I herby confirm that I have received the following documents from CAPRISA in relation to the **CAPRISA 002 Viral Set Point and Clinical Progression in HIV-1 Subtype C Infection: The Role of Immunological and Viral Factors during Acute and Early Infection. 19th June 2007, Version 4.00 S.S. Abdool Karim, CAPRISA (Ref: E013/04)** study (***please tick all received***):

Tick	
☑	Letter dated 2nd December 2008 signed by Professor Salim S Abdool Karim, in response to a request for more information from BREC in a letter dated 4th November 2008.

[signature] Administration 02/12/08

Signature | Print Name & Position | Date

RESEARCH OFFICE
2008 -12- 02
RECEIVED

RESEARCH OFFICE
2008 -12- 02
RECEIVED

CAPRISA is supported by the National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), US Department of Health and Human Services (Grant #AI51794)

A multi-institutional collaboration, incorporated as an independent non-profit AIDS Research Organisation

Registration Number: 2002/024027/08

www.caprisa.org

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20th November 2008

Professor Douglas Wassenaar
Chairperson, Biomedical Research Ethics Committee
Faculty of Health Sciences
University of KwaZulu-Natal

Dear Professor Wassenaar,

RE: CAPRISA 002 Viral Set Point and Clinical Progression in HIV-1 Subtype C Infection: The Role of Immunological and Viral Factors during Acute and Early Infection. 19th June 2007, Version 4.00 S.S. Abdool Karim, CAPRISA (Ref: E013/04)

Many thanks for your letter dated 4th November 2008 providing permission for us to use tissue collected under the Acute Infection study, on student work and their associated collaborations. The committee requested two additional pieces of information:

1. Reference Numbers
   The laboratory studies listed in our letter are all contained within the host genetic components of the CAPRISA 002 Acute Infection study protocol. The reference number for this study is E013/04 and all the laboratory studies listed have been conducted under this study.

2. Proof of Postgraduate Studies
   All the students listed on our letter are registered at the Nelson R Mandela School of Medicine, University of KwaZulu Natal, and their registration numbers are listed below with their postgraduate registration status:

   a. Kavidha Reddy. PhD. *The Role of Apobec3g in Early and Acute HIV-1 C Infection*. Registration number: 983180970.
   b. Veron Ramsuran. PhD. *Chemokine Receptor Expression and Genetic Poly Morphisms in South Africa: Assocation with HIV - 1 Infection and Disease Progression*. Registration number: 200275942.
   c. Paradise Madlala. PhD. *Genetic Polymorphisms of Lens-Epithelium Derived Growth Factor (Ledgf)/P75 and Their Role in HIV Replication)*. Registration number: 982205632.
   d. Ramona Moodley. PhD. *Role of KIR Genes in Acute and early HIV-1 infection*. Registration number: 204524187.
   e. Shamman Sewram. M.Sc. *Characterization of Human TRIM5α and Investigation of its Role in Susceptibility to HIV and in Disease Progression*. Registration number: 992237869.
   f. Ravesh Singh. PhD. *Screening and Characterisation of HIV Resistance and Infection of Host Genes by Microarray Technology*. Registration number: 204519807.

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UNIVERSITY OF CAPE TOWN

National Institute for Communicable Diseases

UNIVERSITY of the WESTERN CAPE

CAPRISA was established in 2002 through a CIPRA grant from the NIH, as a multi-institutional collaboration, incorporated as an independent non-profit AIDS Research Organization

Registration Number: 2002/024027/08

www.caprisa.org

Please do not hesitate to contact me should you require additional clarification.

Yours sincerely

Professor Salim S. Abdool Karim
Director: CAPRISA
Pro Vice-Chancellor (Research), University of KwaZulu-Natal
Professor of Clinical Public Health, Columbia University
Adjunct Professor of Medicine, Cornell University

CAPRISA

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National Institute for Communicable Diseases

Columbia University

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Registration Number: 2002/024027/08

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16th September 2008

Professor Douglas Wassenaar
Chairperson, Biomedical Research Ethics Committee
Faculty of Health Sciences
University of KwaZulu-Natal

Dear Professor Wassenaar,

RE: CAPRISA 002 Viral Set Point and Clinical Progression in HIV-1 Subtype C Infection: The Role of Immunological and Viral Factors during Acute and Early Infection. 19th June 2007, Version 4.00 S.S. Abdool Karim, CAPRISA (Ref: E013/04)

I refer to the above protocol, in which we aim to define immunological and virological factors during acute and early HIV-1 subtype C infection. Specific blood draws were included in the protocol for host genetics studies, and participants gave consent for specimens to be stored for additional studies. Participants have consented to these stored specimens being used for additional studies, and to be sent abroad for additional assessments. The BREC approved consent form states that "We may test your cells, proteins, other chemicals in your body and your genes (DNA). Some of the samples will also be tested to see how your nutritional status may be interacting with HIV-1 infection. Your samples may be analyzed in laboratories outside of South Africa.", Storage consent form, page 1, "Use of Stored Samples". (Version 4.00, dated 19th June 2007).

The purpose of this letter is to notify the committee that we have since embarked on a number of studies that involve analysis of cells, proteins, chemicals and DNA, in an attempt to gain better understanding of the mechanisms involved in relative resistance to HIV-1 infection, or in control of viral replication in the acute and early phase. Below is a list of studies we are undertaking, as part of the "host genetics" component of the study. In most of these substudies, the work has been undertaken by UKZN students under the supervision of Professor Thumbi Ndung'u. In cases where noted, we have worked with collaborators outside of South Africa. It should be noted that the consent form specifies that samples may be used outside of South Africa.

1) The role of TRIM5α in susceptibility to HIV-1 and early virus control- undertaken by student Shamman Sewram. No outside investigator involved.
2) The Role of Cytidine Deaminases in Acute and Early HIV-1 Infection- student Kavidha Reddy. Collaborating international scientist, Dr Cheryl Winkler, Laboratory of Genomic Diversity, Science Applications International Cooperation-Frederick, National Cancer Institute-Frederick, Frederick, Maryland, USA.
3) Lens Epithelium Derived Growth Factor (LEDGF) in Transportation of the HIV-1 preintegration complex in vivo- student Paradise Madlala. Outside collaborators: Dr Zeger Debyser, Katholik University, Leuven, Belgium and Dr Cheryl Winkler, USA.
4) Killer Immunoglobulin Receptor molecules and Natural killer cells in HIV-1 Control- student Ramona Moodley. International collaborator- Dr Marcus Altfeld (Massachusetts General Hospital and Harvard Medical School).
5) Chemokine receptor Genes and Whole Genomewide Association Studies- student Veron Ramsuran, collaborator Dr Sunil Ahuja, University of Texas Health Center. Natural Killer Cells.

6) Microarray Technology for Assessment of Host Factors involved in HIV-1 Replication. Student Ravesh Singh, collaborator Rafick-Pierre Sekaly, University of Montreal, Canada
7) Interleukin 10 Gene polymorphisms in HIV-1 Infection- student Dshanta Naicker

These studies are mostly exploratory in nature and we consider them of "high risk, high potential impact" in nature because of the general lack of clarity in the field regarding the contribution of host genetic factors to the heterogeneity seen in HIV-1 exposure and infection. We would be grateful if the committee could take note of these substudies as part of this protocol for your records.

Yours sincerely

Professor Salim S Abdool Karim
Pro Vice Chancellor (Research), University of KwaZulu-Natal
Director: CAPRISA – Centre for the AIDS Programme of Research in South Africa
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UNIVERSITY OF
KWAZULU-NATAL

1 July 2008

Professor K. Dheda
University of Cape Town
Department of Respiratory Medicine
H46, H Floor, Salmouth Building
Anzio Road
Observatory
7925

Dear Professor Dheda,

PROTOCOL: Diagnostic value and performance outcomes of the newer technologies (interferon gamma release assays (IGRA) and TB antigen detection test) in patients with tuberculous meningitis (TIGRA Study) - Dr V B Patel, PHD, Neurology, student number 783786134 (Ref PG045/07)

At a meeting of the Postgraduate Education Committee held 1 July 2008, your request for approval of protocol amendments dated 1 July 2008 have been noted and approved.

Yours sincerely

Professor P Moodley
Dean's Assistant : MMedSc & PhDs
Postgraduate Education Committee
HD/PatelV

cc Dr V. Patel
Dept. of Neurology
IALCH

Professor T. Ndung'u
HIV Medicine

Postgraduate Education administration
Medical School Campus
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Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

UNIVERSITY OF KWAZULU-NATAL

BIOMEDICAL RESEARCH ETHICS COMMITTEE

APPLICATION FOR ETHICAL APPROVAL OF AMENDMENTS

NAME OF RESEARCHER: DR VB Patel

DEPARTMENT: Neurology, IALCH, UKZN

TITLE OF STUDY: The original title was:
The value of gamma interferon response to culture filtrate protein 10 (CFP10), early secreted antigen 6 (ESAT 6) and heparin binding hemagglutinin (HBHA) as a diagnostic test in patient with tuberculous meningitis(TBM) and the effect of depletion of CD25+ T regulatory cells and memory cells in TBM. The value of this test as a marker for improvement in patients with TBM.
Diagnostic value and performance outcomes of the newer technologies (interferon gamma release assay (IGRA) and TB antigen detection test) in patients with tuberculous meningitis (TIGRA Study).

ETHICS REFERENCE NO: E325/05

DATE OF ETHICAL APPROVAL OF STUDY: 17th May 2006

DATE OF AMENDMENTS: amended to postgraduate committee in 12 th May 2008.

AMENDMENTS REQUESTED:

1. The original protocol had the following title: The value of gamma interferon response to culture filtrate protein 10 (CFP10), early secreted antigen 6 (ESAT 6) and heparin binding hemagglutinin (HBHA) as a diagnostic test in patient with tuberculous meningitis(TBM) and the effect of depletion of CD25+ T regulatory cells and memory cells in TBM. The value of this test as a marker for improvement in patients with TBM. It is changed to **Diagnostic value and performance outcomes of the newer technologies (interferon gamma release assay (IGRA) and TB antigen detection test) in patients with tuberculous meningitis (TIGRA Study).**
This is because we have realised that funding for the additional immunological tests is not available. Further that follow up of patient in this cohort will be inadequate. The new title does not deviate from the previous intent of the study but limits it to diagnostic tests for TBM which is the thrust of the study. There will be impact on patient in that follow up lumbar punctures are unlikely to be done thus reducing effect on patients. Lastly HBHA is not presently available.

The original protocol did not include the following. Following discussion with colleagues

1. The T Cell profile in blood and CSF in TBM patients (CD4/CD8/CD20/CD17/CD4 RO/CD4RO/Dendritic cells, CD25 fox p3, macrophages) in terms of percentages
2. Cytokine profiles such as TH1 (IFN gamma, IL2, TNF alpha), TH2 (IL4, IL10), TH3 (TGF beta), TH5 (CD17)
3. Characterisation of HIV viral genome in blood and CSF
4. Measurement of viral loads in blood and CSF
5. Detecting TB specific genomic sequence in CSF by PCR
6. Possibly longitudinal cytokine patterns in a selection of patients with TBM
7. Original protocol did not include LAM studies which has been included in the subsequent extensively revised protocol already submitted.

These tests will be considered provided funds are available. All these amendments will not have any effect on the patient as these will be done on already acquired samples.

SIGNATURE OF PRINCIPAL RESEARCHER: .....DATE: .....

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Website: http://research.ukzn.ac.za/ResearchEthics11415.aspx

15 July 2009

Dr V B Patel
Level 3, D Block
Albert Luthuli Central Hospital
Cator Manor
Durban
4041

Dear Dr Patel

PROTOCOL: The value of gamma interferon response to culture filtrate porotein 10 (CFP 10), early secreted antigen 6 (ESAT 6) and heparin bindin haemagglutinin (HBHA) as a diagnostic test in patients with tuberculousis meningitis (TBM) and the effect depletion of CD 25T regulatory cells and memory cells in TBM. The value of this test as a marker for improvement in patients treated for TBM, VB Patel, Medicine, Neurology. Ref: E325/05.

PROTOCOL AMENDMENT RATIFICATION

Further to our letter to you dated **04 June 2009**, this letter serves to notify you that at a full sitting of the Biomedical Research Ethics Committee Meeting held on **14 July 2009**, the Committee RATIFIED the sub-committee's decision to approve Protocol Amendments.

Yours sincerely

Ramnarain

Ms D Ramnarain
Senior Administrator: Biomedical Research Ethics
DR/dl

Appendix II: Published paper arising from study

JOURNAL OF VIROLOGY, Jan. 2011, p. 208–216 Vol. 85, No. 1
0022-538X/11/$12.00 doi:10.1128/JVI.01810-10

Association of TRIM22 with the Type 1 Interferon Response and Viral Control during Primary HIV-1 Infection[▽]

Ravesh Singh,[1] Gaurav Gaiha,[2] Lise Werner,[3] Kevin McKim,[2] Koleka Mlisana,[3] Jeremy Luban,[4] Bruce D. Walker,[1,2,5] Salim S. Abdool Karim,[3] Abraham L. Brass,[2] Thumbi Ndung'u,[1,2,3]* and the CAPRISA Acute Infection Study Team

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Type 1 interferons (IFNs) induce the expression of the tripartite interaction motif (TRIM) family of E3 ligases, but the contribution of these antiviral factors to HIV pathogenesis is not completely understood. We hypothesized that the increased expression of select type 1 IFN and TRIM isoforms is associated with a significantly lower likelihood of HIV-1 acquisition and viral control during primary HIV-1 infection. We measured IFN-α, IFN-β, myxovirus resistance protein A (MxA), human TRIM5α (huTRIM5α), and TRIM22 mRNA levels in peripheral blood mononuclear cells (PBMCs) of high-risk, HIV-1-uninfected participants and HIV-1-positive study participants. Samples were available for 32 uninfected subjects and 28 infected persons, all within 1 year of infection. HIV-1-positive participants had higher levels of IFN-β ($P = 0.0005$), MxA ($P = 0.007$), and TRIM22 ($P = 0.01$) and lower levels of huTRIM5α ($P < 0.001$) than did HIV-1-negative participants. TRIM22 but not huTRIM5α correlated positively with type 1 IFN (IFN-α, IFN-β, and MxA) (all $P < 0.0001$). In a multivariate model, increased MxA expression showed a significant positive association with viral load ($P = 0.0418$). Furthermore, TRIM22 but not huTRIM5α, IFN-α, IFN-β, or MxA showed a negative correlation with plasma viral load ($P = 0.0307$) and a positive correlation with CD4+ T-cell counts ($P = 0.0281$). *In vitro* studies revealed that HIV infection induced TRIM22 expression in PBMCs obtained from HIV-negative donors. Stable TRIM22 knockdown resulted in increased HIV-1 particle release and replication in Jurkat reporter cells. Collectively, these data suggest concordance between type 1 IFN and TRIM22 but not huTRIM5α expression in PBMCs and that TRIM22 likely acts as an antiviral effector *in vivo*.

Tripartite interaction motif (TRIM) E3 ligases represent a recently described family of proteins with potent antiviral activity (39). There are approximately 70 TRIM family members, and they are characterized by the presence of a tripartite motif, which consists of a RING domain, one or two B-box motifs, and a coiled-coil region (21, 29, 39). The presence of the RING domain suggests that these proteins function as E3 ubiquitin ligases and mediate ubiquitylation events (6). The E3 ubiquitin ligase activity of the RING domain is important for the antiretroviral function of many TRIM proteins (29, 38).

The prototype member of this family, TRIM5α, is responsible for the complete block of HIV-1 replication in Old World monkey cells (33, 36). This effect is mediated through the interaction of rhesus monkey TRIM5α (TRIM5αrh) with the HIV-1 capsid (36). Further studies suggested that in addition to the effects of TRIM5αrh on HIV via binding to capsid, other mechanisms of viral inhibition are possible (27, 31). TRIM5α is responsible for the species-specific postentry restriction of retroviruses, such as N-tropic murine leukemia virus (N-MuLV) and HIV-1, in primate cells (36, 45). Other TRIM E3 ligases with antiviral activity have been described (45). TRIM family proteins affect specific steps in the HIV life cycle (13). TRIM proteins appear to mediate their antiviral activities via diverse mechanisms: interference with the uncoating of the viral preintegration complex was noted for TRIM5α (24), and an inhibition of viral budding has been described for TRIM22 (26).

Although the antiretroviral activity of TRIM E3 ligases is established, the contribution of this family of proteins to protection against HIV-1 infection or to the control of disease progression is largely unknown. Many *in vitro* studies have suggested that human TRIM5α (huTRIM5α) has little effect on HIV replication. However, some huTRIM5α genetic variants have been associated with reduced susceptibility to HIV infection (14, 35), suggesting that huTRIM5α may have a protective role in infection. Modest effects of huTRIM5α genetic polymorphisms on the rate of disease progression have also been reported (9, 41), and it was suggested previously that human TRIM5α may select for HIV-1 escape mutants after a prolonged duration of infection (17).

In a prospective cohort study of HIV-1-negative individuals at high risk for HIV-1 infection, we have recently shown that elevated levels of expression of huTRIM5α are associated with decreased susceptibility to HIV-1 infection (34). Furthermore,

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we found that huTRIM5α mRNA levels were neither actively downregulated nor upregulated among individuals in this cohort who eventually became HIV-1 infected. The latter finding was surprising, because previous studies demonstrated that type 1 interferons (IFNs) are dysregulated during HIV-1 infection (10, 15), and TRIM5α is a well-known type 1 IFN-inducible gene (1, 31). Therefore, we would have expected to observe trends for huTRIM5α similar to those reported for type 1 IFN. In addition to TRIM5α, several TRIM proteins with antiviral activity were recently described and were shown to be IFN inducible (2, 39). However, there are also notable exceptions to this IFN inducibility rule (4, 28). IFNs are themselves the main mediators of innate immunity to viral infection, and they play a significant role by upregulating the expression of many antiviral effectors within the cell (5, 30). Our understanding of the type 1 IFN regulation of TRIMs is complicated by the fact that IFN-α has 13 functional isoforms. It is unclear whether all these isoforms have the same effect on IFN-stimulated genes (23).

Here we investigated the expression of the type 1 IFNs IFN-α (IFN-α2b isoform), IFN-β, a surrogate interferon-inducible gene (myxovirus resistance protein A [MxA]), and TRIM22 in a longitudinal cohort of black African females at high risk for HIV-1 infection, for which we have previously demonstrated that enhanced TRIM5α mRNA expression is associated with reduced susceptibility to HIV infection (34). TRIM22 was selected for this analysis because in addition to TRIM5α, it is one of the relatively well-characterized TRIM E3 ligases, has been shown to be type 1 IFN inducible *in vitro*, and appears to possess anti-HIV-1 activity (2, 4, 25). Specifically, we tested the hypotheses that the increased expression of type 1 IFNs and TRIM22 is associated with a significantly lower likelihood of HIV-1 acquisition and lower viral loads or higher $CD4^+$ T-cell counts during primary HIV-1 infection. We tested whether there are differences in mRNA levels of select type 1 IFNs, huTRIM5α, and TRIM22 in peripheral blood mononuclear cells (PBMCs) from HIV-negative versus HIV-positive individuals. We used multivariate analysis models in order to better understand the kinetics and antiviral implications of the expression of these genes *in vivo*. Finally, we performed *in vitro* experiments to better understand the relationship between type 1 IFNs, TRIM22 expression, and antiviral activity.

MATERIALS AND METHODS

Subjects. Study subjects were part of the CAPRISA 002 Acute Infection Study, which is an observational natural history study of HIV-1 subtype C infection established in Durban, South Africa, in 2004 (34, 40). The cohort consisted of 245 high-risk seronegative women who were monitored to identify acute or recent infections. Participants were enrolled into the acute-infection phase if they were antibody positive within 5 months of a previous antibody-negative test or if they had evidence of viral replication without HIV-1 antibodies, as assessed by rapid tests and PCR testing. Women from other seroincident cohorts in Durban were enrolled into CAPRISA 002 if they met the above-described criteria. Time of infection was defined as the midpoint between the last HIV antibody-negative test and the first HIV antibody-positive test or 14 days prior to the first positive HIV RNA PCR assay for those identified as being antibody negative but HIV RNA PCR positive. The study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal, and participants provided written informed consent.

PBMCs from a total of 32 HIV-1-uninfected and 28 recently HIV-1-infected individuals from the CAPRISA study cohort were available for use in this study.

Sample processing, viral load quantification, and CD4 cell enumeration. PBMCs were isolated by Ficoll-Histopaque (Sigma) density gradient centrifugation from blood within 6 h of phlebotomy and frozen in liquid nitrogen until use. Viral load was determined by using the automated COBAS Amplicor HIV-1 Monitor Test v1.5 (Roche). $CD4^+$ cells were enumerated by using the Multitest kit (CD4/CD3/CD8/CD45) on a four-parameter FACSCalibur flow cytometer (Becton Dickinson).

Lentivirus production. Lentiviruses containing short hairpin RNAs (shRNAs) expressed under the control of the U6 promoter in a lentiviral vector (pLKO.1) that also confers puromycin resistance were generated in 293T cells as previously described (22).

Plasmids pLKO.1, pLKO.1/scramble$_{shRNA}$ (Harvard Institute of Proteomics), and pLKO.1/TRIM22$_{shRNA}$ 3′UTR (5′-CCGGTATTGGTGTTCAAGACTAT ATCTCGAGATATAGTCTTGAACACCAATATTTTTG-3′; Sigma Aldrich) were used. 293T cells were transfected with a packaging plasmid (pCMVdR8.2 dvpr; Addgene), a pRSV-Rev (Addgene) envelope plasmid (VSV-G/pMD2.G; Addgene), and the corresponding pLKO.1 vector.

Viral infection. Peripheral blood mononuclear cells (1×10^6 cells) isolated from healthy HIV-negative donors were placed into a 12-well plate in the presence or absence of CD3.8 antibody (0.5 μg/ml) and incubated for 3 days at 37°C in 5% CO_2. Following stimulation, cells were washed with R10 medium and then infected with HIV-IIIB (NIH AIDS Reagent Repository) by spinoculation (2 h at 2,500 rpm at 37°C) at 2×10^5 cells/well in a 96-well plate. Virus was subsequently removed, and cells were washed once and then allowed to incubate for an additional 3 days before analysis of TRIM22 expression by Western blotting.

For Jurkat LTR-G cell experiments (JLTR-G; NIH AIDS Reagent Repository), cells were transduced by using spinoculation as described above, and the cells were puromycin selected 48 h later. Stably transduced JLTR-G cells in 96-well plates (2×10^5 cells/well) were incubated with IFN-α2a (1,000 U/ml; Perths Biomedical Laboratories, NJ) for 24 h. Cells were then infected by spinoculation with HIV-IIIB. Cells were allowed to incubate for 2 h and then analyzed for HIV long terminal repeat (LTR)-dependent green fluorescent protein (GFP) expression by flow cytometry on days 2 and 7. Cell culture supernatants from day 7 samples were harvested and analyzed by a p24 enzyme-linked immunosorbent assay (ELISA) (Becton Dickinson).

Type 1 IFN stimulation of immune cell lines. CEM, Jurkat, and THP1 cells in 96-well plates (2×10^5 cells/well) were stimulated with IFN-α for 6 h, whereupon gene expression was assessed for TRIM5α and TRIM22.

RNA isolation and analysis. For all samples, RNA was extracted immediately after thawing and counting of PBMCs without *in vitro* stimulation. RNA was extracted from 2×10^6 PBMCs by using TRIzol LS reagent (Invitrogen). The total RNA concentration was quantified, and samples were used only if the optical density at 260 nm (OD_{260})/OD_{280} ratio was 1.90 or greater. All RNA samples were DNase treated. One microgram of total RNA from each sample was reverse transcribed by using the iScript cDNA synthesis kit (Bio-Rad).

RNA quantitation by real-time PCR. The PCR primers and cycling conditions used for IFN-α, IFN-β, MxA, huTRIM5α, and TRIM22 real-time quantitative PCR are provided in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined to be the most suitable reference gene based on PCR efficiency. Each PCR mixture consisted of 3 mmol/μl $MgCl_2$, the respective primers (0.5 pmol/μl for MxA, huTRIM5α, and TRIM22 and 0.25 pmol/μl for IFN-α, IFN-β, and GAPDH), 1 μl Fast Start SYBR green I (Roche), 1 μg cDNA, and water (10-μl total volume). Reactions were run with a Roche Light-Cycler v1.5 instrument (1 cycle at 95°C for 10 min and then 45 cycles of denaturation, annealing, and extension [Table 1]). To confirm amplification specificity, the PCR products were subjected to a melting-curve analysis and agarose gel electrophoresis. Serial dilutions of cDNA from total RNA were performed for each target gene. These served as standard curves for quantitative analysis.

Western blotting. Antibodies used in this study were rabbit polyclonal anti-TRIM22 (Prestige, catalog number HPA008575; Sigma), mouse monoclonal anti-Ran (catalog number R4777; Sigma), or rabbit polyclonal anti-IFITM1 (catalog number ab70477; Abcam). Antibody-antigen complexes were detected by using enhanced chemiluminescence reagents (Invitrogen).

Statistical analysis. The generation of dot plots, nonparametric statistical analysis, and correlations (Pearson) were performed by using the statistical programs Instat Graphpad Prism V.5. and SAS. Values are expressed as medians. Differences between groups were evaluated by using a Student's *t* test. We correlated huTRIM5α and TRIM22 to type 1 IFN (MxA, IFN-α, and IFN-β) gene expression values and to each other. Pearson correlations were performed on log-transformed data. Univariate and multivariate generalized estimating equation (GEE) models were fitted to huTRIM5α, TRIM22, MxA, IFN-α, and IFN-β expressions; viral loads; and CD4 cell counts. Viral loads and expression

TABLE 1. Primers used in this study

Gene	GenBank accession no.	Sequence (5′–3′)*	Cycling conditions (denaturation, annealing, and extension)
MxA	NM_0024462	5′-AAGCTGATCCGCCTCCACTT-3′ (F) 5′-TGCAATGCACCCCTGTATACC-3′ (R)	95°C for 6 s, 60°C for 6 s, and 72°C for 10 s
IFN-α	NM_000069	5′-GAAACCACTGACTGTATATTGTGTGAAA-3′ (F) 5′-CAGCGTCACTAAAAACACTGCTTT-3′ (R)	95°C for 6 s, 60°C for 6 s, and 72°C for 10 s
IFN-β	L41942	5′-AGTCAGAGGGAATTGTTAAGAAGCA-3′ (F) 5′-TTTGGAATTAACTTGTCAATGATATAGGTG-3′ (R)	95°C for 6 s, 60°C for 6 s, and 72°C for 10 s
huTRIM5α	NM_033034	5′-AGGAGTTAAATGTAGTGCT-3′ (F) 5′-ATAGATGAGAAATCCATGGT-3′ (R)	95°C for 6 s, 60°C for 15 s, and 72°C for 6 s
TRIM22	NM_006074	5′-GGTTGAGGGGATCGTCAGTA-3′ (F) 5′-TTGGAAACAGATTTTGGCTTC-3′ (R)	95°C for 6 s, 60°C for 6 s, and 72°C for 10 s
GAPDH	NM_002046	5′-AAGGTCGGAGTCAACGGATT-3′ (F) 5′-CTCCTGGAAGATGGTGATGG-3′ (R)	95°C for 6 s, 65°C for 6 s, and 72°C for 6 s

* F, forward; R, reverse.

levels were log transformed, while square root transformation was applied to CD4 count data to ensure normality.

RESULTS

Relative expression levels of type 1 IFN (IFN-α and IFN-β), MxA, and huTRIM5α in PBMCs from HIV-1-uninfected versus HIV-1-infected subjects. We have previously shown that HIV-negative patients have higher levels of huTRIM5α than do HIV-1-positive patients (34). However, in matched pre- and postinfection samples, we did not see a significant dysregulation of TRIM5α. This result was surprising, as TRIM5α is a type 1 IFN-responsive gene, and type 1 IFN is dysregulated in primary HIV-1 infection (15). Furthermore, we found that women at high risk for HIV-1 infection who did not seroconvert following 2 years of follow-up had significantly higher TRIM5α mRNA levels in PBMCs than did seroconverters (34). A possible explanation for the latter finding is that high-risk nonseroconverter study participants have generally higher levels of innate antiviral defense mechanisms, perhaps mediated through type 1 IFN, thus providing an explanation for our observation of elevated huTRIM5α levels among nonseroconverters. We therefore sought to better understand the relationship between TRIM5α, IFN-α, IFN-β, and a type 1 IFN-inducible gene, MxA.

We compared mRNA levels of IFN-α, IFN-β, MxA, and TRIM5α in PBMCs from HIV-1-negative versus HIV-1-infected samples collected within the first 12 months postinfection. There were 32 individual HIV-1-negative samples available and 28 HIV-1-infected samples. Samples from HIV-1-positive individuals were available at multiple time points postinfection, and samples closest to the 12-month-postinfection time point were included in the analyses presented here. Only patients that remained HIV-1 negative upon 2 years of follow-up were used for this analysis ($n = 19$). The expression values were log transformed to ensure normality. Median expression levels between HIV-negative and HIV-positive samples were compared by using an unpaired Student's t test. There were no significant differences in IFN-α expression between HIV-1-negative and -positive participants (Fig. 1A). HIV-1-positive participants had significantly higher levels of IFN-β ($P = 0.0005$) and MxA ($P = 0.007$) (Fig. 1B and D). As we have previously reported, HIV-1-negative PBMCs had significantly higher levels of TRIM5α than did HIV-1-positive PBMCs ($P < 0.0001$) (Fig. 1C).

We next investigated the relationship between TRIM5α and type 1 IFN expression in HIV-1-negative and -positive samples. There was no correlation between TRIM5α and IFN-α or MxA (Fig. 1E and G) for both negative and positive time points. All HIV-1-negative ($n = 32$) and HIV-1-positive ($n = 75$) samples available at multiple time points were used for this analysis. We found a significant inverse correlation between IFN-β and TRIM5α in both HIV-1-negative ($r = -0.49$; $P = 0.004$) and HIV-positive ($r = -0.39$; $P = 0.0008$) samples (Fig. 1F). As expected, our data also indicated that MxA is a suitable surrogate for type 1 IFN induction, because MxA mRNA levels showed a significant positive correlation with IFN-α in both HIV-1-negative ($r = 0.8$; $P = 0.0001$) and HIV-1-positive ($r = 0.81$; $P = 0.0001$) PBMCs. MxA mRNA levels were also significantly correlated with IFN-β levels in both HIV-1-negative ($r = 0.7$; $P = 0.0001$) and HIV-1-positive ($r = 0.8$; $P = 0.0001$) samples (Fig. 1H). Thus, in this cohort of individuals at high risk for HIV-1 infection in a high-prevalence setting, the level of TRIM5α was higher in HIV-1-negative than in HIV-1-positive PBMCs, and surprisingly, there was an inverse correlation between TRIM5α and IFN-β.

Expression of TRIM22 in PBMCs from HIV-1-uninfected versus -infected subjects and association between type 1 IFN and TRIM22. We next wished to evaluate the expression of the TRIM22 gene, which is located downstream of TRIM5α on chromosome 11 (location 11p15) (32), because it was shown previously to be type 1 IFN inducible *in vitro* and has known antiviral activity (2). HIV-1-positive participants had higher mRNA levels of TRIM22 than did HIV-negative patients ($P = 0.01$) (Fig. 2A). IFN-α mRNA levels positively correlated with TRIM22 expression levels in both HIV-negative ($r = 0.91$; $P < 0.0001$) and HIV-1-positive ($r = 0.9$; $P < 0.0001$) subjects (Fig.

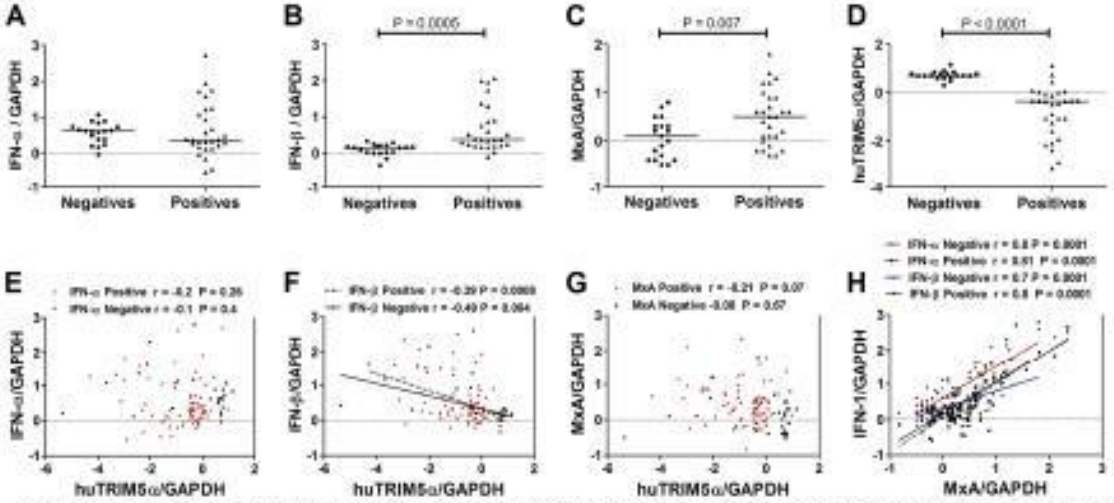

FIG. 1. Expression of type 1 IFN (IFN-α, IFN-β, and MxA) and huTRIM5α in PBMCs from HIV-1-uninfected versus HIV-1-infected subjects and association between type 1 IFN and huTRIM5α. The samples from infected participants were all collected within 12 months of infection. At least two time points were available postinfection for the primary infection samples. For the HIV-1-positive group we compared one time point, closest to the set point of 12 months postinfection ($n = 28$). Only patients that remained HIV-1 negative upon follow-up were used for this analysis ($n = 19$). Data are depicted as the normalized ratio of huTRIM5α, IFN-α, or IFN-β versus GAPDH. The expression values were log transformed to ensure normality. Median expression levels between HIV-negative and HIV-positive samples were compared. The differences between groups were evaluated by using an unpaired Student's t test. A P value of <0.05 was considered statistically significant. Pearson correlations were performed for huTRIM5α and IFN-α, IFN-β, or MxA for both negative and positive patients. Pearson correlations were also performed for MxA and IFN-α or IFN-β for both negative and positive patients.

2B). IFN-β also showed a significant positive correlation with TRIM22 in both HIV-negative ($r = 0.93$; $P < 0.0001$) and HIV-1-positive ($r = 0.87$; $P < 0.0001$) subjects (Fig. 2C). Likewise, MxA mRNA levels correlated positively with TRIM22 mRNA levels in both HIV-1-negative ($r = 0.81$; $P < 0.0001$) and HIV-1-positive ($r = 0.92$; $P < 0.0001$) subjects (Fig. 2D). Thus, TRIM22 positively correlates with type 1 IFN expression in both HIV-1-negative and HIV-1-positive PBMCs *in vivo*.

Expression of type 1 IFN (IFN-α and IFN-β), MxA, and TRIM22 mRNA in PBMCs at baseline (study enrollment) from nonseroconverters versus seroconverters. We next addressed whether preinfection samples from seroconverters differed from those from nonseroconverters in IFN-α, IFN-β, MxA, and TRIM22 expression levels. Although seroconverters showed generally higher mRNA levels of IFN-α and MxA than nonseroconverters, the differences between the groups did not

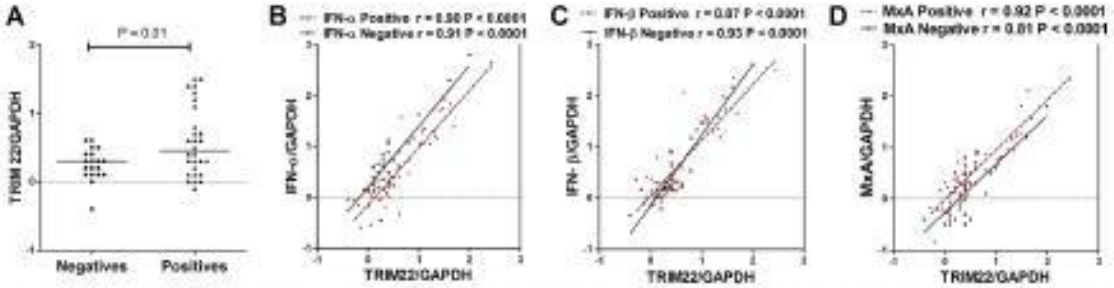

FIG. 2. Expression of TRIM22 in PBMCs from HIV-1-uninfected versus HIV-1-infected subjects and association between type 1 IFN and TRIM22. The samples from infected participants were all collected within 12 months of infection (primary infection phase). At least two time points were available postinfection for the primary infection samples. For the HIV-1-positive group we compared one time point, closest to the set point of 12 months postinfection ($n = 28$). Only patients that remained HIV-1 negative upon follow-up were used for this analysis ($n = 19$). Data are depicted as the normalized ratio of TRIM22 versus GAPDH. The expression values were log transformed to ensure normality. Median expression levels between HIV-negative and HIV-positive samples were compared. The differences between groups were evaluated by using an unpaired Student's t test. A P value of <0.05 was considered statistically significant. Pearson correlations were performed for TRIM22 and IFN-α, IFN-β, or MxA for both negative and positive patients.

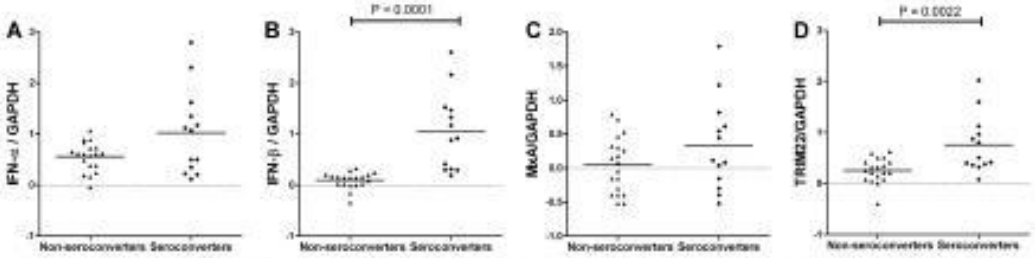

FIG. 3. Expression of type 1 IFN (MxA, IFN-α, and IFN-β) and TRIM22 mRNA in PBMCs at baseline (study enrollment) for nonseroconverters versus seroconverters. Participants included in this analysis were all enrolled as high-risk HIV-1-uninfected individuals and were longitudinally monitored for at least 36 months each at the time of analysis. Data are depicted as the normalized ratio of IFN-α, IFN-β, MxA, and TRIM22 versus GAPDH. The expression values were log transformed to ensure normality. The horizontal line represents the median. The differences between groups were evaluated by using an unpaired Student's *t* test. A *P* value of <0.05 was considered statistically significant.

reach statistical significance (Fig. 3A and C). Individuals who became HIV-1 positive ($n = 13$) during the 2-year study follow-up period had significantly higher IFN-β ($P < 0.0001$) and TRIM22 ($P = 0.0022$) mRNA levels preinfection than did those who remained HIV-1 negative ($n = 19$) ($P < 0.0001$; $P = 0.0022$) (Fig. 3B and D).

Association between antiviral gene expression, viral load, and CD4 T-cell counts. To determine if IFN-α, IFN-β, MxA, huTRIM5α, and TRIM22 gene expressions had functional implications for viral control during primary infection, we used a generalized estimating equation (GEE) model to evaluate viral load or $CD4^+$ T-cell counts, adjusting for repeated measurements for the same individual. In the univariate models, MxA displayed a statistically significant association with HIV-1 plasma viral load. For every log increase in MxA mRNA levels, the viral load increased by 0.29 log copies/ml ($P = 0.0444$). IFN-β also showed a positive association with viral load; however, this was not statistically significant ($P = 0.0995$).

Following adjustment for the other antiviral factors included in this study, MxA and TRIM22 maintained a statistically significant association with viral load. The association between MxA and HIV-1 viral load increased after adjusting for the other antiviral factor expression variables, with every log increase in MxA increasing the viral load by 0.85 log copies/ml ($P = 0.0418$). On the other hand, for every log increase in TRIM22, the viral load decreased by 0.98 log copies/ml ($P = 0.0307$) (Table 2).

$CD4^+$ T-cell counts are an important correlate of disease progression rate and outcome in HIV-1 infection. We therefore investigated whether IFN-α, IFN-β, MxA, huTRIM5α, and TRIM22 had any association with $CD4^+$ T-cell counts during primary HIV-1 infection. A GEE model was fitted to $CD4^+$ T-cell counts, adjusting for repeated measurements for the same individual. In the univariate models, MxA, TRIM22, IFN-α, and IFN-β all had significant a negative association with $CD4^+$ T-cell counts; thus, as the expression increased with 1 log, $CD4^+$ T-cell counts decreased by 2.12, 1.79, 1.75, and 2.04 square root $CD4^+$ T cells/μl for these factors, respectively. However, in the multivariate model, only TRIM22 remained statistically significant ($P = 0.0281$), showing a positive association with $CD4^+$ T-cell counts (Table 3).

TRIM22 expression is induced in HIV-negative PBMCs by infection with HIV-1. We next sought to determine whether infection of PBMCs isolated from HIV-negative donors could induce TRIM22 expression *in vitro*. Infection of PBMCs in the presence or absence of stimulating bispecific CD3.8 antibody (11) resulted in the upregulation of the TRIM22 protein in comparison to uninfected controls (Fig. 4A and B). The stim-

TABLE 2. Association between gene expression and viral load[a]

Gene[b]	Unadjusted model		Adjusted model	
	Effect estimate (SE)	*P* value	Effect estimate (SE)	*P* value
MxA	0.2887 (0.1436)	**0.0444**	0.8539 (0.4195)	**0.0418**
IFN-α	0.2163 (0.1363)	0.1126	0.2512 (0.6476)	0.6981
IFN-β	0.2216 (0.1345)	0.0995	0.1693 (0.6087)	0.7343
huTRIM5α	0.0368 (0.1308)	0.7785	0.1539 (0.1439)	0.2851
TRIM22	0.2154 (0.1532)	0.1597	−0.9807 (0.4539)	**0.0307**

[a] A GEE model was fitted to viral load, adjusting for repeated measurements for the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on viral load. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. Viral load and expression level were log transformed to ensure normality. Boldface type indicates significance.
[b] Versus GAPDH.

TABLE 3. Association between gene expression and $CD4^+$ T-cell counts after HIV infection[a]

Gene[b]	Unadjusted model		Adjusted model	
	Effect estimate (SE)	*P* value	Effect estimate (SE)	*P* value
MxA	−2.1227 (0.8552)	0.0131	−4.1024 (2.3833)	0.0852
IFN-α	−1.7526 (0.7378)	0.0175	−2.9797 (2.4699)	0.2277
IFN-β	−2.0405 (0.6065)	0.0008	−0.8828 (2.3954)	0.7125
huTRIM5α	−2.0405 (0.6065)	0.0008	−0.3175 (0.5683)	0.5764
TRIM22	−1.7936 (0.8945)	0.0450	6.0974 (2.7761)	**0.0281**

[a] A GEE model was fitted to $CD4^+$ T-cell counts, adjusting for repeated measurements for the same individual. Unadjusted models were fitted for each expression level in order to determine the effect on $CD4^+$ T-cell counts. An adjusted model was fitted, including all expression variables in the model to determine whether they have an effect while adjusting for other expression levels. A square root transformation was applied to $CD4^+$ T-cell counts to ensure normality. The expression level was log transformed. Boldface type indicates significance.
[b] Versus GAPDH.

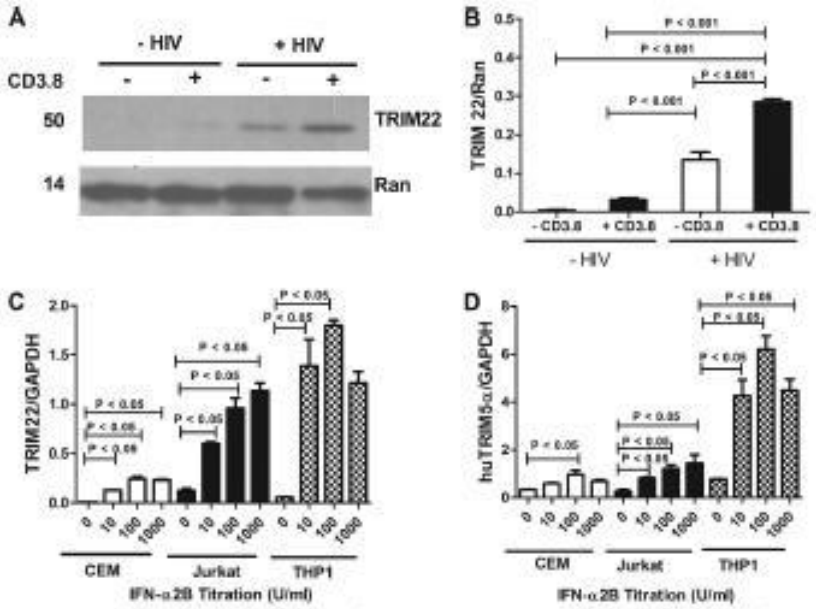

FIG. 4. Induction of TRIM22 expression by HIV and IFN-α. Shown is a representative example of data for TRIM22 induction in PBMCs from a healthy HIV-negative donor following infection with HIV-IIIB in the presence or absence of stimulating CD3.8 antibody. (A) Expression of TRIM22 following HIV infection was assessed by Western blotting. Ran levels are shown as a loading control. (B) Relative fold induction of TRIM22 by HIV averaged over three HIV-negative donors as determined by densitometric analysis (NIH Image). Values were normalized to TRIM22 expression following CD3.8 stimulation and HIV infection in each donor. (C and D) Dose-dependent increases in TRIM22 (C) and huTRIM5α (D) in various immune cell lines as determined by RT-PCR.

ulation of PBMCs with CD3.8 antibody with no infection also resulted in a slight increase in the TRIM22 expression level, indicating that activation alone was enough to alter TRIM22 expression.

To validate the role of IFN-α in TRIM22 induction, we stimulated a number of cell lines used in HIV infection assays (CEM, Jurkat, and THP-1) with increasing amounts of IFN-α and examined TRIM22 and huTRIM5α expression by reverse transcription (RT)-PCR. IFN-α significantly upregulated TRIM22 and huTRIM5α expression in a dose-dependent manner in all three cell lines tested (Fig. 4C and D).

Silencing of TRIM22 increases HIV infection and virus release in the presence of IFN-α. To determine its functional role in HIV infection, we tested the role of TRIM22 in HIV infection. JLTR-G reporter cells were stably transduced with empty pLKO vector, control scrambled shRNA (control), or an shRNA directed against the 3′ untranslated region (3′UTR) of TRIM22 and then challenged with HIV-IIIB (a fully infectious laboratory strain), with our without stimulation by IFN-α. At day 7, the percentage of HIV-infected cells (GFP-positive staining) in the 3′UTR cells treated with type 1 IFN exhibited a significantly higher percentage of infected cells (51.2%) than did vector-treated (22%) and control (24.5%) cells in the presence of IFN-α (Fig. 5A). Furthermore, the percentage of infected TRIM22-depleted cells was nearly equivalent regardless of whether cells had been treated with IFN-α or not, strongly demonstrating a significant functional role for TRIM22 in the anti-HIV IFN-α response. Data for culture supernatants collected on day 7 were consistent with these observations when assessed for p24 levels by ELISA (Fig. 5B). The knockdown of TRIM22 by the 3′UTR shRNA in the presence of IFN-α was validated by both RT-PCR and Western blotting (Fig. 5C and D).

DISCUSSION

For species other than humans, it has been demonstrated that restriction factors can completely block or partially restrict retroviral infection (7, 8, 12, 24, 36). In contrast, little is known about the *in vivo* regulation of restriction factors or their possible role in protecting or controlling retroviral infections in humans. In this study, we used a well-characterized clinical cohort of high-risk seronegative and acute or primary infection samples to investigate the association of the expression of select type 1 IFN isoforms, two well-characterized TRIM E3 ligases (TRIM5α and TRIM22), and the impact on HIV-1 susceptibility and viral control during primary HIV-1 infection.

Our earlier study revealed that lower huTRIM5α mRNA expression levels were associated with increased susceptibility to HIV-1 infection in a cohort of high-risk black African females (34). In addition, we found that in matched samples of

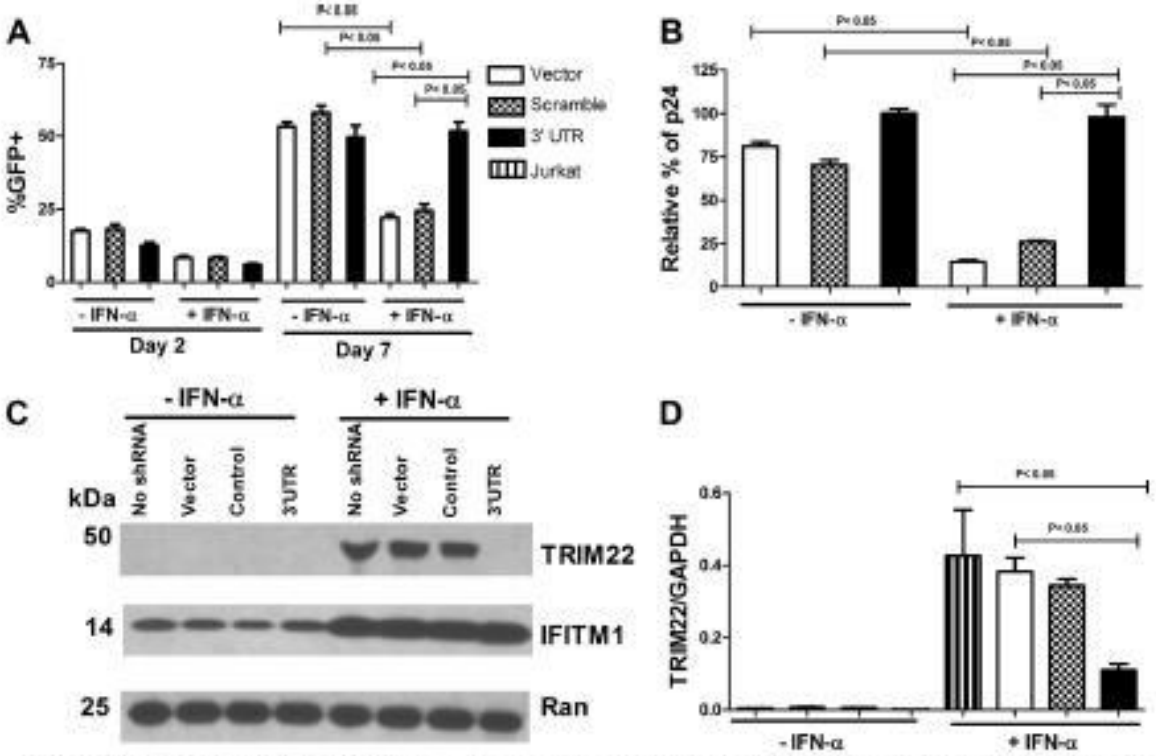

FIG. 5. TRIM22 silencing increases HIV infection and virus accumulation. (A) Jurkat reporter cells (LTR-G) transduced with the empty pLKO vector, control shRNA, or an anti-TRIM22 shRNA targeting the 3'UTR were infected with HIV-IIIB following a 1-day stimulation with IFN-α. Infection of JLTR-G cells (on day 7 postinfection) was assessed by GFP expression using flow cytometry. (B) TRIM22 silencing enhances accumulation of HIV particles in culture supernatants as determined by p24 ELISA on day 7 postinfection. (C and D) JLTR-G cells transduced with the indicated lentivirus were assessed for TRIM22 expression by RT-PCR and Western blotting. IFITM1 was used as a control (3a) for IFN induction, while Ran was used as a loading control.

HIV-1-negative individuals who later became HIV-1 positive, huTRIM5α levels were not dysregulated following infection.

Here we sought to understand the relationship between huTRIM5α and type 1 IFN in HIV-1-negative and -positive donor PBMCs, in part because huTRIM5α is type 1 IFN inducible (4, 31). Similarly, we also assessed the expression and activity of the related TRIM22 protein, focusing on this protein because, like TRIM5α, it is IFN inducible and has been demonstrated to have anti-HIV-1 activity (2, 4, 25). We found that PBMCs from HIV-1-positive study subjects had higher levels of IFN-β and MxA, suggesting that these antiviral proteins are actively upregulated following HIV-1 infection. This result is consistent with findings from other groups (42, 44). We did not see significant differences in IFN-α expression between HIV-1-negative and -positive PBMCs, suggesting that there may be differences in the mobilization of the varied type 1 IFN isoforms (10, 23) following HIV-1 infection. However, overall, in both HIV-1-negative and -positive PBMCs, we found a strong positive correlation between the two type 1 IFN isoforms tested and the IFN-inducible gene MxA, as previously described (18). Surprisingly, huTRIM5α showed a significant inverse correlation with IFN-β and had no association with IFN-α or MxA, even though huTRIM5α was shown previously to be an IFN-α-inducible gene *in vitro* (31). These results could reflect the limitations of our methodology here in analyzing global expression in PBMCs rather than in specific constituent cellular compartments or using frozen samples instead of fresh samples. Alternatively, we can speculate that distinct type 1 IFN isoforms may differentially regulate huTRIM5α expression, or huTRIM5α expression *in vivo* may involve more-complex pathways in addition to type 1 IFN. Further studies are needed to comprehensively investigate how different type 1 IFN isoforms and other cellular proteins may function in the regulation of huTRIM5α in different cellular environments and varied cohorts.

Unlike huTRIM5α, TRIM22 mRNA levels were higher in HIV-1-positive participants than in HIV-1-negative ones. We also found that TRIM22 correlated with IFN-α, IFN-β, and MxA in both HIV-1-negative and -positive samples. These results show an association between TRIM22 and type 1 IFN expression *in vivo*, suggesting that TRIM22 is a type 1 IFN-responsive gene *in vivo*, as was shown previously in *in vitro* experiments (2, 3). Higher levels of IFN-α, IFN-β, MxA, and TRIM22 were detected in seroconverters than in nonseroconverters at baseline, with differences reaching significance for IFN-β and TRIM22. These results suggest that there is im-

mune activation or another dysfunction before these persons become HIV-1 positive, and this may have contributed to the increased susceptibility to infection for these study subjects. It was previously demonstrated for a cohort of individuals at high risk of HIV-1 acquisition that participants who remained seronegative had lower levels of $CD4^+$ T-cell activation at baseline (when both groups where HIV-1 negative) (16). Similarly, levels of type 1 IFNs are elevated during immune activation, which in turn has been associated with increased HIV-1/AIDS pathology (19, 20). Further analysis of the dynamics of the expression of these antiviral factors in matched pre- and postinfection samples, especially from longitudinal cohorts of low- and high-risk study subjects with frequent sampling, may further help to better understand how intrinsic immunity is mobilized in acute HIV-1 infection and its possible contribution to antiviral control, especially in the critical acute phase of infection. It may also be useful to investigate the impact of HIV-1 infection on the type 1 IFN pathway response genes in mucosal tissues.

We also investigated the association of antiviral gene expression with viral load and CD4 T-cell counts, two commonly used markers of disease progression. We found that MxA mRNA levels showed a positive association with plasma viral load. For every log increase in MxA, the viral load increased by 0.85 log copies/ml. This is consistent with data from previous studies that demonstrated that levels of type 1 IFN increase as the viral load increases (18). Interestingly, we also observed that TRIM22 has a negative association with plasma viral load and a positive correlation with $CD4^+$ T-cell counts. We found that for every log increase in TRIM22 mRNA levels, there is an associated viral load decrease of 0.98 log copies/ml ($P = 0.0307$). TRIM22 also showed a positive association with $CD4^+$ T-cell counts, as every log increase in TRIM22 expression was associated with a 6.09 square root increase in $CD4^+$ cells/μl ($P = 0.0281$).

The new findings of a paradoxical elevation of TRIM22 levels in HIV-positive versus HIV-negative PBMCs and the favorable association of TRIM22 expression with markers of disease outcome prompted us to investigate whether TRIM22 could be induced by HIV-1 infection of HIV-negative donor PBMCs. Our experiments confirmed that TRIM22 was induced by HIV (Fig. 5A and B) and provided an *in vitro* corroboration of results demonstrating that HIV-1-positive subjects have higher levels of TRIM22 than do HIV-negative donors. This was similar to data from the work of Wang et al. (43), who showed that pseudotyped HIV-1 infection could induce APOBEC3G expression. Since PBMC populations are composed of a number of immune cell types (T cells and monocytes, etc.), we also demonstrated that IFN-α exerts an enhancing effect on TRIM22 expression in a dose-dependent manner in CEM and Jurkat T-cell lines and the monocyte cell line THP1 (Fig. 4C).

In addition, we found that the silencing of TRIM22 in a T-cell line nearly completely abrogated the IFN-mediated restriction of HIV-1 (Fig. 5A). TRIM22 silencing also resulted in an increased accumulation of HIV particles in culture supernatants (Fig. 5B), suggesting a role for TRIM22 in late viral replication activities, such as viral release or budding. Overall, therefore, our results are in agreement with data from several studies that have suggested that TRIM22 is induced by type 1 IFN and that TRIM22 can potently inhibit HIV replication and release (2, 3, 37).

Together, these data are suggestive of both *in vivo* and *in vitro* anti-HIV roles for TRIM22, although it is difficult to prove a cause-effect relationship between TRIM22 expression levels and viral load or CD4 T-cell count variables. Based on our findings, we speculate that the targeted enhancement of the expression of TRIM22 in HIV-1-infected individuals may be beneficial in reducing the viral load and could be employed as a novel antiviral strategy.

In conclusion, we have demonstrated with a cohort of HIV-1-uninfected and -infected individuals in a high-prevalence setting that HIV-1 infection is associated with an increased expression of the antiviral factor genes IFN-β and MxA, key components of the type 1 IFN pathway. However, we did not find a correlation between IFN-α or MxA and huTRIM5α, a previously described type 1 IFN-responsive host restriction factor. Indeed, we found a significant negative correlation between IFN-β and huTRIM5α. In contrast, we found that TRIM22 levels strongly correlated with IFN-α, IFN-β, and MxA expression in both HIV-1-negative and -positive PBMCs and were upregulated in HIV-1-positive study subjects. Remarkably, TRIM22 was associated with lower plasma HIV viral loads and higher CD4 T-cell counts in multivariate models adjusted for multiple antiviral factors analyzed, suggesting that TRIM22 could have antiviral effects *in vivo*. We show *in vitro* that TRIM22 is induced by type 1 IFN and HIV-1 infection. Furthermore, we demonstrate that TRIM22 plays a critical role in type 1 IFN-induced anti-HIV-1 activity in tissue cultures. This is the first study to provide evidence suggesting an *in vivo* antiviral activity of TRIM22. Further studies will be needed to address what specific cell types in the PBMC milieu express TRIM22 and the other members of the TRIM family, to better define how the expression of these proteins is regulated and to address whether these proteins can be harnessed as antiviral therapies or prophylactics.

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